The Blood-Brain Barrier and Its Microenvironment





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Basic Physiology to Neurological Disease

Edited by

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À mes trois garçons: Gregoire, Lucas, and Robin —A.P.

Preface_

The cerebral capillaries are organized in a way that they form a continuous cellular barrier that isolates the brain from the systemic circulation. By its unique properties, the blood-brain barrier (BBB) restricts the entrance of blood-borne large and hydrophilic compounds into the brain, controls the supply of nutrients to the brain and restricts immune cell entry within the CNS. The inner biochemical and immunological environment of the CNS is thus closely regulated by the BBB. This phenomenon is largely due to the intrinsic nature of the cerebral vasculature, which differs in a number of properties from other non-CNS vascular beds, as well as to the unique cellular and molecular environment surrounding the BBB-endothelial cells.

The blood-brain barrier has now been acknowledged as a neuronal component of the central nervous system and actively regulates brain homeostasis by its specialized nature. It is only recently recognized that the dynamics of the blood-brain barrier are of crucial importance in the development of various neuro-pathological conditions. In this book, experts in the field address the latest advances in both the cellular and molecular biology of this highly specialized structure and discuss its functional characteristics under pathological conditions.

This book is divided in two sections; in the first section, the role and structure of the blood-brain barrier are addressed. In Chapter 1, Stefan Liebner and Britta Engelhardt describe the development of the blood-brain barrier and what special features are formed at the barrier during embryogenesis and in the neonatal period. Ultrastructural alterations at the level of the tight junctions during development will be elucidated. In chapter 2, Eric Shusta gives insight into the specialized nature of the brain endothelial features by discussing differences between peripheral and cerebral endothelium by the use of genomics and proteomics, allowing the identification of brain endothelial specific genes and proteins. In Chapter 3, Gijs Kooij, Jack van Horssen and Elga de Vries provide a detailed description on the protein composition of the tight junction and describe in detail how the junction is regulated by a number of signalling molecules. In Chapter 4, Eric Ronken and Guus van Scharrenburg present their view on how trophic factors may influence the blood-brain barrier and discuss potential therapeutic strategies to modulate barrier permeability. Lars Edvinsson describes the neuronal influence on the blood-brain barrier in Chapter 5 and offer information on how various neuronal pathways affect the dynamics of the barrier and vice versa. Since the BBB has the capacity to actively regulate the passage of solutes and drugs from the brain-to-blood and blood-to-brain direction. pharmacologists are searching for ways to allow drugs to enter the CNS by facilitating their transport across the BBB. In Chapter 6, Wandong Zhang and Danica Stanimirovic describe in detail the various transport systems that exists at the BBB and how these influence the transport of compounds across the BBB. In Chapter 7, Béatrice Heurtault and Jean-Pierre Benoit address the potentials of the use of liposomes and other non-viral vectors and the described transport mechanisms for the delivery of drugs through the BBB to the CNS.

In section II, we have decided to present the current knowledge on CNS-directed inflammatory diseases and other neuropathological conditions that either affect the BBB or are a consequence of BBB dysfunction. In Chapter 8, Jack Antel, Katarzyna Biernacki and Alexandre Prat discuss whether brain endothelial cells themselves have a role in the inflammatory response and debate whether brain endothelial cells can function as antigen presenting cells. In Chapter 9, Zsuzsana Fabry and co-workers present the various mediators involved in the process of T lymphocyte infiltration into the CNS. Additionally, John Greenwood, David Wateridge and Patric Turowski describe the dynamic role of the brain endothelium in cellular migration in Chapter 10 and address how adhesion molecules actively participate in this process by initiating signal transduction events. In Chapter 11, Alexandre Prat and Elga de Vries introduce the molecular mechanisms of monocyte migration across the brain endothelium and discuss how these cells are of importance in the development of new lesion during the chronic neuro-inflammatory disease multiple sclerosis. Babs Fabriek, Ian Galea, Hugh Perry and Christine Dijkstra discuss the role of a relatively new cell type of the blood-brain barrier, the perivascular macrophage, in the neuro-inflammatory response in Chapter 12. The role of inflammatory mediators in controlling blood-brain barrier permeability and cellular migration are discussed in Chapter 13 by Melissa Callahan and Richard Ransohoff, who address the role of the family of the chemo-attractant chemokines and by Gary Rosenberg who shares his view on how the family of the matrix metalloproteinases and other proteolytic enzymes influence BBB permeability. Hartwig Wolburg and Arne Warth describe in Chapter 15 the interplay

Preface

of the brain endothelium and its neighbouring cells, in particular astrocytes. In Chapter 16 Melissa Fleegal, Sharon Horn and Thomas Davis describe the effect of stroke on the BBB and the role of the BBB on post-anoxic CNS damage. Monique Mulder and Raj Kalaria in Chapter 17 describe the role of apolipoproteins in controlling barrier permeability, which is a class of proteins that so far has not been taken into consideration in terms of influence to barrier properties. Not only stroke and multiple sclerosis influence BBB permeability, severe alterations in the barrier properties are also observed after brain trauma as discussed in Chapter 18 by Maria Cristina Morganti-Kossmann and co-workers. Elegant imaging techniques have revealed that the blood brain permeability may be altered in time under neuro-inflammatory conditions. In Chapter 19, Erwin Blezer describes the use of various imaging techniques to monitor BBB permeability in vivo. In the last chapter, Natalie Arbour will elucidate the mechanisms of non-HIV viral entry into the central nervous system through interaction with the brain endothelium.

This new and exciting book provides a complete overview of the dynamics of the blood-brain barrier. We hope to stimulate both clinicians and researchers to look upon the BBB as an active player in a number of physiological and pathological conditions affecting the CNS. The contributors have done an excellent job in making their chapters of high scientific merit by presenting their latest results and sharing their hypotheses and we firmly believe that this will open new avenues of collaboration for BBB research.

Elga de Vries Alexandre Prat

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The editors wish to thank Machteld Polfliet for her wonderful drawing on the cover of this book. We further wish to thank our dear colleagues for their support and enthusiasm and helpful discussion for the generation of this exciting book. We would also like to thank the editor Geoffrey Greenwood and Rosemary Doherty for their support and patience. Finally, we would like to thank all contributors who have put a lot of effort into the writing of their outstanding chapters.

> Elga de Vries Alexandre Prat

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Development of the Blood–Brain Barrier

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1. INTRODUCTION

The vertebrate central nervous system (CNS) is considered to be the most complex organ in the animal kingdom. Nerve cells communicate with each other through electrical signals, which are generated through ion-based concentration gradients. The brain is endowed with brain fluids, i.e., cerebrospinal fluid (CSF), bathing neurons, and glia and provides a unique milieu, essential for normal CNS functioning. The CSF and extracellular fluids of the brain are in a steady state. For example, the concentrations of K^+ , Ca^{2+} , bicarbonate, and glucose in the CSF are lower than in blood plasma, whereas the pH is more acidic (1). Unlike in all other organs of the vertebrate body, the brain needs to be protected from the free diffusion between blood plasma and the interstitium in order to maintain the homeostasis necessary for its proper function. The discovery of a vascular barrier between the blood circulation and the CNS dates back to more than 100 years, when in the 1880s Paul Ehrlich discovered that cationic vital dyes, which bind to serum albumin, were rapidly taken up by all organs after injection into the vascular system with the exception of the brain and spinal cord (2). Ehrlich himself interpreted these findings as a lack of affinity of the nervous system for these dyes and could not believe that the cerebral vascular endothelium might selectively exclude them. However, shortly

afterwards Edwin E. Goldman, an apprentice of Ehrlich, could show that the very same dyes, when injected into the CSF exclusively, stained the nervous tissue whereas all other tissues remained unstained. This suggests that these dyes were prevented from getting access to the blood circulation (3). The concept of a vascular blood-brain barrier (BBB), which also functions as a brain-blood barrier, was born (4). The term "blood-brain barrier" was coined, however, by Lewandowsky (5) after he, and later Briedl and Kraus (6), had performed experiments demonstrating that neurotoxic agents affected brain function only when directly injected into the brain but not when injected intravenously. The exact location of the BBB, however, remained unknown at this point and it took another 70 years until Reese and Karnovsky (7) and Brightman and Reese (8) identified the barrier to be located in brain capillary endothelial cells, using electron microscopy studies. By injecting the small electron dense tracers horseradish peroxidase (40 kDa) or lanthanum nitrate (433 Da) either into the blood or into the CSF, they could demonstrate the diffusion of these molecules into the intercellular clefts between brain capillary endothelial cells up to the tight junctions (TJs) and identify the interendothelial TJs as the morphological correlate of the BBB. The localization of the BBB at the level of endothelial cell TJs applies to all vertebrates with the exception of elasmobranch fishes, which have a BBB formed by TJs located in between glial cells (9) as do many invertebrates (10).

Not all cerebral blood vessels are entirely impermeable. Leaky areas, lacking an endothelial BBB, are located at strategic positions in the midline of the ventricular system, and are collectively referred to as circumventricular organs (CVOs). Most often, these regions not protected by the BBB for reasons that relate to physiological functions. For example, in the pituitary, neurosecretory products have to diffuse into the blood stream, whereas in the subfornical organ, a chemoreceptive area, the transcellular transport is required for regulation of homeostatic functions (11). Yet another structure where endothelial cells do not form a barrier within the CNS is the choroid plexus (12). The choroid plexus is a villous structure consisting of an extensive capillary network enclosed by a single layer of cubical epithelium.

It extends from the ventricular surface into the lumen of the ventricles. Its major known function is the secretion of cerebrospinal fluid. The capillaries within the CVOs are fenestrated allowing free diffusion of proteins and solutes between the blood and the CVOs. These leaky regions are isolated from the rest of the brain by specialized ependymal cells (tanycytes), which apically form complex TJs sealing off the CNS from the CVOs (11,13).

Interestingly, the vertebrate retina, as an external part of the brain, shows a similar organization in which the intraretinal blood vessels exhibit blood-retina barrier (BRB) properties. The retinal pigment epithelium creates an outer barrier, protecting the retina from blood-borne substances of the fenestrated vessels of the choroid.

Finally, it has to be mentioned that the outer blood-cerebrospinal fluid barrier is formed by leptomenigeal cells, providing the outer most cavity of CSF circulation. The TJs structure of leptomeningeal cells in the arachnoids show some differences, as compared to TJs in ECs (14), however, no information about molecular differences is available so far.

2. PHENOTYPE OF THE MATURE BLOOD-BRAIN BARRIER

Besides the bare endothelial barrier function, the mature BBB consists of a complex cellular system with a highly specialized morphology. Brain capillaries are formed by a single endothelial cell connected to itself and also connected to neighboring endothelial cells by complex TJs. These capillaries are regularly covered by a high number of pericytes (also called perivascular macrophages, see Chapter 13) embedded in a common basal membrane, forming the vascular entity. Further on the abluminal side, the vessels are almost completely covered by astrocytic end feet and some perivascular macrophages. While the endothelial cells form the barrier proper, the onion shell-like interaction with adjacent cells seems to be a prerequisite for barrier function.

Nevertheless, the barrier itself is a consequence of the formation of complex TJs between endothelial cells, establishing—unlike the rudimentary TJs of peripheral blood vessels—a high electrical resistance across the endothelial barrier (about 2000 Ω cm²) (15). TJs can be visualized best by freeze-fracture electron microscopy, where strands of particles can be observed to be associated with the protoplasmic fracture face (P-face) of the plasma membrane or with the exo-cytoplasmic fracture face (E-face) (16). In mammalian BBB endothelial cells, TJ particles are found to be associated at a higher degree with the P-face of the plasma membrane than in non-BBB endothelial cells. Thus, the P-face association was considered to correlate with the barrier function of BBB endothelium in mammals (17). This observation is also consistent with the low P-face association in TJs of peripheral, non-barrier endothelial cells, in which E-face associated tight junctional particles clearly predominate (18,19). Therefore, the P-face association has become a morphological criterion of endothelial barrier properties in mammals and it has been hypothesized that the particle distribution is presumably the result of the cytoplasmic anchoring of TJ-proteins, which differs in BBB and non-BBB endothelia (17). Indeed, the presence of TJ-proteins, forming the particle strands on P- and E-face freeze-fracture preparations could be elegantly demonstrated by the freeze-fracture immunogold technique (20-22).

Which proteins are involved in TJ-formation? Occludin was the first integral membrane protein found to be localized exclusively within TJs including those of the BBB (23). However, mice carrying a null mutation in the occludin gene are viable and develop morphologically normal TJs

in most tissues including those of brain endothelium (24). These observations suggest that occludin, although localized to TJ particles in freezefracture preparations, is not essential for proper TJ formation. In contrast to occludin, the claudins have been shown to be sufficient for TJ-strand formation (20). Claudins comprise a more recently discovered gene-family of integral membrane TJ-proteins with more than 20 members and exhibit no sequence-homology to occludin. Besides the endothelial specific claudin-5, claudin-3 was also shown to localize to endothelial TJs in the CNS of mice and man (25,26). Additionally, claudin-12 has been described in CNS endothelium (26). It should be mentioned that contradictory results have been reported on the expression of claudin-1 in brain endothelial cells (25,27,28). These discrepancies might result from original work using an anti-claudin-1 antibody that was subsequently shown to cross-react with claudin-3. Specific antibodies and molecular biology techniques should help to clarify the exact claudin makeup of BBB TJs in the near future. It is interesting to note that transfection of claudin-1 and claudin-3 into TJ-lacking fibroblasts induces P-face associated TJs (29), whereas transfection of fibroblasts with claudin-5 induces E-face associated TJs in the absence of occludin (21). This observation demonstrates that different claudins induce structurally different TJs suggesting that at the BBB, claudin-3 and claudin-5 may be responsible for the presence of P-face and E-face associated TJ particles, respectively. Recently, mice deficient for claudin-5 have been described (26). Lack of claudin-5 leads to lethality of neonates after 10 hr, an event shown to be due to a size selective loosening of the BBB for molecules < 800 Da (26). These observations suggest that within TJs, different claudins might regulate paracellular permeability such that each claudin inhibits the diffusion of molecules of a given size by the formation of pores. which prevent the passage of these molecules. Whether this specificity correlates directly with the appearance of TJ-protein particles on the P- or E-face has yet to be elucidated. It should be noted that in general, endothelial cells of submammalian species show TJs with high P-face association (30). This may reflect differences in their molecular composition.

In addition, during BBB-development in the chicken, only TJ-strand complexity is up-regulated, whereas in the rat, both P-face association and complexity of TJs increase (31–33). It would be interesting to understand if species diversity in TJ-morphology also correlates with different barrier properties.

Claudins and occludin are linked to the cytoskeleton by a PDZbinding domain, which confers indirect adhesion to the actin cytoskeleton via linker proteins of the family of membrane associated guanylate kinases (MAGUK), such as ZO-1, ZO-2, and ZO-3 (reviewed in Refs. 16 and 34). In addition to conferring the cytoskeletal anchorage of transmembrane TJ-proteins, MAGUKs seem to be important for the correct localization of the transmembrane TJ-protein occludin in the apical cell membrane of epithelial cells (35), whereas for claudin-1 and claudin-5, it is the Cterminus, but not the PDZ-binding domain that seems to be important (36). Transmembrane proteins, which localize to TJs also seem to be involved in membrane-targetting of TJ components. Namely, junctional adhesion molecule (JAM) (37) and the recently discovered endothelial cell-selective adhesion molecule (ESAM) are localized in BBB TJs (38). For JAM-A, the formation of an intracellular complex with atypical PKC (aPKC) and Par-6 through the PDZ-dependent interaction with Par-3/ ASIP, is a prerequisite for the correct localization of occludin (39–41). Indeed, JAM-A is found in primordial, spot like adherens junction, so called puncta, together with E-cadherin and ZO-1 before TJs have been formed. If the JAM-A-attached intracellular complex is disturbed through dominantnegative mutants of Par-3, aPKC, and Par-6, TJ-formation is severely disturbed. JAM-A may therefore have a function to localize the Par-3-aPKC-Par-6 complex to the junction of the early puncta, a prerequisite for cell polarization and the formation of TJs (for review see Ref. 42). (For more details on the dynamic regulation of tight junctions, please see Chapter 3.)

As previously indicated, junctional systems are stabilized via interaction with the cytoskeleton. The importance of the cytoskeleton in the establishment and maintenance of the BBB becomes evident in mice lacking the actin-binding protein dystrophin. The mdx-mouse, which is the animal model for Duchenne muscular dystrophy (DMD), exhibits an increase in brain vascular permeability due to the disorganization of the α -actin cytoskeleton in endothelial cells and astrocytes, leading to altered subcellular localization of junctional proteins in the endothelium, as well as in the astrocytic water channel aquaporin-4 (AQP4) (43).

Another member of the Ig-supergene family, named HT7-antigen/basigin/ neurothelin (44,45), is specifically expressed in BBB endothelial cells and is believed to act as a receptor involved in carrier and/or surface recognition mechanisms (46). However, its precise function at the BBB remains to be defined.

In cerebral endothelial cells, non-occluding adherens junctions (AJs) are found intermingled with TJs (47). In AJs, the endothelial specific integral membrane protein VE-cadherin (48) is linked to the cytoskeleton via catenins (49). In endothelial cells, expression and localization of β -catenin, γ -catenin, and p120^{cas} have been described as crucial for the functional state of adherens junctions, including those found in the brain (50,51). The contribution of VE-cadherin in maintenance of BBB integrity remains to be investigated. In contrary to observations made in non-CNS tissue, VE-cadherin mRNA was shown to be down-regulated in brain microvessels during brain angiogenesis (52). PECAM-1 has also been localized in endothelial cell contacts outside of either TJs or AJs including those of the brain. In PECAM-1 deficient mice, however, no primary defect in BBB integrity has been reported (53). In the case of chronic inflammation, as occur

in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, mice deficient for PECAM-1 showed an early infiltration of mononuclear cells, as compared to wild-type control animals (54). Furthermore, PECAM-1 deficient mice showed a prolonged and exaggerated vascular permeability of CNS vessels during EAE, suggesting that PECAM-1 is a negative regulator of leukocyte migration across the BBB and a positive regulator of BBB maintenance.

Endothelial paracellular impermeability in the brain implies the necessity for specific transport systems, to ensure access of nutrients to the brain parenchyma. The glucose transporter Glut-1 is specifically expressed on brain endothelial cells, with specific localization at the abluminal vs. the luminal membrane (55). Additionally, due to the metabolic needs of the brain, transferrin receptors have been shown to be expressed on endothelial cells forming the BBB, but not on those of the choroid plexus or the CVOs (56). Furthermore, P-glycoprotein is expressed at the BBB and its role seems to secrete the toxic and lipophilic metabolites from the neuroectoderm to the blood (57). Finally, the phenotype of brain endothelial cells is characterized by specific metabolic pathways controlling the substances that leave or enter the brain (for detailed reviews see Refs. 58–63).

The cell morphology, biochemistry, and function of brain endothelial cells make these cells unique and clearly distinguishable from any other endothelial cell in the body. To understand how brain endothelial cells acquire the unique features of the BBB, it is crucial to understand the development of the brain vasculature.

3. DEVELOPMENT OF THE BRAIN VASCULATURE

During vertebrate embryogenesis, the development of the vasculature of the head begins when angioblasts, which originate solely from the lateral splanchnic mesoderm (64,65), enter the head region and form the perineural vascular plexus by de novo formation of vessels, a process named vasculogenesis. In a 2-day-old chick embryo and a 9-day-old rodent embryo this vascular plexus covers the entire surface of the neural tube (62,66,67). Subsequent vascularization of the CNS starts at day 4 of embryonic development in the chicken and day 11 in rodents by vascular sprouting from preexisting vessels of the perineural vascular plexus, a mechanism called angiogenesis (62,64,66,68). These sprouting vessels grow radially into the neuroectodermal tissue, elongate, give rise to manifold branches and finally anastomose with adjacent sprouts, forming an undifferentiated network of capillaries near the ventricular zone of the developing brain (66,69). Interestingly, brain vascularization follows a stereotyped temporal and spatial pattern, with a peak of angiogenetic activity in early postnatal stages (66,70,71).

Driven by the question of what makes endothelial cells grow into the neuroectoderm, Werner Risau hypothesized that soluble factors produced

by the brain and recognized by specific receptors on endothelial cells of the perineural plexus were responsible for brain angiogenesis. Trying to isolate this/these factor/s, acidic and basic fibroblast growth factors (aFGF, bFGF) were the first potential candidates to be identified in the brain (72). Although aFGF/bFGF meet the requirements to be potent inducers of endothelial cell proliferation in vitro and of angiogenesis in vivo (72-74), developmental studies on their expression pattern in the brain and on their corresponding receptors on endothelial cells could not be superimposed with the spatio-temporal pattern of brain angiogenesis. Besides the fact that many different cell types are activated by aFGF and bFGF, the expression of these molecules remains high in the adult brain. when angiogenesis has ceased (75-77). When the vascular endothelial growth factor (VEGF) was identified, it became clear that this was the candidate paracrine factor specifically stimulating endothelial proliferation and sprouting via its high affinity receptors VEGF receptor 1 (VEGFR1, flt-1) and VEGF receptor 2 (VEGFR2, flk-1/KDR) (78). Gene deletion studies of VEGF, as well as its receptors-VEGFR1/2-revealed that VEGF is important for endothelial cell proliferation, survival as well as for vascular remodeling during embryonic perivascular cells (86). The platelet derived growth factor B (PDGF-BB) has also been shown to be important for pericyte recruitment. Interestingly, mice deficient for PDGF-BB show a complete lack of pericyte within brain vessels (87).

Furthermore, the TGF β -pathway, ubiquitously important during embryogenesis, has been shown to have specific effects in angiogenesis through the endothelial specific receptors Alk-1 and endoglin, and their intracellular effectors Smad5 and Smad6 (for review see Ref. 88). Membrane bound ligand-receptor systems, such as delta-Notch or ephrinB2-Eph, have been demonstrated to play an important role in CNS vascular differentiation (for review see Refs. 89 and 90). Additionally, adhesion molecules such as VE-cadherin have also been involved in early vascular maturation, using gene targeted mice (91).

Although many molecular components involved in brain angiogenesis have now been identified, their exact mechanisms of action are not yet fully understood. The biggest remaining obstacle for the understanding of the roles involved in the development of the CNS vascular bed is that mutations in their genes invariably lead to lethal phenotypes early during embryogenesis and sometimes before the beginning of BBB differentiation.

4. DIFFERENTIATION OF THE BLOOD-BRAIN BARRIER

4.1. Structure and Function

When vascular sprouts enter the neuroectoderm—in chick at 4 days, in rodents at 11 days of embryonic development—vessels show a simple

sinusoidal morphology, with a large diameter and an irregular shape. At this point during embryogenesis of the CNS, the endothelial lining shows fenestrations and a high number of vesicles, and these early vessels are considered to be permeable to small hydrophilic substances. Along with their permeable phenotype, endothelial cells exhibit rudimentary TJs indicated by substantial junctional clefts (92). As development proceeds, the vessels lose their fenestrae, become smaller and thinner walled and acquire a more regular shape (55,93). An important step in BBB development is the recruitment of perivascular cells and astrocytes to the growing blood vessels. Pericytes grow into the brain together with endothelial cells, a process largely dependent on the presence of the PDGF-BB growth factor (87). The specific deletion of this factor in endothelial cells leads to perinatal death due to persistent microaneurisms and hemorrhages (94). The morphology of astrocytes and the status of the BBB in these animals however, have not yet been investigated. As vessels forms and cellular arrangement develops, endothelial cells acquire barrier properties, which mirrors in their TJ-morphology and trans-cellular resistance. In early fetal brain capillaries, the TJ-strands of endothelial cells are short with low complexity, a pattern which changes dramatically during development as the strands become longer and interconnected, i.e., complex, and the outer leaflets of adjacent membranes within junctional contacts seem to be fused in so called "membrane kisses" (33,95). As initially described the P-face association of particles in freeze-fracture analysis is another indicator of the TJ-maturity in mammals. Indeed, it could be demonstrated that the density of particles within TJ-strands increases late during embryonic development (after day E18 in the rat) as well as after birth and that a significant increase in the P-face association could be observed, representing a transition to the adult conformation of TJs (33).

Although all morphological studies are in favor of a gradual development of endothelia barrier function, it has been debated whether the fetal BBB is leaky or not (for review see Ref. 96). However at this point, it seems more likely that the permeability of the fetal BBB to macromolecules resembles that of the adult. In contrast, in some species, small molecules access the fetal and newborn brain more readily as compared to the adult brain. The latter findings are supported by the recent observation in mice deficient for the endothelial specific TJ-protein claudin-5; such mice exhibit a selective loosening of the BBB for molecules smaller than 800 Da (26). In addition, the electrical resistance of small pial blood was shown to be several times higher in fetus brain when compared to the adult brain. Electrical resistance was also shown to decrease just before birth (97,98). Barrier permeability to small molecular weight tracers, such as γ -amino isobutyric acid (AIB), was shown to decrease in ovine fetuses late during gestation (99) and as long as 17 days after birth, in rabbits (100).

It is worth noting that along a the region specific "schedule" of brain angiogenesis, the BBB tightness develops regionally and gradually increases in the course of angiogenesis rather than being "switched on" at a specific time point (70).

4.2. Phenotypic Changes

During BBB development, capillary brain endothelial cells acquire a characteristic set of markers that are thought to be related to an impermeable phenotype. The glucose transporter Glut-1 is one of the earliest markers expressed by endothelial cells during vasculogenesis. Interestingly, in the premature BBB. Glut-1 distribution is balanced on the luminal and abluminal membranes of blood vessels. When the BBB matures, there is a re-distribution of Glut-1 on the cell surface with a preferential abluminal distribution of the glucose transporter (55,101). Up-regulation of the transferrin receptor can also be observed on endothelial cells forming the BBB, while it is not expressed by ECs of the choroid plexus or the CVOs (56). Furthermore, P-glycoprotein expression becomes evident on endothelial cells early during brain angiogenesis (102), and likely functions to remove from the neuroectoderm the noxious lipophilic metabolites that crossed the placenta and gained access to the fetus brain trhough the immature BBB (57). The localization of P-glycoprotein to the luminal endothelial membrane and/or to the membrane of astrocytic end feet has recently become a matter of debate (103,104). Up-regulation of other markers, such as the non-receptor tyrosine kinase lyn (105) and the Ig-superfamily member HT7 can be phenomenologically correlated with the development of BBB vessels. However, the function of these markers in CNS ECs remains to be elucidated (46,106,107).

In contrast to BBB markers that are up-regulated during embryogenesis, the pan-endothelial cell antigen MECA-32 is down-regulated in brain ECs during BBB maturation (108). As a consequence, MECA-32 antigen is absent on mature cerebral endothelium, whereas it remains present on vessels outside of the CNS, and on capillaries within the CVOs (Fig. 1). Based on the sequence comparison using the BLAST algorithm, the murine MECA-32 antigen was identified as the mouse orthologue of the rat plasmalemma vesicle associated protein (PV)-1. PV-1 was shown to be specifically localized to the diaphragms of fenestrated endothelia (109,110). This might then explain the specific down-regulation of MECA-32/PV-1 antigen observed on CNS-ECs during BBB maturation, as brain ECs lose their fenestrations (see "Structure and Function"). Therefore, it seems likely that MECA-32 antigen is involved in the formation of diaphragmed fenestrations, directly responsible for the efflux of blood-borne molecules to non-CNS tissues. Other organs, such as cardiac and skeletal muscle, also lack MECA-32, at least in resting and non-inflamed conditions (111) supporting the notion that organ-specific variations in trans-endothelial transport occurs.







Step III

Figure 1 Differentiation of the BBB in three steps. Step I. Angiogenesis: vascular sprouts radially invade the embryonic neuroectoderm toward a concentration gradient of VEGF-A, which is produced by neuroectodermal cells located in the ventricular layer. VEGF-A binds to its endothelial receptor, the receptor tyrosine kinase flk-1/ KDR. The endothelial cell specific receptor tyrosine kinase Tie-2 and its ligand Ang-1 are involved in angiogenic sprouting early during embryogenesis. The cerebral endothelial cells express Glut-1 and the MECA-32 antigen. The TJs are permeable to small molecules. Step II. Differentiation of the BBB: the phenotype of cerebral endothelial cells changes such that they loose expression of the MECA-32 antigen and start to express the HT7 antigen. Glut-1 is now enriched on the abluminal surface of the endothelium. De novo expression of P-glycoprotein and the nonreceptor tyrosine kinase lyn can be observed. The TJs become complex, P-face associated and thus also tight for small polar molecules. Phenotypic changes of endothelial cells are accompanied by their close contact with pericytes and astroglial cells. The molecular mechanims involved in the interaction between pericytes and endothelial cells are partially characterized and have been shown to be important for vessel maturation within the CNS. Recruitment of pericytes along the differentiating BBB vessels is ensured by several mechanisms. PDGF-BB produced by endothelial cells binds to its receptor PDGFR-b on pericytes; N-cadherin enriched on the respective membranes facing the neighboring cell type interact with each other; Ang-1 expressed by pericytes binds to the endothelial receptor tyrosine kinase Tie-2. Only recently, some light has been shed on the molecular interactions between endothelial cells and astroglial cells in the developing CNS, endothelial cells produce LIF, which induces the maturation of astrocytes via the LIF-Rb. Furthermore, due to the presence of vessels the oxygenlevel increases and endothelial cells produce PDGF-BB, both leading to an upregulation of SSeCKS in astrocytes. In turn, SSeCKS upregulates Ang-1 expression in astrocytes, which acts as an endothelial differentiation marker and positively, influences the membrane localization of junction protein as ZO-1 and claudin-1. Step III. The cellular elements of the mature BBB: despite the fact that the cerebral endothelial cells form the barrier proper, close contact with pericytes, astrocytes, and maybe neuronal cells is required for the maintenance of the BBB. The molecular mechanisms involved in this crosstalk required for BBB maintenance in the mature CNS remain unknown to date.

5. PUTATIVE MECHANISMS OF BLOOD-BRAIN BARRIER INDUCTION AND DIFFERENTIATION

Although several aspects of BBB-phenotype in brain capillary endothelial cells have been monitored during development, the crucial question concerning the induction of this differentiation process remains to be elucidated. During brain angiogenesis. ECs come in contact with numerous and diverse neuroectodermal cells, such as neuroblasts, as well as with mature and immature glial cells (80). The notion that endothelial cells are not committed or predetermined to the BBB-phenotype, was elegantly demonstrated using chick-quail xenograft experiments in which vessels of the coelimic cavity of the embryonic chick acquired BBB characteristics while growing into the developing transplanted quail brain (65,112). Because of the close apposition of astrocytic end feet to the vessel wall, astrocytes have immediately been considered to be an important neuroectodermal cell for EC differentiation and BBB maintenance (113,114). Indeed, in vivo experiments demonstrated that astrocytes can induce some BBB-characteristics in vessels of the anterior eye chamber (115), although these results could not be reproduced by other groups (116). It is now widely accepted that in vitro, astrocytes or conditioned medium derived from astrocyte cultures induce BBB-like characteristic in endothelial cells grown in vitro (17,117,118). Until recently, little was known about the nature of the astrocyte-derived signals involved in brain EC maturation. Lee et al. (27), however, shed some light on the astrocyteendothelial interaction and identified the tumor suppressor src-suppressed C-kinase substrate ("SSeCKS", human ortholog is gravin), as a putative molecular effector of BBB induction. Over-expression of SSeCKS in astrocytes markedly reduced expression of VEGF in vitro. This result nicely fits with a low in vivo expression of SSeCKS in early embryonic stages when VEGF is high, whereas in late embryogenesis and in postnatal stages the expression of SSeCKS progressively increases, leading to the downregulation of VEGF, thereby decreasing or blocking angiogenesis. On the other hand, SSeCKS induced the expression of Ang-1 in astrocytes, a known pro-differentiation factor for endothelial cells, leading to an increased localization of ZO-1 and claudin-1 to cell-cell junctions in endothelial cells. Furthermore, SSeCKS can be induced in astrocytes by PDGF-BB, which in turn is produced by endothelial cells and plays an important role in pericyte recruitment to blood vessels (119). Interestingly, there is also evidence that ECs support the astrocyte differentiation from their precursors through the secretion of leukemia inhibitory factor (LIF) (120). Taken together, these data suggest that angiogenic ECs in the brain first induce a step the differentiation of astrocytes through LIF, and in a second step induce the expression of SSeCKS by astrocytes through secretion of PDGF-BB. Astrocytes will then down-regulate VEGF and up-regulate Ang-1 production, stopping angiogenesis and inducing BBB-EC maturation, respectively. Although astrocytes play

an important role in BBB differentiation, it seems unlikely that astrocytes are sufficient to induce the complete spectrum of BBB-properties in endothelial cells, especially since some of the BBB characteristics in brain endothelial cells appear prior to astrocyte differentiation, but also because astrocytes are present in the CVOs, where capillaries do not form a barrier. However, pial blood vessels are vascular structures where astrocytes do not contact ECs, but which possess features that are characteristic of the BBB.

As regard the involvement of neuronal cells in the development of the BBB, a possible involvement of neuronal precursor populations in inducing BBB characteristics of immature endothelial cells has also been suggested (121).

Some evidence also points to a role of the extracellular matrix (ECM) in brain vascular development. For example, α V-integrin deficient mice develop an abnormal vasculature and cerebral hemorrhages due to an inappropriate association of ECs with brain parenchymal cells such as glia and neuronal precursors. This phenomenon seems to occur in the presence of normal endothelial-pericyte interaction (122). The α v-integrin subunit interacts with β 3, β 5, β 6, and β 8, but also with β 1 subunits. The resemblance of the phenotype of β 8-integrin deficient mice (123) to that of the α V-integrin deficient mice suggests that loss of α V β 8-integrin renders brain parenchymal cells incapable of normal adherence to the basal membrane of brain microvessels, which contain the α V β 8-ligand vitronectin. These data point to the possible importance of the ECM either in mediating heterologous cell-cell contact or in storing soluble factors acting as inducers of BBB differentiation.

Pericytes are a cell population found in close association with endothelial cells even at very early stages of development and seem to be more prevalent in CNS capillaries than in other peripheral capillaries (18). The in vivo function of pericytes has been unclear for a long time (124), but this issue was recently revisited and pericytes were found to be required for vessel maturation (87,94,125). During brain angiogenesis several factors such as Tie2/Ang-1 (126), PDGFR-B/PDGF-B (87), TGFB-1, as well as receptors and transcription factors of the TGFβ-signaling pathway, such as TGF_β-receptor II, Alk-1, Alk-5, endoglin, and Smad5 have been shown to partake in the process of endothelial-pericyte interaction (127-132). Furthermore, the adhesion molecule N-cadherin (133) is required for the correct pericyte recruitment along the angiogenic capillaries in the developing chicken brain (133,134). At the EC-pericyte interface, Ncadherin is exclusively linked to the actin cytoskeleton through β-catenin, which along with N-cadherin becomes down-regulated during BBB maturation in the chicken (51). Besides the role of β -catenin as a binding partner for N-cadherin, it is also possible that β-catenin is involved in transcriptional events.
Besides VEGF, PDGF-BB, Ang-1/-2, and TGF β , all factors known to play a role in angiogenesis and vascular differentiation, some evidence points to an involvement of the Wnt-family of growth factors in this process. In the canonical Wnt-signaling, binding of the growth factor located in the ECM (135,136) to the frizzled receptor leads to the stabilization of cytoplasmic β -catenin, through proteins like disheveled and glycogene synthase kinase 3 β (137–140). In endothelial cells, little is known about the role of Wnt-signaling, in particular during vasculogenesis and angiogenesis. However, the dual role of β -catenin as a scaffolding protein on the one hand, linking cadherins to the actin cytoskeleton, and as a transcription factor on the other hand, leading to target gene transcription in the nucleus, makes it an interesting protein candidate for study in the course of vascular development. In particular it is believed that cadherins, at least indirectly, regulate the free cytoplasmic amount of β -catenin, which is able to translocate to the nucleus (139,141).

It has recently been shown that the endothelial cell-specific deletion of the β -catenin gene leads to embryonic lethality around mid-gestation due to vascular fragility, and due to placenta and heart defects (142,143). It has also been demonstrated that the transcriptional activity of β -catenin is involved in this effect (142).

Regarding BBB development, Wnt-signaling may be of great interest, since brain ECs are able to undergo canonical Wnt-signaling. Mouse brain ECs grown in vitro in primary cultures respond to Wnt-1 stimulation with activation of the canonical Wnt-pathway involving β -catenin (144). ECs grown into the neuroectoderm were also found to be positive for a Wnt-signaling reporter, whereas other angiogenic vessels in the embryo were negative for Wnt-signaling. This was true for all developmental stages investigated so far (145). Support for a specific role of canonical Wnt-signaling in brain vascularization also comes from genetic diseases such as familial exudative vitroretinopathy (FEVR), in which the presumptive Wnt-receptor frizzled-4 (FZD4) is mutated, leading to a lack of vascularization in the peripheral retina (146). In addition to FEVR, the FZD4 knock-out mouse shows regression of vessels in the cerebellum, suggesting a role for the Wnt-pathway in vascular maintenance (147).

Although the exact role of Wnt-signaling during brain vascularization remains to be elucidated, results obtained so far need to be considered in the light of previous reports claiming that β -catenin protein expression is high during brain vascularization and becomes down-regulated after BBB establishment (51). Whether this coincides with Wnt-signaling in endothelial cells and, at least to some extent, with the induction of the BBB phenotype is still unclear, but among the known target of canonical Wnt-signaling is claudin-1, a TJ-protein possibly involved in endothelial barrier establishment (148).

Currently available information on Wnt-signaling in ECs suggests that this pathway becomes activated in a very restrictive temporal and spatial manner. So far, the data obtained from knock-out and reporter mice in vivo and from brain endothelial cells in vitro strongly favor a specific role of Wnt-signaling in brain ECs, making it a good candidate pathway involved in BBB establishment, maturation, and maintenance (Fig. 2).

6. MAINTENANCE OF THE BLOOD-BRAIN BARRIER

Under physiologic conditions the BBB is in a steady state. Under pathological situations within the CNS, such as ischemia, inflammation or tumor growth, BBB dysfunction can be seen. This emphasizes that the barrier is not static, but rather depends upon continuing maintenance signals provided by a physiologically intact environment for its proper function. Eventually, leakiness of the BBB is accompanied by loss of the differentiated phenotype of BBB endothelial cells as supported by the re-expression of the MECA-32 antigen on brain endothelial cells in inflamed vessels surrounded by inflammatory cuffs (149) or by the selective loss of the immuno-detectable TJ-molecule claudin-3 (25). In the latter one might speculate that TJs are less tight,



Figure 2 Hypothetical role of Wnt Signaling in brain endothelial cells: the immature neuroepithelial cells produce Wnt-growth factors, which may activate specific frizzled receptors (Fzd) on endothelial cells, activating the intracellular cascade of the canonical Wnt-signaling. In this pathway, disheveled (Dsh) is activated by Fzd and in turn inhibits glycogen synthase kinase 3b (GSK-3b), leading to the disassembly of the b-catenin degradation complex. This favors the nuclear translocation of b-catenin and its interaction with TCF/Lef transcription factors, possibly activating the transcription of TJ-genes like claudin-1.

which is an inversion of the scheme known from TJ-development (33) as suggested by a shift from P-face to E-face associated TJs (28).

Furthermore, the exctracellular matrix might contribute to BBB maintenance. In particular, agrin, a heparan sulfate proteoglycan, was found in the basement membranes of vessels with special barrier properties like those found in the brain, and was proposed to be involved in the development of the BBB (150). Astrocytes have been shown to express agrin in vivo and in vitro. Therefore agrin could serve as a basal membrane factor maintaining barrier functions in brain endothelium in vivo. In any case, it is noteworthy that the extra cellular matrix of leaky blood vessels in malignant human brain tumors was found to be devoid of agrin (151).

Taken together, development and maintenance of the BBB is tightly regulated by the permanent interaction of endothelial cells with the neuroectoderm. Supporting this notion is the fact that when brain endothelial cells are isolated and cultured in vitro they rapidly lose many of their BBB characteristics including P-face associated TJs. They also acquire peripheral markers such as the MECA-32 antigen, indicating that integrity of the BBB strictly depends on signals provided by the CNS microenvironment (152). However, the maintenance of complex TJs and the continued expression of lyn in these primary cultures suggest that brain-derived endothelial cells do not completely "forget" their commitment to form a BBB (63).

7. OUTLOOK

The vascular system of the brain develops in three phases: vasculogenesis, angiogenesis and barriergenesis (153). The last decade has seen an explosion in our understanding of the molecules involved the formation of blood vessels during vasculogenesis and angiogenesis. Although we know that inductive signals within the embryonic neural tube are responsible for committing the endothelium to barrier formation, inductive molecules and their receptors are still largely unidentified. Even the cellular sources providing the inductive signals, i.e., astrocytes, pericytes or neurons remain a matter of debate.

Barrier genesis, i.e., differentiation of brain ECs to BBB-ECs is a long and complex process involving a series tightly regulated temporal and spatial events. This might be one reason why in vitro BBB models have not yet succeeded to define the precise factors involved in BBB formation. The identification of a single putative factor instrumental in the induction of BBB characteristics might depend on more sophisticated genetic models, such as conditional and inducible mouse mutants as well as reporter mouse models. It is obvious that the multitude of factors involved so far will only be dissected by the careful analysis of members of specific signaling cascades, junctional molecules, and extracellular matrix components. Thus, in summary, the apparent complexity of BBB biology necessitates thorough and time-consuming approaches even in this second century of BBB research.

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2

Blood–Brain Barrier Genomics and Proteomics

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1. INTRODUCTION

The endothelium of the brain vasculature is so impermeable that it is referred to as the blood-brain barrier (BBB). Although the endothelial cells form the primary barrier between the bloodstream and the brain parenchyma, many other cells of the so-called neurovascular unit interact dynamically with the endothelial cells to create a unique brain microenvironment. It is this microenvironment that elicits the barrier phenotype of brain blood vessels. Perivascular cells including smooth muscle, pericytes, astrocytes, neurons, and microglia interact with the endothelial cells to help maintain brain homeostasis. A complicated network of physical contacts and soluble messengers mediates the interactions between multiple cell types. In addition, information does not flow only from perivascular cells to endothelial cells, but the endothelial cells can also promote responses from its surroundings. The former interaction paradigm has been the principal focus of BBB researchers to date, while the latter has been studied to a much lesser extent. The barrier also responds in a spatial and temporally discriminate fashion under neurological disease conditions. The fact that the brain vasculature is restrictive has been known for over 100 years, and significant research effort has identified many BBB attributes. However, the complexity of the interconnected neurovascular unit has thus far limited advances in BBB knowledge. Because of this, the detailed structure-function relationship of this critical interface remains largely unrefined.

With the advent of global gene and protein expression profiling techniques (genomics and proteomics), the BBB field is poised to address prevailing questions about the BBB. Some goals of BBB research include discerning how the BBB interacts with surrounding cell types in healthy and diseased brain, how the BBB forms a transport barrier, and how the BBB interacts with circulating immune components. Global profiling techniques can help accomplish these goals. These technologies allow for much more comprehensive gene and protein expression analyses than those that can be provided by traditional biochemical techniques. Thus, instead of examining a few genes or proteins and their effects on the BBB under certain experimental conditions, thousands of molecules can be assayed simultaneously. Time-dependent BBB responses can also be resolved using these global techniques.

Genomics techniques such as gene microarrays (1), serial analysis of gene expression (SAGE) (2), and suppression subtractive hybridization (SSH) (3) have all been applied to BBB analysis (4-7). These techniques can be used with little prior experience as they are widely practiced and have been optimized quite extensively. However, elucidation of the cellular gene expression profile (transcriptome) does not completely characterize the protein expression profile (proteome) of the cell. First, the expression level of messenger RNA (mRNA) does not correlate directly with the expression levels of proteins in the cell (8,9), and this is a result of differential regulation of mRNA and protein in the cell. Second, genomics techniques do not yield information about modifications such as glycosylation, phosphorylation, and proteolytic processing that are critical to protein function in the cell. Thus, protein expression profiling is a necessary complement to genomics techniques to fully characterize the gene/protein expression programs of the cell. However, in contrast to genomics techniques, proteomics technologies are much more difficult to apply to BBB study. Since the BBB severely restricts paracellular transport via tight junctions and exhibits a low level of pinocytosis, molecules and cells must first interact with the endothelial cell membrane in order to signal or transport across the endothelium. This indicates that membrane proteins present on the luminal and abluminal endothelial cell membranes are significant functional components of the blood-brain barrier. Although the most common proteomics methodology, two-dimensional gel electrophoresis, has been applied to the BBB, it is not ideally suited for the analysis of membrane proteins (10). Therefore, in order to analyze the BBB membrane proteome, new techniques need to be developed and employed. In this chapter, a recently developed technique called subtractive antibody expression cloning will be discussed. This technique was developed with the blood-brain barrier in mind, and has been used to begin profiling the membrane

protein constituents of the BBB (11). In addition, a new technology known as isotope-coded affinity tagging (ICAT) profiles membrane proteins reasonably well (12). The ICAT technique has also recently been applied to proteomic analysis of the BBB (13–15), and these preliminary efforts will be presented.

To date, the use of genomics and proteomics techniques in BBB research has been somewhat limited. However seminal BBB studies that used these techniques have demonstrated that they are invaluable assets to any BBB research program. These studies have confirmed the molecular and functional diversity of the blood-brain barrier, and emphasize the fact that the BBB functions as a dynamic communication interface between the blood and the brain. Global approaches have also helped us to confirm a BBB role in immune function and developmental regulation. In addition, these techniques have helped clarify the molecular constituents of tight junctions and the expression profile of molecular transporters. Finally, these methods have been used to determine the role of the BBB in neurological disease progression with the goal of identifying therapeutic targets. Throughout this chapter, the impacts of genomics and proteomics on BBB research will be discussed, and views regarding future directions will be presented.

2. MOLECULAR PROFILING OF THE BBB USING GENOMICS

As mentioned above, the BBB is an impermeable interface that separates the bloodstream from the interstices of the brain, yet it allows selective communication between these two compartments. The epithelial-like tight junctions, asymmetric transporter distribution, and expression of immune mediators contribute to the unique endothelial phenotype observed at the BBB. In order to characterize the BBB in normal brain, two main genomics strategies have been employed. In the first, SSH was applied to distinguish BBB attributes that are not present in the liver or kidney (4-6). In other words, these studies were designed to determine the differentially expressed genes that confer BBB phenotype. The second, more recent study, employed SAGE technology for a comprehensive view of gene expression at the BBB (7). As a stand-alone investigation, the SAGE analysis does not unearth BBB characteristics that are unique, but instead generates a complete BBB transcriptome. These two approaches are complementary, have contributed significantly to the current understanding of the BBB, and are discussed in detail below.

2.1. Sources of mRNA for BBB Genomics

In order to appropriately study the BBB transcriptome, it is important to generate the mRNA directly from isolated brain capillaries. Since the brain vasculature comprises only about 0.1% of the total brain volume (16),

molecular level analyses would likely miss all but the most highly expressed BBB transcripts if whole brain tissue were used. As an approximation by volume, a BBB transcript would be diluted to 1 in 1000 if whole brain tissue were used for genomics approaches. However, microarray sensitivity is only on the order of 1 in 10,000 (1), so the approach would be biased to only those BBB genes that are highly expressed. Although several studies have applied genomic/proteomic methods to whole brain tissue and certain highly expressed BBB transcripts are identified, only those studies that focus primarily on the brain capillary component will be addressed in this chapter.

Because of a robust basement membrane, brain capillaries can be readily separated and purified from surrounding brain tissue using a combination of mechanical homogenization, density centrifugation, and size fractionation techniques. The purified "capillary" product does contain some larger pre-capillary arterioles and venules, and these contributions will be present in the genomic analysis. In addition, the isolated vessels contain pericytes that share a basement membrane with the endothelial cells, and a low level of smooth muscle cells will also be present (Fig. 1) (4). For the genomics approaches described in this section, the data contain contributions from each of these sources in addition to capillary endothelial cells. Since multicellular contributions are also important for neurovascular unit function, their presence simply adds to the scope of the data. Alternative isolation techniques do have the potential to generate a more pure preparation of endothelial cells, however the preparation is generated by enzymatic means ex vivo at physiological temperature (17). This leads to changes in cellular mRNA content, and thus these mRNA samples may not be fully representative of the in vivo situation. In contrast, the mechanical isolation procedure can be performed under refrigeration, increasing the likelihood



Figure 1 Bovine brain capillaries isolated by mechanical homogenization techniques. (A) Isolated bovine brain capillaries stained with o-Toluidine blue. (B) Isolated bovine brain capillaries probed immunocytochemically for smooth muscle actin. Note the pre-capillary arteriole staining of smooth muscle that abruptly ends when the capillaries are reached. *Arrowheads* indicate pericytes dotting the abluminal surface of the capillaries. Scale bars are 20 μ m.

that the mRNA is representative of the situation observed in vivo (18). Laser capture microdissection is another technique that can be used to extract in vivo-like capillary mRNA samples from frozen tissue sections. Due to technical constraints, these samples also contain additional cell types. In addition, one has to take into account the discrete nature of vessel sampling as it is only reasonable to isolate a small number of vessel fragments (~100) with this method. Laser capture methods have been applied to proteomic analysis and will be described later in this chapter.

2.2. Genomics Methods

Once an in vivo-like mRNA sample has been generated from the freshly isolated capillaries, it is suitable for gene microarrays, SSH, and SAGE methods. Gene microarrays can have large transcriptome coverage of around 10,000 genes, but the human genome project has generated conservative estimates of 30-40,000 human transcripts (19,20). Thus, one is limited by whether or not a gene is included in the pre-printed array. On the other hand, SSH analysis relies on solution phase hybridization kinetics between two mRNA populations to selectively enrich for tissue-specific gene expression (3). In this way, SSH generates a *differential* gene expression profile without prior knowledge of gene identity. Thus, any gene expressed in the tissue could be isolated by this method regardless of its novelty. In addition, SSH is well suited for the analysis of low abundance transcripts-like transcription factors (3). Similar to the SSH technology, the SAGE method does not rely on prior knowledge of the gene products of interest. Instead, the SAGE process involves the comprehensive sequencing of small gene tags that allow for transcript identification (2). Unlike microarray and SSH, the result of the SAGE process is a complete catalog of gene expression in the given tissue or cell type. Each genomics approach has its inherent advantages and disadvantages. However, the first BBB studies utilized SSH and SAGE technology to analyze the molecular constituents of the BBB. The main benefit of these analyses was that many BBB-specific gene targets encoding proteins with unknown function were identified (4–7). Thus, when trying to profile the differences in a tissue to gain insights about its specialized origins, it was particularly advantageous not to limit the investigations to pre-printed microarrays.

2.3. Differentially Expressed BBB Genes by SSH Analysis

In order to determine the genes that have differentially elevated expression levels at the BBB with respect to peripheral tissue, a BBB genomics program was initiated using SSH technology. The goal of these experiments was to elucidate molecular level determinants of BBB phenotype by comparing mRNA expression profiles of freshly isolated brain capillaries to those found in kidney and liver tissues. Transcripts extracted from the brain capillaries were subtracted with transcripts isolated from kidney and liver tissue to remove commonly expressed transcripts. This generated pools of differentially expressed transcripts present in human (4) and rat (5,6) BBB, but not liver or kidney. The results of these three studies are compiled in Table 1, and illustrate the rich functional diversity of the BBB. Transcripts have been categorized according to the putative functions of their protein products, and since many of the proteins have multiple functions or are involved in several pathways, the ordering is by no means unique. The data suggest that the BBB has an important role in development, immune response, molecular transport and establishment of a tight barrier.

The data implicate the BBB as a regulator of brain plasticity, given that BBB-enriched expression of growth factors and antiproliferative genes was observed. The Heparin affinity regulatory peptide (HARP) can promote neurite outgrowth and angiogenesis (21), while FGF-19 is up-regulated in fetal brain and exhibits angiogenic and neurotrophic effects (22). Insulinlike growth factor binding-protein-3 is a carrier for insulin-like growth factors (23), and insulin-like growth factor 2 was determined to be enriched at the BBB. In situ hybridization methods have indicated preferential expression of IGF-2 at the BBB (5) and choroid plexus (24). Osteonectin can inhibit angiogenesis through sequestration of vascular endothelial growth factor (25), and PC3 is induced by the p53 protein and can elicit antiproliferative effects (26). The differentially expressed genes also reaffirm the presence of unique barrier properties. Endothelial cell-selective adhesion molecule is localized to tight junctions (27) and in one of the rat SSH studies was found to have enhanced expression at the BBB. In addition, claudin5 helps to regulate paracellular transport by its presence in tight junctions (28), and lack of this protein in knockout mice led to increased BBB permeability that was size dependent (29). Finally, the presence of tight junctions that regulate paracellular transport demands the existence of a broad array of molecular transport systems to supply nutrients to the brain and regulate ion homeostasis. Enriched expression of transport systems that shuttle a variety of substrates including energy (MCT1), organic anions (oatp2), proteins (TfR), and sodium and potassium ions (Na, K ATPase) was observed. Caveolin- 1α , also determined to be enriched at the BBB, is a key component of caveolae that function in endocytotic trafficking (30). The BBB-specific anion transporter type 1 (BSAT1) was isolated with a very high frequency in the two rat studies and was subsequently identified to be the 14th member of the organic anion transporter family (oatp14). Protein expression of oatp14 was then confirmed to be enriched in brain endothelial cell membranes (31). Taken together, these studies have begun to generate the global differential gene expression profile that defines the characteristics of the BBB in vivo.

Blood–Brain Barrier Genomics and Proteomics

Gene functional category	H^{a}	R1 ^b	R2 ^c
Growth factors			
Platelet-derived growth factor receptor β , PDGF-R β	_	_	Х
Insulin-like growth factor 2, IGF-2	_	Х	
Fibroblast growth factor 19, FGF19	Х	_	
Heparin affinity regulatory peptide, HARP	Х		
Insulin-like growth factor binding protein 3, IGF-BP-3	Х		
Tomoregulin	Х		
Signal transduction			
HERC2	Х	_	
Grb-2 associated binder-2, Gab2	Х	_	
AU-rich RNA binding factor, LaAUF-1	Х		
G-Protein signaling regulator-5, Rgs5		Х	Х
Prostaglandin D synthase, Ptdgs			Х
S100 calcium binding protein		Х	
Transport			
Caveolin-1a			Х
Organic anion transporting peptide type 2, oatp2		Х	Х
Na, K ATPase $\alpha 2$	Х	_	Х
Monocarboxylate transporter 1, MCT1	Х	—	_
BBB-specific anion transporter type 1, BSAT1 (oatp14)	—	Х	Х
Cationic amino acid transporter 1, CAT1		_	Х
FXYD domain-containing ion transport regulator 5, FXYD5	—	—	Х
Transferrin receptor, TfR		Х	
Immunology			
Major histocompatibility complex I. MHCI		Х	
Podocalyxin-like protein	Х	_	
Membrane cofactor protein, CD46 ^d		_	
52 kDa ribonucleoprotein, Ro52 ^e		_	
Platelet/endothelial cell adhesion protein, PECAM-1		_	Х
Tight junctions/extracellular matrix			
Endothelial cell-selective adhesion molecule, ESAM	_	_	Х
Claudin5	Х	_	_
Prominin			Х
Serglycin	Х		
Lutheran ^f	_	_	_
Transcription factors			
ElonginA	_	_	Х
Vascular endothelial cell-specific protein 14			Х
EZH1		Х	
ІкВ		Х	

(Continued)

Gene functional category	H^{a}	R1 ^b	R2 ^c
Human homolog of yeast SW12 transcription factor	_	Х	_
B-cell translocation gene-2	Х	Х	
Secretion			
Carboxypeptidase E, Cpe		Х	Х
Secretory granule proteoglycan core protein precursor, Pgsg	—		Х
Amyloid			
Amyloid precursor-like protein 2, APLP2			Х
Sperm membrane protein related to A4 amyloid protein, YWK-II	—	_	Х
Integral membrane protein 2a, Itm2a	Х	—	Х
Hemostasis			
Factor 8	—	—	Х
Serine protease inhibitor 4, Spi4	—	—	X
Tissue plasminogen activator, tPA	—	—	Х
Myelin			
Myelin basic protein, MBP	—	Х	Х
Protein zero-related protein 1, PZR related	—	—	Х
Proteolipid protein 1, PLP-1 Lipids			Х
Phospholipid transfer protein, PLTP	_	_	Х
Paraoxonase	Х	_	
α2-Macroglobulin	Х		
Stearoyl-CoA desaturase 2		_	Х
Vascular remodeling			
Vascular endothelial growth factor receptor type 1, Flt-1	_	Х	Х
Hypoxia inducible factor 2α , HIF- 2α	_	—	Х
Osteonectin	Х	—	
Vascular endothelial receptor-type protein tyrosine phosphatase, VE-PTP		—	Х
Cytoskeleton			
Regulatory myosin light chain isoform C, MLC20			Х
Connexin-45	_	Х	_
Utrophin	_	Х	_
β-Actin	Х		

 Table 1
 Functional Grouping of BBB-Enriched Genes Resulting from SSH

 Studies (Continued)
 Functional Grouping of BBB-Enriched Genes Resulting from SSH

^aH, human SSH study, data compiled from Ref. 4.

^bR1, rat SSH study 1, data compiled from Ref. 5.

^cR2, rat SSH study 2, data compiled from Ref. 6.

^dBovine proteomic study (41).

^eBovine proteomic study (46).

^fBovine proteomic study (11).

2.4. A Comprehensive BBB Transcriptome by SAGE Analysis

Whereas differentially expressed BBB transcripts were identified in the SSH studies, a recent investigation attempted to generate a full transcriptome view of the BBB using the SAGE technology. Messenger RNA was generated from freshly isolated rat brain capillaries, and the SAGE process resulted in the sequencing of nearly 80,000 gene tags. Of these 80,000 genes expressed at the BBB, approximately 11,000 were unique, and only 17% of the BBB-expressed genes encoded proteins with known function (7). When the SAGE-generated BBB transcriptome was compared with publicly available SAGE libraries for cortex and hippocampus, it was found that nearly 700 genes were enriched in microvessels. These enriched genes were clustered into functional groups of transporters (11%), receptors (10%), vesicle trafficking (7%), structural proteins (12%), and signal transduction pathways (18%). It is important to note that although the SAGE method generates a more comprehensive view of the BBB transcriptome than SSH, it requires the availability of SAGE libraries from other tissues to perform differential profiling. However, SAGE libraries are consistently being generated and a significant amount of differential analysis will soon become possible.

2.5. Microarray Analysis of BBB Attributes

Gene microarrays have also been used in an attempt to determine the factors governing BBB phenotype. In vitro gene expression profiles of human brain endothelial cells were compared to those generated from HUVEC cells by gene microarray (32). Thirty-five genes were preferentially expressed in the human brain endothelial cells that appear crucial in conferring BBB phenotype. The preferentially expressed genes included vasculogenic factors, immunoregulators, and growth factors (32). In a related study, the effects of flow-generated shear stress on in vitro brain endothelial cultures were monitored by gene microarray. By comparing static and dynamic in vitro models, it was concluded that flow induces cytoskeletal genes and can contribute to the antioxidant capacity of endothelial cells (33). Other studies have used gene microarray to address disease phenotypes of the BBB, and these will be covered in the final section of this chapter.

2.6. Future Directions of BBB Genomics

The BBB transcriptome and list of differentially expressed BBB genes have many potential uses. First, as is evident from the data presented above, gene expression profiles that mediate unique BBB characteristics were generated. These data sets give BBB researchers a basis for future experiments. Example research directions include in depth investigation of the BBB role in brain development, elucidation of the molecular architecture of tight junctions, and determination of the molecular transporter network. Next, nearly half of the genes identified in the SSH studies, and an even larger percentage of the genes extracted in the SAGE screen, encode genes with no known function (4–7). Thus, a large number of BBB attributes are unknown and need to be elucidated.

In this vein, a particularly intriguing hypothesis can be formulated regarding BBB transport proteins. Given the impermeable nature of the BBB, one might expect an overabundance of molecular transport systems to regulate the bi-directional flow of substrates between blood and brain. Indeed, the SSH and SAGE studies indicated that between 10% and 15%of the known genes encoded transporters (4-7). This contrasts markedly with the predictions that 3% of the total human genome encode transporters (19), and suggests that a disproportionate number of the genes encoding proteins with unknown function may actually be molecular transporters. As an example, the BSAT1 transporter was first identified to be present at the BBB in the SSH screens. Subsequent experimentation indicated that it was in the organic anion transporter family and the hormone thyroxine was a substrate (31). Of course, one of the most challenging aspects of genomics analyses is unearthing the function of the proteins encoded by these novel genes. Therefore, any technological breakthroughs for the assignment of protein function would be significant and would add to the power of genomics approaches.

The SSH studies described above resulted in sequencing of only a small percentage of the differentially expressed library. Continued sequencing of the differentially expressed BBB clones is warranted, and will likely contribute to added understanding of BBB form and function. In addition, the continued generation of SAGE databases will allow in silico differential gene profiling. Such computer database-driven strategies have already been used to clone novel endothelial-specific genes (34). However, since the databases are all generated using different experimental approaches, some discrepancies can result and care should be taken (35). This emphasizes the point that genomics techniques can generate scientific leads, but detailed biochemical investigation is still required for validation. The SSH and SAGE methods can also be applied to disease targets. These studies will be described in the last section of the chapter.

3. MOLECULAR PROFILING OF THE BBB USING PROTEOMICS

Protein expression profiling is a necessary complement to gene expression profiling if full understanding of the BBB is desired. As described earlier, membrane proteins comprise a particularly important subset of BBB proteins since the BBB endothelial cell is a physical barrier separating the blood and brain. Thus, molecular and cellular communication mediated by mem-

brane proteins will be a significant contributor to overall BBB function. The current gold standard of proteomic technologies is two-dimensional gel electrophoresis coupled to mass spectrometry. Unfortunately, membrane proteins are underrepresented when using this methodology because of solubility problems inherent to the hydrophobic, lipid bilayer-spanning segments of membrane proteins (10). In addition, many membrane proteins are glycosylated and result in diffuse banding in gel electrophoresis. A potential alternative to gel-based methods includes probing membrane proteins in a cellular context with a tissue-specific antiserum. In this way, proteins that are differentially expressed in a given tissue can be readily identified. Subtractive expression cloning using phage display has been used in this capacity (36). A cDNA library generated from a tissue of interest is expressed on the surface of a phage particle. This library of proteins is then probed with a tissue-specific antiserum in order to identify differentially expressed proteins. Although this technique can be successful for soluble proteins, applicability to membrane proteins is unlikely. This is due to the fact that the protein of interest is expressed as a fusion to a solvent accessible phage protein that may not be adequate for displaying hydrophobic regions of membrane proteins.

In addition, phage utilize the folding machinery of their bacterial host, and that is not always sufficient for high fidelity processing of mammalian proteins. Thus, there is a low likelihood that a properly folded mammalian membrane protein could be generated on the surface of a phage particle. A new methodology called subtractive antibody expression cloning has been developed to overcome some of these obstacles (11). This method was initially applied to analyzing the membrane proteome of the BBB and is described in the next section.

3.1. Subtractive Antibody Expression Cloning for BBB Membrane Proteomics

This novel proteomic method combines techniques of traditional expression cloning with a component of the phage display technique, the tissue-specific polyclonal antiserum. In a normal expression cloning strategy, a cDNA library is expressed in a surrogate cell line and the collection of cells is probed with an antibody or substrate for a known target. In this way, it is possible to clone a full-length cDNA for the protein of interest. However, when performing BBB proteomics, the target genes are not known a priori and expression cloning in its classic form is of little utility. Therefore, the newly developed subtractive antibody expression cloning method combines expression-cloning strategies with a tissue-specific antiserum. In addition, unlike the phage display technology described above, the proteins are expressed in the native membrane context of a mammalian cell, making the method amenable to identification of differentially expressed membrane proteins (11).



Figure 2 Characteristics of BBB membrane protein-specific antiserum. (A) Isolated bovine brain capillaries stained with o-Toluidine blue. (B) Isolated bovine brain capillaries probed with the BBB-specific polyclonal antiserum. The antiserum was pre-adsorbed with liver and kidney acetone powders. Note the continuous staining indicative of antigens with endothelial membrane origin, and the paucity of pericyte staining (*arrows*). (C) Western blot of total capillary protein probed with (1) BBB-specific polyclonal antiserum, or (2) pre-immune control serum.

First, a polyclonal antiserum was raised against purified bovine brain microvessel plasma membranes (Fig. 2) (37). In order to identify membrane proteins that were specifically expressed at the BBB, the antiserum was depleted of antibodies recognizing common antigens also found in the liver and kidney. This was accomplished by adsorbing the antiserum with kidney and liver acetone powders, and the depleted antiserum was used in subsequent steps. The subtracted antiserum specifically recognizes a host of BBB proteins (Fig. 2C) that are differentially expressed compared to the kidney and liver. In parallel, a bovine cDNA library generated from freshly isolated capillaries was expressed in mammalian COS-1 cells (Fig. 3). Thus, in principle, all membrane proteins normally expressed at the BBB were now being expressed in the COS-1 surrogate. Since a mammalian host was used, membrane protein solubility and glycosylation complications that hinder other proteomic analyses are minimized. Next, monolayers of BBB cDNA library-transfected COS-1 cells were probed with the subtracted BBB membrane-specific antiserum to identify those cells that harbor BBB-specific membrane proteins. These cells were then extracted from monolayer and plasmid DNA recovered. After several rounds of enrichment, purified BBB-specific clones were isolated (Fig. 3).

The subtractive antibody expression cloning method was validated by the recovery of three membrane proteins that exhibit enriched BBB expression compared with that found in the liver and kidney. The first protein that was extracted was the Lutheran membrane glycoprotein (11). This protein functions in basal cell adhesion (38) is a laminin receptor (39), and was



Figure 3 Flowchart of subtractive antibody expression cloning strategy. *Lower right panel*: Cells of a transfected monolayer were probed with BBB-specific antiserum.

previously shown to be a BBB marker in brain tissue (40). In addition, it is specifically expressed at the BBB compared to the liver and kidney (40). Next, the regulator of complement activation, CD46, was cloned using this technique, and confocal microscopy demonstrated significant expression of this protein at the BBB (41). The CD46 has also been shown to bind the measles virus (42) and could therefore potentially act in measles virus entry into the brain parenchyma (43). Finally, Ro52, an autoantigen in Sjogren's syndrome (44) and systemic lupus erythematosus (45) was identified as BBB-enriched (46). Both of these diseases can have CNS involvement and preferential BBB expression of Ro52 may indicate BBB participation in autoimmune responses.

Recovery of three BBB-enriched membrane proteins illustrates the utility of the subtractive antibody expression cloning methodology. These proteins would not likely be identified using any of the other proteomic analyses described above. However, the subtracted antiserum recognizes many other proteins that have yet to be cloned (Fig. 2C) and continued cloning of BBB membrane proteins is necessary to fully deconvolute molecular characteristics of the BBB.

3.2. ICAT for BBB Proteomics

Isotope-coded affinity tag (ICAT) technology is useful for differential proteomic profiling. When using the ICAT methodology, two independent protein samples are labeled with isotopically distinct tagging reagents. After proteolytic digest, the proteins are analyzed by mass spectrometry (12). The slight differences in tag molecular weight allow resolution of mixed protein samples by mass spectrometry, and the peak size gives quantitative information about differences in protein expression levels. The technique most commonly requires attachment of the tagging ligand to cysteine residues, and

this somewhat limits the applicability of ICAT because not all proteins or proteolytic fragments have cysteine residues. However, reagents that allow tagging via other amino acids or sites of post-translational modification are rapidly being developed (47). In addition, this technique can allow for membrane protein profiling because the solvent mixtures required for the isotope tagging reaction are also compatible with membrane protein solubilization (48).

Very recently, the ICAT technique has been applied to BBB research to analyze differential protein expression in two disease states, cerebral ischemia (14,15) and Alzheimer's disease (13). Haggani and co-workers have examined the protein expression profiles of brain endothelial cells in response to ischemia both in vitro and in vivo. Rat and human brain endothelial cells were cultured in vitro and subjected to ischemia (14). Both two-dimensional electrophoresis and ICAT were used to identify nearly 200 proteins that were up or down-regulated by at least 2-fold. Proteins such as antioxidative enzymes and membrane transporters were represented in the list of differentially regulated proteins. This study was then extended to an in vivo rat stroke model (15). After inducing stroke by carotid artery clamping, animals were allowed to recover for 1 to 24 hours. Brain tissue was isolated, and vessels recovered using laser capture microdissection. Then ICAT was used to profile the protein expression changes from the captured vessels because the total protein necessary for the ICAT method is significantly less than that needed for gel methods. Results of this experiment indicated changes in protein expression profiles that were consistent with the physiological and pathological changes observed after incidence of stroke. In a similar study with Alzheimer's disease brain vessels, ICAT was used to compare laser captured Alzheimer's vessels to non-demented controls (13). The study was successful in identifying several proteins that were up-regulated (tubulin β-1 chain, CD27BP, RNF18) and down-regulated (RGS8, IL-6R, fibringen γ -A chain precursor). Although each of these three studies is preliminary in nature, they illustrate the potential of using ICAT and laser capture microdissection to analyze differences in protein expression upon pathological insult.

3.3. Future Directions of BBB Proteomics

The use of proteomics to profile the brain vasculature is admittedly in its early stages. However, it is an important component in understanding BBB function in both normal and diseased brain. Several new proteomic approaches are being refined and applied to the BBB. This includes the continued cloning of membrane proteins using the subtractive antibody expression cloning methods. The laser capture and ICAT combination for analysis of differential protein expression is in its nascent stages, but early returns are very promising. Comparison of the BBB protein expression profile to the peripheral vasculature using ICAT is also an intriguing possibility. Finally, the BBB would be an excellent system for another recently described proteomic technique targeted to membrane proteins. This technology employs custom membrane-solubilizing solvent conditions followed by proteolytic digest and mass spectrometry (49). This technique could be especially powerful for BBB research since it has the ability to resolve membrane protein topology as a consequence of sequential steps that selectively process extracellular regions.

4. GENOMIC AND PROTEOMIC ANALYSIS OF BBB INVOLVEMENT IN NEUROLOGICAL DISEASE

The earlier portions of this chapter were primarily focused on profiling the BBB under normal conditions. However, genomics and proteomics techniques have also been applied to analyzing changes in BBB expression profiles during disease. These methods have the potential to identify single molecules or cellular pathways that undergo altered expression behavior, and can serve as the template for subsequent analysis targeted to these pathways. As mentioned earlier, many studies have used genomics and proteomics methods to profile whole brain tissue, but this section will focus only on those studies having isolated capillaries or endothelial cell culture as source tissues.

The BBB responds to tumor necrosis factor alpha (TNF α) and this response has been demonstrated to affect BBB permeability. Using gene microarray and two-dimensional electrophoresis techniques, the effects of TNF α on human cerebral endothelial cells were evaluated (50). The study identified differential expression of apoptosis, cell adhesion, and chemotaxis genes. When HUVEC cells were profiled under the same conditions, many responses were similar, but several TNF α responses were clearly brain endothelial cell specific. This reinforces the importance of using appropriate cell sources for genomic/proteomic approaches.

Another study indicated that glioblastoma cells with mutant tumor suppressor protein p53 exhibited no change in VEGF expression upon radiation treatment, whereas tumor cells with wild-type p53 exhibited increased VEGF production (51). Both glioblastoma cell types were co-cultured with HUVEC cells, and it was discovered that HUVEC cells subject to glioblastoma cells expressing mutant p53 were much more sensitive to radiation treatment. Thus, it was suggested that altered intercellular communication could lead to altered endothelial cell response, and this effect was dependent on the p53 status of tumor cells. More evidence that the BBB is involved in immune mechanisms was generated by microarray analysis of endothelial cells that were cultured in the presence of group B *streptococcus* (52). The bacteria induced chemokine (Gro α and Gro β), interleukin (IL-6 and IL-8), and adhesion molecule (ICAM-1) expression due to an inflammatory response mediated by the bacterial B-hemolysin/cytolysin toxin.

Microarray profiling of epileptic vessels compared with normal brain vessels indicated that several classes of genes could be responsible for the resistance to epileptic drugs (53). Over-expression of MRP1, MRP2, MRP5, and cisplatin resistance protein was observed in the vessels of epileptic tissue helping to explain the drug-resistant epileptic phenotype. In addition, stroke can effect BBB permeability properties and lead to cerebral edema. In order to define the molecular signature of stroke-prone spontaneously hypertensive rats, SSH was used to compare brain capillary gene expression profiles from these rats to those found in brain capillaries of stroke-resistant spontaneously hypertensive rats (54). It was determined that the rat sulfonylurea receptor 2B was up-regulated in stroke prone rats while at the same time, G-protein signaling 5 regulator was down-regulated. These results show that genomics and proteomics can be applied to the BBB in pathological conditions, and can generate insight into the immune, barrier, and transport statuses of the BBB.

5. PERSPECTIVES

Global genomics and proteomics techniques are of great utility for BBB researchers and should be incorporated where possible to extend scientific understanding. Genomics analyses are beginning to be used routinely in the BBB field, and several researchers are creating and applying specialized proteomics analyses to BBB research. Armed with this technical tool set, researchers will continue to redefine what makes the BBB equipped to both protect and supply the CNS. These techniques also have the power to clarify disease mechanisms, and thus identify therapeutic targets. In addition, genomics and proteomics could assist in drug delivery efforts by identifying novel BBB-specific transport systems that could function as conduits for non-invasive drug delivery. Finally, although most studies described in this chapter focused solely on the brain endothelium, the BBB research community is now poised to evaluate the endothelial cell–perivascular cell interactions that truly define the neurovascular unit.

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3

Tight Junctions of the Blood–Brain Barrier

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1. TIGHT JUNCTIONS

1.1. Introduction

Multicellular organisms are primarily required to establish a distinct internal environment to maintain homeostasis. As a result, all internal and external surfaces of organs, such as skin, stomach, and intestines, are covered with various kinds of epithelia. In order to work efficiently as a barrier, intercellular spaces of these epithelial sheets are strictly sealed by junctional complexes. Likewise, microvascular-associated brain endothelial cells are linked by intercellular junctions. The main structures responsible for the barrier properties are tight junctions (1,2). These intercellular structures are located at the most apical section of the plasma membrane of adjacent cells (Fig. 1A), whereas adherens junctions are found in the basal part of the cell membrane. Tight junctions also act as an intramembrane fence that prevents the intermixing of apical and basolateral lipids in the exocytoplasmic leaflet of the plasma membrane. Points of cell-cell contact are sites where integral proteins of two adjacent membranes meet within the cellular space (Fig. 1B). The adjoining membranes make contact at intermittent points, rather than being fused over a large surface area. The integrity/permeability of tight junctions can be assessed by transendothelial electric resistance



Figure 1 Morphology of tight junctions. (A) Electron micrograph of a section through the apical region of two adjacent epithelial cells. The tight junction is located at the apical part of the plasma cell membrane. (Courtesy of Daniel S. Friend) (B) Schematic diagram of tight junctions.

(TEER) measurements. In this chapter we describe the molecular composition of tight junctions and their role in several intercellular signaling pathways and leukocyte migration.

1.2. Structure

The main transmembrane molecules mediating intercellular contacts are occludin and the endothelial claudin family. These proteins bind to the cytoskeleton via the zonula occludens-1, -2, and -3 complexs (ZO-1, ZO-2, and ZO-3) (Fig. 2).



Figure 2 Schematic representation of endothelial tight junction- and adherens junction- associated proteins and their linkage to the cytoskeleton. *Abbreviations:* EC, endothelial cell; JAM, junctional adhesion molecule; ZO, zonula occludens; PECAM, platelet endothelial cell adhesion molecule.

1.2.1. Transmembrane Proteins

Tight junctions consist of at least three types of transmembrane proteins, including occludin (3), claudins (4,5), and members of the CTX family (cortical thymocyte marker in Xenopus) (6) of the immunoglobulin superfamily: junctional adhesion molecules (JAMs) (7,8), coxsackievirus and adenovirus receptor (CAR) (9), and endothelial cell-selective adhesion molecule (ESAM) (10,11).

Occludin was the first tight junctional transmembrane molecule to be identified (3). It is a 65 kDa transmembrane phosphoprotein that spans the membrane four times (12). Both the amino (NH2) and carboxy (COOH) terminus are located intracellularly and the two extracellular loops of occludin comprise 45 amino acids. Tyrosine and glycine residues are abundant in the first loop, indicating a role of this domain in cell-cell adhesion (13). The carboxy-terminal cytoplasmic tail of occludin directly forms a complex with ZO-1, ZO-2, and ZO-3, which on its turn is linked to the actin cytoskeleton (14,15). Occludin is ubiquitously expressed at tight junctions in epithelial and endothelial cells and its expression correlates with its barrier function. Occludin-deficient mice (16) still had the capability to develop normal tight junction strands, and no differences were measured in transendothelial electric resistance compared to wild-type mice. These findings implicate that occludin is not required to maintain the structural integrity of tight junctions, however histological abnormalities were observed in occludindeficient mice, suggesting that the role of occludin is more complex than previously proposed. Occludin physically interacts with a variety of structural proteins at the tight junction and may therefore regulate a wide array of signaling pathways.
Claudins are essential components for the formation of tight junctions by their homophilic and heterophilic binding to adjacent cells (4,17). Twenty-four types of claudins have been identified so far and they all share similar membrane topology with occludin, including two extracellular loops and four transmembrane domains. However, they are considerably smaller with 22 kDa and share no sequence homology (4,5,18). Some members of the claudin family are expected to form extracellular aqueous pores in paracellular spaces (19). Claudin-1, -3, -5, and -15 are thus far the only claudins detected in mammalian endothelial cells (20–23). Claudin-3 and -5 are particularly expressed in brain capillary endothelial cells and not in epithelial cells (20.23). Recent findings point to a promoter function of claudin-5 in pro-matrix metalloproteinase (MMP)-2 activation by membrane-type MMP (MT-MMP), suggesting an important role in vessel permeability and angiogenesis (24). Specific claudin-knock out models and genetic disorders demonstrate the crucial role of claudins in the formation of tight junctions (25).

JAM-A, -B, -C, and -D, according to the nomenclature of the JAM family proposed by Bazzoni (26), together with CAR and ESAM are also localized within the tight junction region. These members of the CTX family consist of extracellular variable (V-type) and constant (C2-type) immunoglobulin domains, a single transmembrane region and a cytoplasmic tail (6). To date, four distinct JAM molecules have been identified, with JAM-A being the most extensively characterized. JAM-A is expressed in endothelial and epithelial cells (7) and codistributes with tight junction components at the apical region of the junction (27). It has been shown that the carboxyterminal cytoplasmic tail of JAM-A interacts with the guanylate kinase and/or the acidic domain of occludin, the PDZ [Postsynaptic density protein (PSD95), the Drosophila tumor suppressor dlg-A, and ZO-1] domain of ZO-1 and the amino-terminal globular head of cingulin (27). PDZ domains are protein-protein interaction modules that recognize motifs of three amino acids at the carboxy-terminus of transmembrane proteins. It has also been suggested that JAM-A plays a role in the generation of cell polarity. Ebnet et al. (28) have recently shown that the cell polarity protein PAR-3 directly associates with JAM and together with the atypical protein kinase C (PKC) is crucial for tight junction formation. In contrast to JAM-A, JAM-B, -C, and -D (8,29,30) are only observed in endothelial cell-associated tight junctions and not in epithelial cells. JAM-molecules mediate homophilic and probably also heterophilic interactions in the tight junction region and are thought to be involved in the organization of the tight junction structure (31) and modulation of leukocyte extravasation (7, 29).

ESAM (10,11) and CAR (9) are also components of tight junctions. The cytoplasmic domain of ESAM is homologous to CAR, but longer than the cytoplasmic domain of JAM. Hirata et al. (10) have shown that ESAM is selectively expressed by cultured human and murine vascular endothelial cells and that it colocalizes with cadherins and catenins in cellcell junctions. The authors suggest that ESAM mediates cell-cell adhesion through homophilic interactions. Exogenous expression of CAR in epithelial cells increases the junctional barrier function of Chinese hamster ovary (CHO) cell monolayers, and soluble CAR inhibits the formation of tight junctions (9). Recently, a similar member of the CTX family was discovered. Coxsackie- and adenovirus receptor- Like Membrane Protein (CLMP) (32) is also localized to junctional complexes between endothelial and epithelial cells. It can mediate cell aggregation and can regulate transendothelial electric resistance across polarized epithelial cells, suggesting that CLMP is also actively involved in cell-cell adhesion.

Like JAM, other molecules (e.g., PECAM-1 and CD99) that are concentrated at the lateral borders of endothelial cells have been implicated in the process of transmigration. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a member of the immunoglobulin (Ig) superfamily and is concentrated at the apical domain of intercellular junctions (33). PECAM-1 is involved in cell-cell adhesion through either homophilic interaction (34,35) or heterophilic interaction (36), such as its interaction with β-Catenin (37). Neutralizing antibodies against PECAM-1 effectively inhibit paracellular movement of leukocytes (38). However, the exact function of PECAM-1 remains unclear as PECAM-1 knock out mice are viable and undergo normal vascular development (39), but their vascular permeability is altered (40). CD99 is a 32 kDa molecule that is expressed on the surface of hematopoetic cells and is concentrated at the borders between confluent endothelial cells. CD99 functions in a homophilic manner in transmigration. Blockade of CD99 on either leukocytes or endothelial cells blocks diapedesis of monocytes as well as blockade of CD99 on both cell types (41). Detailed information about leukocyte extravasation is given in Section 2, Chapters 9-11.

1.2.2. Cytoplasmic Proteins

The first tight junction-associated protein to be identified was the cytoplasmic 210–225 kDa protein ZO-1 (42). It has been shown that ZO-1 is present within tight junctions of epithelial cells (42) and endothelial cells (43,44). Several years later, ZO-2 and ZO-3 were identified and showed to form a submembranous plaque of tight junctions (45,46). ZO family members belong to the membrane-associated guanylate kinase (MAGUK) protein family and are likely involved in intracellular signaling (47). Gumbiner et al. (48) have shown that ZO-1 associates with ZO-2, which is a 160 kDa protein, related to ZO-1 (45) and is also found in brain endothelial cells (49). ZO proteins can form heterodimeric complexes with one another, e.g., ZO-1 interacts with the PDZ motifs of both ZO-2 and ZO-3 (46). These proteins belong to the MAGUK family and contain three PDZ domains, one SH3 domain and a guanylate kinase homology domain. This SH3 (sulfhydryl group; -SH) side-chain is only present in cysteine, glycine, or proline amino acids and can be oxidized into a disulfide bond with other cysteine, glycine, or proline residues. Both the SH3 domain and the PDZ regions are essential for the linkage of ZO proteins to the cytoskeleton. Furthermore, it has been shown that PDZ domains of ZO-1, ZO-2, and ZO-3 directly bind to the C-terminals of several claudins (50). In addition, the C-terminal cytoplasmic tail of occludin also directly interacts with the ZO protein complex (14,15). Several other PDZ-containing proteins have been identified in the tight junctional complex, such as membrane associated guanylate kinase-1, -2, and -3 (MAGI-1, MAGI-2, MAGI-3) (51), AF-6/ s-afadin (52), multi-PDZ domain protein 1 (MUPP1) (53), and protein associated to tight junctions (PATJ) (54). Hirabayashi et al. (30) reported the interaction of MAGI-1 with JAM-4, which may regulate epithelial permeability. Recently, Jeansonne et al. (55) have shown that MUPP1 interacts with claudin-8 in tight junctions on epithelial cells. The authors suggested that this interaction is involved in the tight junction barrier function. Over-expression of PATJ disrupted the tight junction localization of ZO-1 and ZO-3, suggesting that PATJ might be involved in regulating the integrity of tight junctions (54). Furthermore, other proteins lacking the PDZ domain are also recruited to tight junctions, including cingulin (140-160 kDa) (56) and 7H6 antigen (155 kDa) (57). Cingulin directly interacts with ZO-1 (58) and shows structural similarity to myosin, indicating that it may interact with actin filaments. 7H6 is a phosphoprotein and is thought to play a crucial role in the regulation of paracellular barrier function of both epithelial and endothelial cells (59). Additional proteins that localize at tight junctions such as rab 3B (60), rab 13 (61), and Sec 6/8 (62) may be involved in vesicle transport processes. However, these proteins have only been identified in epithelial cells, and it is yet unclear whether they are present in endothelial tight junctions. Several other proteins also play a role in tight junction regulation, including symplekin (63), the transcription regulator ZO-1 associated nucleic acid-binding protein (ZONAB) (64), huASH1 (65), Pilt (protein incorporated later into tight junctions) (66), PTEN phosphatase (67), junction-enriched and -associated protein (JEAP) (68), and protein phosphatase 2A (69). These cytoplasmic proteins are thought to play a role in tight junction signaling, which will be discussed in detail in paragraph 2.

2. REGULATION OF TIGHT JUNCTIONS

2.1. Tight Junction Signaling

Several cytoplasmic signaling molecules are involved in the assembly and disassembly of tight junctions (70). Molecules involved in virtually all

intracellular signaling pathways have been reported to affect paracellular permeability, including tyrosine kinases, protein kinase C (PKC), Ca^{2+} , calmodulin, heterotrimeric G proteins, cyclic adenosine monophosphate (cAMP), lipid second messengers, and phospholipase C (71,72). Here we describe the role of these signaling molecules in the regulation of tight junction signaling and the interactions of these molecules in tight junction biogenesis (summarized in Fig. 3).

Although signaling molecules are used to control assembly and disassembly of the junction, they may also correlate with changes in actin organization (73). It has been proposed by Madara (74) that contraction of peri-junctional actin filaments and the resulting centrifugal traction on tight junction membranes regulates tight junction permeability.

PKC is the major regulator of tight junction formation and regulation (75). It plays an important role in ZO-1 migration to the plasma membrane and there are 34 PKC phosphorylation consensus sequences in the ZO-1 protein, suggesting that ZO proteins serve as a scaffold for PKC signal transduction pathways on the cytoplasmic surface of intercellular junctions (76). Several studies have shown that ZO-1 can be directly phosphorylated (75,77), however, the implications for tight junction maintenance and biogenesis are unknown. Although the kinase(s) responsible for phosphorylating ZO-1 have not been identified, a serine protein kinase has been partially characterized that selectively interacts with the SH3 domain of ZO-1 and is associated with the junctional complexes extracted from Madin-Darby canine kidney cells (MDCK) (78). Another study demonstrates that activation of PKC causes disruption of tight junctions through activation of mitogen-activated protein (MAP) kinases (79). The authors suggested that the MAP kinase signaling pathway plays a key role in the regulation of the tight junction barrier function. Two atypical PKC isotypes, PKCE and PKC, and their specific binding protein, atypical PKC isotypespecific interacting protein (ASIP) are concentrated at tight junctions and play an important role in the establishment of cell polarity, which is fundamental for the fence function of tight junctions.

Both extracellular and intracellular Ca^{2+} regulates tight junction activity. When extracellular Ca^{2+} is removed, there is a concurrent decrease in electrical resistance across the membrane and an increase in permeability (80). Intracellular Ca^{2+} plays a role in cell–cell contact (81) and leads to an increased transendothelial electric resistance (82) and tight junction assembly (83). Furthermore, increase in intracellular free Ca^{2+} activates myosin light chain kinase (MLCK), which leads to unfolding of myosin II and endothelial-cell retraction that might facilitate leukocyte passage (84). Ca^{2+} acts together with a number of calcium-binding proteins. One of these calcium-binding proteins, calmodulin, may play a role in the barrier function of tight junctions, since calmodulin inhibitors (trifluoperazine and calmidazoline) inhibited transendothelial electric resistance (85).



Figure 3 Potential interactions of signaling molecules and pathways in tight junction biogenesis. Tight junction signaling can be initiated by G protein coupled receptors, receptor tyrosine kinases and/or cellular adhesion molecules (CAMs). This may lead to an activation (*black arrows*) or inhibition (*grey arrows*) of several signaling pathways by a variety of molecules, including G α subunits, small GTPases (Rho), and tyrosine kinases (src) or phosphatases. These signaling events might eventually lead to cytoskeletal rearrangements and tight junction protein translocations. As a result of these signaling events, the paracellular permeability of endothelial cells will be altered. *Abbreviations*: CAM, cellular adhesion molecule; cdc42, cell-division control protein 42; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/ extracellular regulated kinase; PLCK, myosin light chain kinase; P, phosphorylated; PAK, p21-activated kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; ROCK, Rho kinase.

Brain endothelial cells contain actin fibers located throughout the cytoplasm and are sensitive to changes in cAMP levels. After treatment with cAMP, stress fibers become less abundant and belts of filamentous actin sites of cell junctions become more apparent (86). These effects are reversible as cAMP returns to normal levels and junctional resistance decreases. The rate of resistance modulation by cAMP suggests that certain proteins are phosphorylated by protein kinase A (PKA). These phosphoproteins control tight junction properties, the strength of cell–cell adhesion, and regulate interactions between the plasma membrane and cytoskeleton (87).

Confocal microscopy studies have demonstrated that $G\alpha$ subunits (i2, i3, and l2) (88) are present at tight junctions. Additionally, the $G\alpha$ 12 subunit co-localized with PKC ξ at ZO-1 intercellular contacts (89), and its activation is involved in electrical resistance development across the membrane (88). Recently, a family of G-protein-regulating proteins has been identified. These so-called RGS proteins (regulators of G-protein signaling) interact with the $G\alpha$ subunit of heterotrimeric G-proteins. They are key elements inducing receptor desensitization by inactivating the $G\alpha$ subunit via their ability to accelerate GTP-hydrolysis (90).

Signaling molecules that directly control actin cytoskeleton organization are particularly intriguing with regard to tight junction function regulation. The family of Ras-related small GTP-binding proteins RhoA, Rac1, and cell-division control protein 42 (Cdc42) are regulators of the actin cytoskeleton (91). Microinjection of RhoA, Rac1, or Cdc42 induces stress fiber formation, membrane ruffling, and extension of filopodia in serum-starved Swiss 3T3 fibroblasts (92). Several studies have implicated RhoA GTPases in tight junction functions. Nusrat et al. (93) found that the organization of the peri-junctional actin cytoskeleton and ZO-1 was disrupted after exposing T84 intestinal epithelial cells to recombinant Clostridium botulinum exotoxin C3. This toxin ADP-ribosvlates Rho-family proteins and thereby disrupts their functions and subsequent tight junction organization (94,95). Takaishi et al. (96) examined MDCK cells constitutively expressing RhoA mutant genes, and reported no effect on tight junction morphology at steady state except during PKC-induced tight junction assembly in the presence of low extracellular calcium concentrations. To study the role of RhoA and Rac1 in the structural and functional organization of tight junctions, Jou and Nelson (97) developed RhoA and Rac1 mutants. These mutants were characterized by severe disorganization of tight junction strands and proteins (occludin, ZO-1, and actin) ultimately leading to an increased fence function of tight junctions. Interestingly, the GTPasedependent pathways regulating the intercellular permeability seem to operate in an opposite way in epithelial and endothelial cells. In cerebral endothelial cells, the activation of the Rho pathway in vitro by lysophosphatidic acid (LPA) (98) disrupted the paracellular barrier (49), whereas inhibition of the Rho pathway prevented LPA-induced hyperpermeability (99).

Alternatively, Rho might function as a regulator of myosin light chain kinase (MLCK), an enzyme which plays an important role in maintaining closure of the peri-junctional actin ring in epithelial tight junctions (100). Moreover, MLCK-mediated contraction of lung endothelial cells can be influenced by Rho proteins (101), potentially acting at the level of Rho kinase (ROCK) inhibition of myosin phosphatase activity. Other in vitro studies using human umbilical vein endothelial cells (HUVEC) demonstrated that the ROCK-dependent pathway is a central target for inflammatory agents like bacterial toxins to induce vascular permeability (102). Furthermore, Walsh et al. (103) have shown that ROCK inhibition in T84 intestinal epithelial cells induced reorganization of apical F-Actin structures and enhanced paracellular permeability, suggesting that ROCK regulates tight junction organization via its effects on the actin cytoskeleton.

Ras is a small G protein residing at the inner surface of the plasma membrane, and is involved in several signaling pathways. The only evidence that Ras may directly interact with tight junction-associated proteins came from a study of AF-6 (52). AF-6, which has been identified as a Ras target (104), can directly associate with ZO-1 at tight junctions. Activated Ras perturbed cell-cell contacts, inhibited the interaction between AF-6 and ZO-1, and decreased the AF-6 and ZO-1 expression at cell-cell contact sites (52). Therefore, it is possible that Ras regulates tight junction functioning via direct modulation of AF-6. Alternatively, Ras affects other intracellular signaling pathways (105), including Raf/MEK/ERK kinase cascade. Activation of PKC by phorbol ester treatment of pulmonary artery endothelial cells led to barrier dysfunction via sequential activation of Ras/ Raf/MEK signaling pathways followed by the activation of ERK (106). Furthermore, transfection of oncogenic Raf-1 into Pa-4 cells resulted in a complete loss of tight junction function and acquisition of a stratified phenotype that lacked cell-cell contact growth control (107). In addition, the expression levels of occludin and claudin-1 were down-regulated, and the distribution patterns of ZO-1 and E-Cadherin were altered. Together these findings demonstrate that the Ras/Raf/MEK/ERK signaling cascade plays an essential role in the regulation of endothelial tight junctions.

2.2. Tight Junction Proteins as Signaling Units?

Phosphorylation of both transmembrane and accessory proteins plays an important role in the establishment and regulation of tight junctions. Occludin and ZO-1 can be phosphorylated on Ser, Thr, and Tyr residues (108). Ser/Thr phosphorylation or dephosphorylation of occludin has been implicated in the biogenesis of tight junctions in MDCK cells (108) and Xenopus laevis embryos (109). In addition, Wong (110) has revealed that hyperphosphorylated forms of occludin played an important role in the functional assembly of tight junctions. Moreover, it has been shown that

after tight junction disruption, their re-assembly correlates with increased phosphorylation of occludin, particularly of Ser and Tyr residues (111). Inhibition of tyrosine phosphatases in tight junctions results in occludin proteolysis and increased permeability (112). Regulation of tight junctions is also dependent on tyrosine phosphorylation of other proteins at cell-cell contacts. Development of tight junction barrier functions has been correlated with decreased tyrosine phosphorylation of proteins at the tight junction complex (113). Furthermore, tyrosine phosphorylation of junctional proteins decreased after endothelial cell-cell contact and maturation of junctions (114). Several studies suggested that tyrosine phosphorylation was correlated with decreased transendothelial resistance and increased permeability (112).

Ras might function via multiple intracellular signaling pathways (105), including the MAP kinase (MAPK) cascade. Once activated, the last protein kinase in the cascade is translocated to the nucleus where it can phosphorylate and activate specific transcription factors. Chen et al. (115) have demonstrated that occludin, claudin-1, and ZO-1 were absent from cell–cell contacts but present in the cytoplasm of Ras-transformed MDCK cells. After inhibition of the MAPK pathway by specific inhibitors, occludin, claudin-1, and ZO-1 were recruited to the cell membrane and tight junctions were assembled. Inhibition of the MAPK pathway also resulted in tyrosine phosphorylation of occludin and ZO-1. Taken together, many studies have shown that tyrosine phosphorylation of tight junctions.

Several membrane-associated guanylate kinase-like proteins (GUKs) are involved in organizing signal transduction at tight junctions. In subconfluent epithelial cells, ZO-1, and symplekin are localized to the nucleus, suggesting that they might have a role in regulating transcription (116,117). Balda and Matter (64) have identified the transcription regulator ZO-1 associated nucleic acid-binding protein (ZONAB), which is localized to the nucleus and tight junctions. ZO-1 and ZONAB control endogenous ErbB-2 expression and regulate paracellular permeability indicating that ZO-1 is implicated in a signal transduction pathway that leads to specific gene expression. Furthermore, Traweger et al. (118) reported that ZO-2 localizes to the nucleus of highly migratory endothelial cells. This tight junction-associated protein can directly interact with the DNA-binding protein scaffold attachment factor-B (SAF-B), which is supposed to be involved in transcriptional regulation (118).

In conclusion, signal transduction cascades are involved in a multitude of tight junction functions, including biogenesis, establishment of cellular polarity and tight junction regulation.

2.3. Lipid Rafts

Over the past decade, several studies have addressed the presence of lateral asymmetric membrane lipid domains, also named detergent-insoluble glycolipid rafts (DIGs) (119,120). These cholesterol-enriched microdomains vary in size from extremely small and near the limit of biochemical and morphological resolution, to much larger sizes (121,122). Besides cholesterol, DIGs also contain (glyco)sphingolipids, specific membrane molecules and glycosylphosphatidyl-inositol (GPI)-anchored proteins. Some DIG-like rafts contain a 21–24 kDa scaffolding protein, caveolin-1. Caveolin-1 is distributed in the apical membrane of polarized MDCK cells as homo-oligomers, and in the basolateral membrane as hetero-oligomers with caveolin-2. DIG-like membrane compartments play a central role in many signaling pathways at the cell surface, since they are markedly enriched in a variety of signal transduction proteins (47,123–126). Non-receptor tyrosine kinases (e.g., src), which associate with membranes in their active state, also associate with DIG-like compartments. This is particularly interesting since src and its related tyrosine kinase yes and PKC are observed in the vicinity of tight junctions (60,71). Nusrat et al. (127) demonstrated that membrane domains that form the tight junction seal exhibit biophysical DIG-like properties, including the presence of caveolin-1, which co-localized with structural tight junction proteins. Furthermore, they found that disorganization of DIGs by cholesterol depletion was accompanied by dislocation of occludin and subsequent disassembly of tight junctions. These observations suggest that the junctional complex represents a unique signaling membrane microdomain that controls and influences fundamental properties of endothelial and epithelial cells. However, it remains unclear how these microdomains are structurally organized.

2.4. Leukocyte Migration and Tight Junction Integrity

Recruitment of leukocytes from the circulation into tissue parenchyma is a crucial event in the development of neuro-inflammatory diseases and the general principles governing leukocyte extravasation have been thoroughly documented. The process by which leukocytes exit the bloodstream and cross the endothelium into the underlying tissue is referred to as either extravasation, diapedesis, or transendothelial migration (TEM; addressed in Chapters 9–11). It is generally accepted that leukocytes transmigrate using a paracellular route between adjacent endothelial cells (128), either via the junctional complex or through tricellular corners in peripheral endothelial cells.

The barrier function of the blood-brain barrier (BBB) can change dramatically during various central nervous system (CNS) diseases, such as multiple sclerosis (MS). Increased BBB permeability might be the result of either opening of tight junctions or enhanced pinocytotic activity and formation of transendothelial channels (129). An in vivo model of CNS inflammation has been used to investigate leukocyte-mediated breakdown of the BBB and subsequent recruitment into the CNS (130,131). Juvenile rats, unlike adult rats, are highly susceptible to interleukin (IL)-1ß induction of BBB enhanced permeability, due to an acute polymorphonuclear cell (PMN)-dependent inflammatory response. IL-1ß injected into the striatum of juvenile rat brains resulted in enhanced expression of intercellular adhesion molecule-1 (ICAM-1) by the endothelium and adhesion of PMNs by a β 2-integrin-dependent interaction. This was paralleled by a large flux of PMNs across the endothelium (131). ICAM-1 is known to act as a binding molecule for circulating lymphocytes via leukocyte function antigen-1 (LFA-1), and is expressed on endothelial cells during inflammation (132). In areas with extensive PMN recruitment, an increase in staining for phosphotyrosine was observed in both PMNs and endothelial cells, suggesting activation of cell signaling events. Alterations in the structural components of the junctional complex were observed in parallel with leukocyte transmigration in the brain (131). In vessels with extensive PMN recruitment, there was a loss of tight junction proteins, occludin and ZO-1, as observed by immunohistochemical staining of brain tissue (131,133). These observations suggest that adhering and/or migrating leukocytes trigger a signaling cascade that results in an acute breakdown of tight junctions along with disorganization of tight junction components. Other studies investigating lymphocyte migration through the BBB have shown that cross-linking of ICAM-1 resulted in reorganization of the endothelial actin cytoskeleton, Rho activation (134), and induction of c-Jun-N-Terminal kinase (JNK) via a Rho-dependent pathway (135). Interestingly, pretreatment of brain endothelial cells (BECs) with a Rho inhibitor, C3-transferase, significantly diminished monocyte migration across brain endothelium (136). Furthermore, Etienne-Manneville et al. (137) demonstrated that ICAM-1 cross-linking also induced calcium signaling, which via PKC mediates phosphorylation of actin-associated proteins (e.g., ZO-1) and cytoskeleton rearrangement in brain endothelial cell lines. These calcium-mediated intracellular events are necessary for lymphocyte migration through the BBB. In addition, ICAM-1 expression is up-regulated on astrocytes during inflammatory conditions, and cross-linking of ICAM-1 on astrocytes in culture resulted in the production of TNF- α (138). TNF- α and other proinflammatory cytokines might influence tight junction permeability at therefore affect BBB integrity (139).

Recent studies demonstrate the complex and dynamic nature of junctional complexes between endothelial cells. Local chemical signals trigger intricate signaling mechanisms, which lead to cytoskeletal reorganization and ultimately modulate paracellular permeability. Under pathological conditions, disruption of BBB integrity might occur, followed by transendothelial migration of activated leukocytes. In future, cell biological imaging techniques may provide new insights in the complex and dynamic nature of junctional complexes between endothelial cells. Further research may also reveal new signaling mechanisms involved in tight junction biogenesis. Finally, these findings will provide additional knowledge about the underlying mechanisms of tight junction functioning, which may ultimately lead to new therapeutic approaches.

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4

Trophic Factors and Cerebrovascular Regulation

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1. INTRODUCTION

The choroid epithelial cells (EC) of the brain form the blood–cerebrospinal fluid barrier (BCB), which is responsible by active and passive transport mechanisms for producing the major part of the cerebrospinal fluid (CSF). The BCB closely regulates the exchange of molecules between blood and CSF. The CSF is somewhat more acidic than blood and contains significantly less protein compared to serum. The CSF flows via the ventricles down to the spinal canal but also upward over the cortex. In this way, the CSF covers the entire brain, reaching into the sulci and depths of the cortex and thus acts as a mechanical cushion. The extracellular environment of neurons and neuroglia is in a steady state with the CSF, and this phenomenon is considered to be an important factor in maintaining homeostasis in the central nervous system.

The vascular system is also important for the transport of nutrients and molecules into the extracellular fluid surrounding neuronal cells. Blood capillaries of the brain are made up by special EC capable of joining via high resistance tight junctions, thus forming an effective "blood-brain barrier" (BBB). Astrocyte end-feet completely surround the basement membranes of the EC of brains capillaries, and provide brain ECs with trophic factors that modulate the permeability of the BBB. These astrocytes are also in direct contact with neurons. It is therefore tempting to believe that in case of a high local metabolic activity and energy demand, the astrocytes will receive inputs from neurons, and thus influence the local permeability of the capillaries.

In the case of a constant high transport demand (e.g., during brain development or within brain tumors) or in specialized brain areas like the pituitary, the ECs of capillaries do not form a BBB, and the EC in these brain capillaries are fenestrated like in the periphery.

Certain brain areas need more energy on a regular basis, as is the case during intensive training for a certain task, and thus will demonstrate extreme plasticity. Neurons in these active brain regions will develop more synapses, the volume fraction of the astrocytes will increase and as a consequence, the local energy demand will grow. Despite long-held beliefs that the brain capillary system is not plastic, several findings suggest that this system is also quite responsive to energy demand. Young rats in a so-called complex environment develop up to 80% higher capillary volume per neuron, as compared to individually caged rats (1). The greatest response is seen in weanlings, but experience-induced plasticity continues clearly into adulthood, although diminishing with age (2).

The central message from studies of neuronal and cerebrovascular plasticity is that the brain is an organ of adaptation. Growth factors play a pivotal role in regulating these experience-based or disease-induced adaptations and are involved in maintenance of established brain functions under normal conditions.

This review will focus on these diffusible morphogenic factors and their role in the development, maintenance and rescue of brain tissue, as well as the integrity of the BBB under both normal and pathophysiological circumstances.

2. TROPHIC FACTORS

Trophic factors have been identified by their key role in the development and long-term survival of tissues. The existence of these factors was first demonstrated by the work of Viktor Hamburger, Rita Levi-Montalcini and Stanley Cohen (3) (for review see Reference 4.) The key observation was that extracts from mouse tumors induced profuse dendritic sprouting of chick dorsal root ganglia in tissue culture. It was concluded that these extracts contained factors, which were able to induce specific properties during development. Moreover, avulsion experiments demonstrated that survival of differentiated dorsal root sympathetic neurons also depended on continuous trophic support from their target. All in all, it was established that trophic factors were able to induce neuronal differentiation, as well as to support survival of neurons. In line with these notions, a trophic factor should comply with these definitions, i.e., it should support survival of cells and induction and maintenance of differentiation or specialization in target cells.

The prototypic neurotrophic factor, Nerve Growth Factor (NGF), was isolated from mouse salivary glands and its crystal structure was elucidated (5). By analogy, other trophic factor families such as the Transforming Growth Factor β (TGF β) superfamilies were further identified. These molecules exert similar effects to those described for NGF, in various tissues, species and models (for reviews see Refs. 6 and 7). Neurotrophins and TGF^β family members are currently referred to as "group-2 cytokines" and known to be primarily involved in growth, maintenance and differentiation of a variety of epithelial, endothelial, and neuronal tissues. These factors act as homodimers and share structural features such as a cystine knot and frequency of β -sheets (Table 1). Figure 1 depicts a cartoon of the dimeric human vascular endothelial growth factor (VEGF), a prototypical trophic factor (Protein Data Bank http://www.rcsb.org; encoded 1VPF). The VEGF monomers contain six beta-sheets and have up to three intramolecular cystine bridges, known as a cystine knot. The fourth disulfide bridge is intermolecular and joins the two monomers, thereby forming a dimer (8).

Most of the seminal findings for trophic factors were obtained from studies of the central nervous system. Currently, it is understood that trophic factors are widely distributed throughout the body. Furthermore, trophic factors usually refer to a limited class of biologically active proteins, but the term may apply to an even wider variety of biologically active compounds provided that they support growth, differentiation, and survival of specific tissues.

3. TROPHIC FACTORS AND THEIR RECEPTORS

The receptors for class-2 cytokine trophic factors are kinases. These receptors have one membrane-spanning domain and operate as dimers. Upon activation by its cognate agonist, specific amino acid residues are autophosphorylated resulting in distinct intracellular signaling cascades. Several trophic factor receptors are the so-called tyrosine kinases as the phosphorylated residues are tyrosine, as it is the case for TrkA, TrkB, TrkC, VEGFR, and PDGFR (Table 1). Neurotrophic factor receptors may display binding preferences for specific neurotrophins. For example, TrkA selectively binds NGF but can also become activated by BDNF or NT3. By contrast, TrkB can be activated by both BDNF and NT4/5, whereas TrkC is reported to specifically bind NT3 (9).

In addition to tyrosine kinases, other sets of trophic factor receptors (auto) phosphorylate serine and threonine residues. These include the so-called TGF β receptor subclass. Obviously, the intracellular signaling consequences will differ since phospho-tyrosine residues are known to

able I Neurotrophic Factors (Class-2 Cytokines) and their Receptors	
Class	Factor	Receptor
Neurotrophins	Nerve growth factor (NGF) Brain-derived neurotrophic factor (BDNF)	TrkA TrkB
	Neurotrophin-3 (NT-3)	TrkC > TrkA > TrkB
Neuropoietins	Neurotrophin-4/5 (NT-4/5) Ciliary neurotrophic factor (CNTF)	Trk B CNTFRα, gp130, LIFRβ
	Leukemia inhibitory factor (LIF)	LIF receptor complex
Insulin-like growth factors	Insulin-like growth factor-I (IGF-I)	IGFR-1 >> insulin receptor
	Insulin-like growth factor-II (IGF-II)	IGFR-1 >> insulin receptor
Transforming growth factor β	Transforming growth factor β (TGF $\beta_{1,2,3}$)	TGFBR I, II, III
	Glial cell line-derived neurotrophic factor (GDNF)	
	Artemin	c-Ret, GDNFRa1
	Persephin	c-Ret, GDNFRa3
	Neurturin	c-Ret, GDNFRα4
	Sonic Hedgehog	c-Ret, GDNFRa2
	Bone morphogenetic proteins (BMP1-9)	Patched, Smoothened
Fibroblast growth factors	Acidic fibroblast growth factor (aFGF; FGF-I)	FGF receptors 1–4 (FGFR)
	Basic fibroblast growth factor (bFGF; FGF-2)	FGFR1-3
	Fibroblast growth factor-5 (FGF-5)	FGFR-1, FGFR-2
Other growth factors	Transforming growth factor- α TGF α)	EGFR
	Platelet derived growth factor (PDGF)	PDGFR- α , PDGFR- β
	Stem cell factor (mast cell growth factor)	c-kit
	Vascular endothelial growth factor (VEGF)	VEGFR1 (flt-1), VEGFR2 (Kdr)
	Macrophage-Colony stimulating factor (M-CSF)(GC-CSF)	c-fms
	Angiopoietin	TIE-1. TIE-2

 Table 1
 Neurotrophic Factors (Class-2 Cytokines) and their Receptor



Figure 1 Human Vascular Endothelial Growth Factor (VEGF) determined from x-ray crystallography, obtained from Protein Data Bank (http://www.rcsb.org); deposited by Muller et al. *Source*: From Ref. 8.

provide anchor points to SH2-domain containing signaling proteins such as Grb2 and Shc and signaling may proceed via activation of Phospholipase- γ , PI-3-kinase and recruitment of JAK/STAT pathways (10,11). In contrast, Ser/Thr receptor kinase signaling is less clear than for tyrosine kinases. Using yeast two hybrid systems, a number of putative signaling proteins have been discovered but their physiological roles are not easily established. For example, TGF β signaling affects cyclins and cyclin-dependent kinases, which may require FKBP12 (12).

To make the receptor issue even more complicated, proteoglycan co-receptors have also been identified. Although trophic factor receptor dimers are able to produce intracellular signals, these co-receptors have specific impact on the final direction of the cellular response. With regard to TrkA neurotrophin receptor, a p75 co-receptor was identified (13). This co-receptor interacts with a number of Trk receptors and has a relatively low affinity for NGF. However, it was postulated that p75 may play a role in potentiating NGF signaling and may confer ligand selectivity to the receptor. However, p75 contains the so-called Death Domain sequence in its intracellular part, a structure that may also force cells into apoptosis upon withdrawal of trophic factors (for review see Refs. 14 and 15).

4. BRAIN VASCULARIZATION AND BLOOD SUPPLY

The brain is a highly vascularized organ. It is estimated that the brain takes up about 20% of our body energy in glucose and oxygen (16). Palkovits and

co-workers demonstrated the extent of brain vasculature by India ink perfusion (17,18). The key trick in these sophisticated experiments was the choice of hexobarbital as an anesthetic. While it is a narcotic, it also opens up cerebral blood vessels allowing maximal access of small colloidal ink particles. Throughout the brain, various networks can be identified including the nucleus accumbens, specific subnuclei in the hypothalamus and various nuclei in the brain stem such as the area postrema and the reflex centers for blood pressure and respiration.

Although heavy vascularization can be seen at a histological level, in vivo labeling studies using ¹⁵O–H₂O, ¹⁸F-deoxyglucose in PET label studies shows that under basal conditions, the brain is not uniformly perfused but rather seems to operate at a resting pace. When challenged with specific tasks or cues, perfusion and activity in the brain rapidly adapt to specific requirements, thereby lighting up specific brain regions in PET scans (e.g., Refs. 19 and 20). From the wealth of imaging data available, it can be concluded that blood flow in the brain is tightly controlled by local demand of energy. This phenomenon is likely to be controlled both by direct nervous input, as well as by changes in acidification and the decrease in oxygen pressure.

Interestingly, microcirculation complexity is determined in part by the frequency of demand. Accordingly, young adult rats challenged with physical exercise showed an increase in cerebellar capillaries, as compared to unchallenged control litter mates (1,21). These changes developed quickly as after 10 days of continuous exposure and are completed in 30 days. These data indicate that novel capillaries develop and mature upon increased demand. However, the ability to develop supportive microcirculation is lost with age (2). Repeating the exercise paradigm in 2-year-old rats failed to show these adaptive changes, suggesting that cerebellar angiogenesis in old rats is significantly reduced. As the cerebellum is particularly involved in co-ordinating motor behavior, it can be argued that intense use of any specific brain function may trigger an increased vascularization in order to support the local demand in nutrients and oxygen.

Formation of blood vessels involves two distinct processes, i.e., vasculogenesis and angiogenesis, which vary in their source of EC. Vasculogenesis occurs during embryonic development where endothelial precursor cells from the splanchnic mesoderm organize themselves into blood vessels. Conversely, in angiogenesis, new EC are being generated through cell division of existing EC. Both processes involve specific trophic factors.

One relevant trophic factor is VEGF and its receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR). This system has been established to be essential in proper formation of the EC lineage (22,23) but they require a balanced act. Indeed, VEGF knockout mice *but also* knockout mice for either receptor are embryonically lethal and show aberrant vascularization of the embryonic yolk sac. Flt1-knockout mice have an uncoordinated

overgrowth of blood vessels (24). However, when blocking KDR/Flk signaling in these mice, angiogenesis can be attenuated (25). *Disruption* of VEGF or its receptors during embryonic development thereby illustrates the role of this system in vascular development (23,26–28).

The key role of VEGF in maintaining or reorganizing brain vasculature has also become apparent from studies on brain tumors. Astrocytomas and gliomas actively secrete trophic factors such as VEGF, thereby stimulating EC to divide in order to support de novo angiogenesis. Increased blood supply supports tumor growth and facilitates metastasis (29,30).

5. TROPHIC FACTORS AND TIGHT JUNCTIONS

When mature, cerebrovascular EC tightly interact with astrocytes. Astrocytes are considered important for the development and maintenance of tight junctions and therefore of endothelial permeability (31,32), for review see Reference 33. A number of trophic factors, such as FGF2, FGF5, and TGF β 1, as well as GDNF in conjunction with sphingosine-1-phosphate, have been implicated in maintenance and regulation of BBB permeability (30,34–36). Furthermore, TNF α and interferon- γ have been described to activate cerebral EC but it is at present unknown whether this leads to the opening or the tightening of BBB function (37). Finally, an oxygenation-sensitive balance between VEGF and angiopoietin-1 is emerging as a regulator of tight junction stability, as well as BBB permeability (38,39).

Cerebral EC are exposed to three different environments, i.e., the plasma side, the CNS side and the neighboring EC. The luminal plasma side involves continuous blood flow shear stress and mechanical stretch stimuli. Blood-borne factors such as PDGF, TNF α , interferon- γ and other inflammatory cytokines may affect barrier function in the endothelium (see above). Also, the endothelium itself may be a source of trophic factors, although there is a paucity of information in this regard. Human EC have been identified as a source of BDNF (40,41), which may be involved in the maintenance of contacts between EC and neurons, as BDNF does not activate human cerebrovascular EC themselves (37).

Finally, glial, and pericytic cells are considered a major source for trophic factors. Under basal conditions astrocytes and glial cells derived from astrocytoma are well described to have low expression levels of trophic factors such as GDNF, BDNF, VEGF, and angiopoietin-1. Changes their in expression is most likely the consequence of a variety of physiological and pathophysiological conditions. Thus, inflammatory stimuli such as treatments with either bacterial lipopolysaccharide (LPS), viral transformation, ischemia or mechanical damage induce the production of a variety of trophic factors, such as TNF α , BDNF, GDNF, and angiopoietin-1 (39,42,43).

6. BRAIN OXYGEN AND TROPHIC FACTOR REGULATION

Brain angiogenesis occurs just as anywhere else in the body through ingrowing EC, which ultimately form blood vessels. With progressive completion of blood vessels and concomitant perfusion and oxygen supply, the trophic factor repertoire changes to FGF and angiopoietin-1 (Ang-1) while VEGF expression is steadily decreasing.

In the adult brain, mature EC adhere to the extracellular basal membrane and start to produce their own trophic factors, including PDGF and Ang-1. The VEGF is then secreted from vessel-associated pericytes. Ang-1 is considered an antipermeability factor, strongly attenuating vascular leakage (44).

In recent literature it is postulated that glial cells may act as an oxygen sensor. Accordingly, under normoxic conditions $(5-6\% O_2)$ VEGF is repressed while angiopoietin-1 and thrombospondin-1 (*TSP-1*) are normally expressed (45). By decreasing oxygen pressure to 1% in order to mimick ischemic conditions, VEGF is quickly elevated while Ang-1 and TSP-1 are repressed (45). Re-oxygenation rapidly re-induces expression of Ang-1 (within 30 minutes), while repression of VEGF and induction of TSP1 takes about 3 hours. Simultaneously, tight junction formation was monitored as a function of VEGF and Ang-1 expression (39) and a strong correlation between the expression and regulation of src-suppressed C-kinase substrate (SSeCKS) and ZO-1 was observed, suggesting that astrocytes are primarily responsible for forming and subsequently controlling tight junctions in brain vasculature (39). In line with these findings, the vasogenic edema observed in stroke, concussion or brain tumors is proposed to be related to the acute rise in VEGF and subsequent opening of tight junctions (46,47).

Partial oxygen pressure is likely to play a role in setting the balance between VEGF and Ang-1. Blood-brain permeability will be controlled accordingly as well, having VEGF as a permeability promoting factor and Ang-1 as an anti-permeability factor. Partial oxygen pressure will also relate to energy requirements. Increased oxygen consumption due to cerebral activity will lower local oxygen pressure thereby increasing permeability. Upon vascular occlusion during stroke cerebrovascular incidents, a similar process will happen. The key difference in consequences is that under physiological conditions (e.g., low local cerebral blood flow) no edema will develop but transport across the BBB will be facilitated. This notion is compatible with a functional BBB (for review see Ref. 33)—it allows BBB to open up at times when local blood flow is reduced.

This oxygen-sensitive trophic factor regulation fits well with the adaptive physiology of brain perfusion and angiogenesis (e.g., according to Refs. 1 and 21): local demand will impact on local cerebral oxygen consumption and set a demand for local supply.

More recently, reactive oxygen species such as superoxide and nitric oxide have been implicated in regulating vascular responses and endothelial permeability (48,49). These factors act acutely on cerebral EC, reorganizing tight junctions and the endothelial cytoskeleton. Interestingly, VEGF expression can be readily induced by superoxide (50,51), which affects the integrity of tight junctions in the BBB as well (52). VEGF counteracts its regulation by superoxide as it activates superoxide dismutase activity (53). In conclusion, VEGF promotes vascular permeability while at the same time, it induces countermeasures to preclude reactive oxygen species from driving vascular permeability too much, particularly when local blood flow is increased.

Similarly, dysregulations of trophic factors have been described in animal models for epilepsy, in which VEGF induction was reported throughout the brain, particularly in hippocampal regions (54).

7. TROPHIC FACTOR RECEPTORS AS PHARMACOLOGICAL TARGETS

It is clear that trophic factors play a role in generating, maintaining, and regulating BBB function under patho-physiological conditions. These mechanisms may thus be targets for therapeutic intervention. Attenuating angiogenesis, as well as promoting and stabilizing tight junctions in BBB EC is an example of potent therapeutic intervention. Two mechanisms may be considered, i.e., interference with specific trophic factor receptors or modulation of glial oxygen sensitivity and reactive oxygen species signaling.

Trophic factor receptors have been under investigation for the development of suitable signaling inhibitors and some trophic factor receptor inhibitors have reached the clinic with success (55). The main focus for anti-vasculogenic trophic factor inhibitors is the treatment of cancer. As various tumors trigger vasculogenesis for growth support, it is important to consider that anti-vasculogenic compound could limit or halt vascularization and impede tumor growth.

Amongst such inhibitors is Imatinib, originally identified as a c-Kit inhibitor, which also displayed potent cytostatic properties in a variety of tumors—it is currently on the market under the trade name of Gleevec (56). Other compounds, such as semaxinib, were tried in the clinic but generated disappointing results due to poor kinetics and to severe thrombocy-topenia (57). Several other anti-angiogenic inhibitors of trophic factors are in various stages of preclinical and clinical evaluation (see Ref. 55). We have listed in Table 2, a few compounds that are currently being investigated for their anti-angiogenic properties.

Receptor	Inihibitor	Details	Reference
PDGFR1	STI571/imatinib	Selectively inhibits c-Kit, PDGFRα, PDGFRβ, c-Abl	56
	ZD6474	PDGFR1 > bFGF	58
VEGF-R1	Vatalanib (ZK222584)	VEGF-R1~VEGF-R2 ~PDGFR β ~c-Kit > c-Fms	57,59
VEGFR2	AAL993	VEGF-R2 > VEGF-R1	57
	Semaxinib (SU6668)		60
EGFR	Erlotinib	EGFR	61
	Gefitinib (ZD1839; Iressa)	—	

 Table 2
 Summary of Developed Inhibitors for Trophic Factor Receptors and their Selectivity

With these emerging trophic factor inhibitors, tools are also becoming available to further study cerebral vasculature and its dependence on trophic factors for maintaining BBB integrity. As was discussed before, VEGF stimulates angiogenesis and increases cerebrovascular permeability. Accordingly, VEGF receptor antagonists may also be applicable in short-term treatment for post-ischemic angiogenesis and to prevent angiogenic edema as a consequence of head trauma or ischemia. Furthermore, VEGF receptor inhibitors may also be effective in the treatment of MS during inflammatory episodes. Again, lowering activity of VEGF or its receptors will support maintenance of strict BBB functions. To that end additional experimental approaches such as a VEGF trap, thrombospondin-1 or angiopoietin-1 mimetics may also prove helpful in the treatment of neuroinflammatory and cerebrovascular diseases.

The second approach in designing therapy with trophic factors in relation to cerebral EC and the BBB would be to modulate oxygen tension and signaling by reactive oxygen species. The VEGF and angiopoietin-1 respond to hypoxia in opposite directions thereby facilitating increased vascular permeability and angiogenesis. Conversely, reactive oxygen species induce VEGF expression, which may again underlie increased vascular permeability.

Anti-oxidant treatment during and after head trauma as well as during MS episodes will scavenge increased reactive oxygen species that may attenuate the induction of VEGF expression and thereby preclude adaptive changes in vascular permeability and maintain tight junctions (48,49,51). However, as usual trophic factors may be a two-edged sword. Data suggest that VEGF can induce Cu/Zn superoxide dismutase, suggesting that by

down-playing VEGF, a long-term decrease in SOD activity and a concomitant increase in superoxide signaling might be expected (53).

8. INTEGRATION OF PHYSIOLOGY AND PATHOPHYSIOLOGY

Physiological regulation of the BBB involves a plastic, adaptive opening and closing of tight junctions according to local needs. The proposed role of trophic factors is consistent with this need, as mentioned earlier. Trophic factors play a role in cell survivals and maintenance, as well as inducing differentiation and specialization of target cells.

A balance between pro- and anti-permeability trophic factors which are regulated in an acute manner by reactive oxygen species and which may relate to CNS control of vasculogenesis occurs at the level of the BBB. In addition, a slow steady-state balance is proposed, which may be driven largely by local oxygen consumption and balances between angiogenesis and maintenance of BBB integrity. As described, this mechanism seems to loose its dynamics with age.

A number of pathophysiological conditions are also involved in the regulation of the BBB in relation to trophic factors. For example, brain tumors actively synthesize and secrete pro-angiogenic trophic factors, primarily VEGF. Angiogenesis further supports tumor growth and may facilitate metastasis as well. Furthermore, VEGF reduces tight junctions expression, thereby further compromising BBB function and thus resulting in cerebrovascular leakage.

The acute response of VEGF to superoxide may also be implicated in MS. Superoxide acutely affects endothelial tight junction and cytoskeletal organization, whereas the induction of VEGF—which occurs within 30 min—may support increased vascular permeability, thereby allowing transmigration of inflammatory cells and leakage of plasma proteins into the brain parenchyma. Its subsequent induction of Cu/Zn superoxide dismutase may be effective in reinstating BBB integrity thereby contributing to remission after an MS episode. Finally, with cerebrovascular incidents such as trauma, stroke, concussion, or epilepsy, a change in the delicate balance of trophic factor is observed and has been implicated as a causal factor in angiogenic edema.

In conclusion, the identification of trophic factor receptor inhibitors and their effects on angiogenesis are still limited to the treatment of cancer but shall soon be relevant to the treatment of BBB-related diseases, such as neuroinflammatory diseases, cerebrovascular diseases, and head trauma. Finally, as cerebrovascular plasticity was found to be related to local demand and as its plasticity seems to wear off with age, additional applications of trophic factors in vascular dementia, Alzheimer's disease and agerelated cognitive decline could be considered.

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Neuronal Influence on the Local Control of Microcirculation

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1. INTRODUCTION

The brain is a very complex structure; it is the origin of thoughts and actions, has 10¹¹ neurons, with more than 1000-fold more dendrites. The neurons not only interact with each other but also with the surrounding glial cells. Electrical impulses propagate along dendrites and axons to communicate between pre- and postsynaptic functions. It is no wonder that these complex functions in the brain require a constant microenvironment to provide the necessary fuel (oxygen and glucose) and to transport away the waste products. In order to secure a constant microenvironment and to maintain adequate metabolism, it is important to have a precise local regulation of the cerebral circulation. Traditionally, cerebral vascular resistance is regulated by metabolic factors, chemical stimuli, perfusion pressure, and perivascular nerves (1). We have recently reviewed the neuronal messengers in the human cerebral circulation (2), with a focus on large arteries belonging to the circle of Willis and their involvement in cerebrovascular disorders. In this chapter, we focus on intracerebral microvessels and their relation to the control of the microenvironment in the brain.

2. INTRACEREBRAL VASCULAR INNERVATION

Several metabolically produced substances (e.g., H^+ , CO_2 , K^+ , Ca^{2+} , and adenosine) have been proposed to mediate the local changes in cerebral

blood flow that accompany neuronal activity. However, none of these seems to fully account for the adaptative responses in vasomotricity of the microvascular bed. In this respect, it has been proposed that regional changes in brain perfusion are in some situations controlled directly by neurons located within the brain parenchyma (3.4). Stimulation of specific brain regions, such as the cerebellar fastigial nucleus (5), the basal forebrain (6,7), or the brainstem raphe nuclei (8–10) to name only a few, elicits changes in cerebral blood flow in specific brain areas. The changes in perfusion are sometimes independent from those in glucose metabolism implying that neuronal pathways can exert direct effects on the microcirculation. Furthermore, a population of neurons has been identified in the cerebral cortex with activity related to spontaneous waves of cerebral blood flow, and suggested to transduce neuronal signals into vasomotor responses (11,12). These observations suggest a neuronal control of the cerebral microvascular bed, which in concert with other mechanisms (vasoactive metabolic substances, ionic gradients, and intrinsic endothelial or myogenic responses within the vessel wall), are important determinants, in the spatial and temporal adaptation of local perfusion to cellular activity (3). This statement infers that: (1) brain neurons send projection fibers to microvessels in target regions, (2) microarterioles and possibly capillaries have the ability to modify their diameters and therein adapt local blood flow in response to changes in the status of brain neurotransmitters/neuromodulators, and (3) mechanisms exist to transmit information to the feeding vessels as well as to circumscribe the extent of the vascular response to the areas in demand (Fig. 1).

Early morphological studies documented the presence of nerve fibers and, occasionally, neuronal cell bodies that contain different neurotransmitters/neuromodulators are associated with intraparenchymal blood vessels (13). At the light microscopy level, these perivascular fibers or neuronal perikarya follow the contours of blood vessels, being apposed to vessel walls or literally encircling the vessel. Subsequently electron microscopy studies of intracerebral arterioles and capillaries found axon terminals on their abluminal walls (14,15). Electron-dense core vesicles (presumably adrenergic) remain after bilateral cervical sympathectomy—the hypothesis being that intracerebral vessels receive an adrenergic innervation of central origin (16–18).

The central innervation of intraparenchymal arterioles may be located primarily at branching sites—a strategic location for the control of local blood flow (19).

However, detailed analyses have been undertaken in order to assess the nature of these neurovascular associations, their pathways of origin and their ability to functionally interact with the microvascular bed. Such studies have indicated that brain intrinsic neurovascular associations are region specific and establish non-junctional appositions, which act in a volume transmission or parasynaptic mode and whose effect on the microcirculation



Figure 1 Possible arrangements between intracerebral neurons and the cerebral microvessels. (A) Classical relationship where the vessels respond secondarily to the products of cellular metabolism. (B) Innervation of the microvessels by collateral fibers from intracerebral neuronal systems. *Source:* From Ref. 3.

relies upon the distribution of appropriate microvascular receptors. Further, some of these neurovascular systems seem to converge towards a common denominator, namely the NO neuron, which appears as a privileged, but not exclusive, intermediary in the transmission of neuronal signals to the local microvessels (20–22).

2.1. Noradrenergic System from the Locus Ceruleus

The first conclusive morphological evidence for nerve fibers of central origin associated with brain intraparenchymal blood vessels was provided for the noradrenergic system by demonstration of perivascular nerve fibers either labeled for catecholamine histofluorescence or immunoreactive for dopamine- β -hydroxylase in the brain of ganglionectomized rats (13,23). Such fibers were later visualized at the electron microscopic level and defined as perivascular on the basis of their close proximity to the basal lamina of small capillaries with their attached pericytes (16). The fact that manipulations (stimulation/lesion) of the locus ceruleus, the seat of central noradrenergic fibers, were found to alter blood-brain barrier permeability (24,25), induces changes, albeit of small magnitude, in local cerebral blood flow (24) or results in up-regulation of microvascular α -adrenergic receptors (26) all support that these fibers could represent a functional innervation of the microvascular bed. More recent studies have confirmed that the locus ceruleus is the exclusive source of cortical perivascular noradrenergic nerve terminals (27), and ultrastuctural analysis has emphasized the frequent association of these fibers not only with capillaries but also with microarterioles, the resistance vessels responsible for the fine regulation of local cerebral blood flow (27,28). Moreover, topometric analysis of perivascular fiber distribution showed their enrichment in the immediate vicinity of the vessel basement membrane, a characteristic that has been found, at least for some neurovascular systems (e.g., serotonin), only in regions that modify their local perfusion in response to neuronal activation (for review see Ref. 29). However, based on the relatively minor effect of this system on local cerebral blood flow and the most frequent association of neuronal and perivascular noradrenergic nerve terminals with astroglial cells and leaflets (27,28), it has been suggested that the primary roles of noradrenergic neuronal-glial or neuronal-glial-vascular associations might be to regulate local metabolic functions and blood-brain barrier permeability (Fig. 2).

2.2. Dopaminergic System

Observations using histofluorescence techniques revealed similar morphological proximity between microvessels and central dopaminergic fibers, similar to what was observed for the catecholamine (Edvinsson, unpublished). Although there are no detailed in vivo functional studies to attest for a primary vascular effect of dopaminergic centers (mesencephalic ventral tegmental area and substantia innominata) on the local microcirculation, it has been shown that perivascular application of dopamine in cortical brain slices caused vasoconstriction in about 50% of the microvessels studied (30). These authors also documented the presence of dopaminergic fibers closely associated with intracortical microvessels, such as capillaries, microarterioles, and penetrating arteries, in a manner similar to that described for functional neurovascular systems such as those containing noradrenaline, serotonin, and acetylcholine, suggesting that dopaminergic fibers are strategically positioned to regulate local perfusion in addition to neuronal activity.



Figure 2 An electron micrograph (A) taken in the paraventricular nucleus to show a small dense-cored vesicle-containing varicosity lying directly on the basal lamina of a pericyte associated with a capillary endothelial cell. The varicosity is shown at a higher magnification in (B), where it is clear that the preterminal axon is surrounded by astrocytic (star) processes. *Source:* From Ref. 16.

2.3. Serotonergic System from the Brainstem Raphe Nuclei

In contrast to the relatively minor effect on local perfusion exerted by central noradrenergic neurons, stimulation of the brainstem raphe nuclei (the source of serotonergic nerve fibers throughout the brain), or the ascending serotonergic pathways results in vascular responses in projection areas such as the cerebral cortex. This corresponds primarily to vasoconstriction not superimposable on underlying metabolic changes (8–10), for review see Reference 29. A strong morphological basis exists to support the presence of functional neurovascular serotonergic fibers of intracerebral origin. In this respect, Golgi analysis, fluorescence histochemistry, and serotonin (10) immunocytochemistry, first revealed an intimate associations between serotonergic neuronal processes and intraparenchymal vessels of the raphe nuclei (31-37). This innervation of local microvessels would appear to embrace all vascular elements: arterioles, capillaries, and venules. In addition, a close relationship between serotonergic nerve fibers and the cerebral ventricles was observed (32-38), and a sensory or neurosecretory role was suggested for these fibers. Neurovascular associations in the terminal field areas of serotonergic neurons were also noted (36) and have been best described in the hippocampus and cerebral cortex (39). When studied at the ultrastructural level, perivascular nerve terminals labeled for the serotonin synthesizing enzyme tryptophan hydroxylase associated with capillaries, and microarterioles of all sizes, including penetrating arteries. They occasionally contacted the basal lamina of the blood vessel directly, but more frequently abutted on the perivascular astroglial leaflet. Perivascular terminals were significantly smaller than non-perivascular terminals possibly because they have reached their final (vascular) target, a situation reminiscent of that previously reported for perivascular terminals in the hypothalamus (15). Most interesting was the observation that the density and vascular proximity of perivascular serotonergic terminals varied depending on the brain region examined. Areas in which local perfusion was most affected by raphe nuclei stimulation were those (e.g., frontoparietal cortex) with the highest density of terminals in the immediate vicinity of the vessel wall (39). Although vascular frequency and proximity are likely not the only factors involved, these observations suggest that any change in brain serotonergic status will be perceived more readily by the microvascular bed in regions such as the frontoparietal cortex which exhibits intimate neurovascular relationships. Interestingly, cortical microvessels are endowed with specific serotonin receptors able to mediate constriction or relaxation, strategically located on the smooth muscle or endothelial cells of the vessel wall to best respond to the central release of neuronal serotonin (40-42). Notably, serotonin has been implicated not only in regulation of cerebral blood flow but also in blood-brain barrier permeability and astrocytic glycogenolysis (for review see Ref. 29).

2.4. Cholinergic Basal Forebrain System

Cholinergic neurons of the basal forebrain represent one of the most studied neurovascular systems both physiologically and morphologically, in part due to their relation to the pathophysiology of dementia (4). Electrical or chemical stimulation of the basal forebrain has been shown to induce cortical increases in cerebral blood flow (vasodilation) that are (i) accompanied by a local release of acetylcholine, (ii) limited to the side of the stimulation, (iii) mediated in part by muscarinic and nicotinic acetylcholine receptors, (iv) sensitive to NO synthase inhibition, and (v) not a secondary response to changes in local cerebral metabolism (see reviews, Refs. 6 and 43). Several anatomical studies have documented the presence of cholinergic fibers in association with the microcirculation of various forebrain regions (44–48), but primarily in the cerebral cortex including in man (49). It has been suggested that the majority of perivascular cholinergic nerve fibers in the rat cerebral cortex originate centrally from the basal forebrain (48), with only a small contribution from the local cortical cholinergic bipolar neurons (44.46). Furthermore, cholinergic basal forebrain fibers were shown to interact not only with cortical microvessels, but also with cortical NO interneurons (49,50), in agreement with the findings that these neurons are cholinoceptive and endowed with muscarinic acetylcholine receptors (51-53). It has thus been suggested that in addition to exerting direct vasomotor effects mediated by specific microvascular muscarinic acetylcholine receptors (54,55), basal forebrain cholinergic neurons could act via an intermediary intracortical NO neuron to adapt local perfusion to changes in neuronal activity resulting from activation of basal cholinergic neurons (50,56). Perivascular cholinergic fibers associated with microvessels of all sizes were smaller than their neuronal counterparts, distributed in all cortical layers, and the perivascular astrocytes appeared to be an important relay in the neurovascular interactions.

2.5. Neuropeptides

Several studies have documented the presence of close associations between neuropeptide-containing neurons and the microcirculation. For instance, cholecystokinin (57), vasoactive intestinal polypeptide (VIP) (44,58), neurotensin (59), neuropeptide Y (NPY) (60), atrial natriuretic peptide (ANP) (61), and galanin (62) containing neurons, have all been shown to contact intracerebral microvessels. However, few studies have tried to quantitate the number of projection. Intracortical VIP and NPY neuronal perikarya and processes have been shown at the electron microscopic level to frequently abut on cortical microvessels in a manner similar to that described above for the other neurovascular systems. Functionally, stimulation of the mesencephalic reticular formation or intracortical injection of VIP both pointed to a role for this peptide in cortical (64,63a,63b) or striatal vasodilatation (63a,b). In this respect, the morphological characteristics and cellular targets of neocortical VIP interneurons, some of which also co-localize acetylcholine (44,46,64), support a role for these neurons in the regulation of regional blood flow (46,47,58). VIP containing cell bodies, dendrites, and axon terminals are often seen directly apposed to cortical microvessels, a situation reminiscent to that reported for NO neurons. The fact that VIP

and NO are not co-localized in cortical neurons (65) may suggest that neocortical VIP interneurons constitute an additional population of local neurons concerned with fine tuning of local perfusion. This statement would agree with the finding that the spontaneous and evoked elevations of local blood flow linked to the activity of a population of cortical interneurons are not affected by inhibition of NO production (12). In addition to neurovascular interactions, several cortical VIP nerve terminals establish neuroglial relationships, including with perivascular astrocytes (47), in support of a role for VIP in the control of glycogen content in astroglial cells (66). Nicotinic and glutamate receptors have been found on VIP cortical interneurons (67,68), which supports a more specific role for these neurons in the intregration of local cortical blood flow as a function of changes in neuronal activity elicited by acetylcholine and glutamate.

The potent vasocontractile peptide NPY, perivascular cortical NPY fibers that surround penetrating arterioles and intracortical microvessels have been observed in rat and human cerebral cortex (69–71). The fact that the content of NPY in microvessels was not decreased following double sympathectomy (70a) demonstrated their central origin, which most likely corresponds to the intracortical and subcortical white matter NPY neurons (72).

Although NPY is a potent vasocontractile agent in the cortical microcirculation (73), which possesses vasocontractile NPY-Y1 receptors (70b,71), close to 50% of cortical NPY neurons reportedly also contain the potent vasodilator NO and all cortical NO neurons also co-store NPY (65). These observations have given rise to the suggestion that these two opposite vasomediators may work in synchrony to limit the changes in blood flow to the region in demand (21,70a).

2.6. Nitric Oxide (NO)

The discovery of the vasorelaxant NO and the finding that it is synthesized constitutively not only by endothelial cells of all blood vessels but also by a population of brain neurons (74) resulted in an explosion of studies aimed at understanding the role of this novel messenger in the regulation of cerebrovascular functions, and more specifically as a signaling molecule in the coupling of local cerebral blood flow to neuronal activity (21,22,75,76). Indeed, the ability of NO to diffuse tens of microns away from its site of synthesis makes it a very attractive candidate to mediate the dilatation of a broad segment of the microcirculation, as compared to what is expected for more classical neurotransmitters released at the terminal sites and acting on membrane receptors. Morphologically, NO neurons have repetitively been found to impinge upon microvessels of several brain regions (Fig. 3) (49,51,75,77,78), including in man (79,80). When compared with cortical cholinergic neurovascular associations, those established by NO interneur-



Figure 3 NOS neurons stained with the NADPH-D technique. Note the close apposition between the neurons and the microvessels and their branches (*arrows*) in the rat cortex. The pial surface is towards the top of each photo. The length of the bar is $50 \,\mu\text{m}$. *Source*: From Ref. 78.

ons were significantly more frequent in all segments of the cortical microcirculation (56). At the electron microscopic level neuronal perikarya, dendritic processes, and axon terminals immunostained for neuronal nitric oxide synthase (nNOS) were found associated with microarterioles and capillaries (75). Altogether these observations indicate that cortical NO neurons, possibly more that any other type of cortical neurons, are strategically located to act upon the microcirculation and orchestrate blood flow and neuronal activity. Moreover, on the basis of the co-localization of NO and the potent vasocontrictor NPY in all cortical NO neurons (65), it has been speculated that such co-localization could be important functionally in offering a means to spatially restrict the vasodilatory response to NO. Hypothetically, NO would diffuse out to the microvessels located in proximity of nitrergic cell bodies and dendrites while a NPY-mediated vasoconstriction would prevail at the terminal level to restrict the extent of the NO-mediated dilation (21,70a). Another interesting characteristic of cortical cholinoceptive NO neurons (51–53) is their innervation by corticopetal fibers such as basalocortical cholinergic (49,50) and NO-synthesizing (81) nerve terminals, suggesting that neocortical NO interneurons act as a relay for the incoming basalocortical input to neuronal and vascular cells in the cerebral cortex. It is also possible, however, that other neuronal systems (e.g., glutamate, serotonin) may converge towards intracortical NO neurons in order to modulate their excitability, hence their ability to alter local perfusion (82,83). If present, such an organization would uncover a role for cortical NO neurons as an important, although not exclusive (22,75,68) relay in adapting local cortical perfusion to neuronal activity in response to various stimuli. This idea is supported by a report that some cortical areas depend more than others on intracortically produced NO (84).

3. CONCLUSION

The presence of frequent and strategically located neurovascular appositions, their region-selective distribution and perivascular proximity in the regions known to modify their local perfusion in response to stimulation of specific neuronal populations, and the exceptional positioning of cortical NO interneurons, provide morphological arguments for a role of neurally produced substances in the control of microvascular tone, local cerebral blood flow, and blood-brain barrier function (24,85).

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6

The Transport Systems of the Blood–Brain Barrier

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1. THE NEUROVASCULAR UNIT

Brain is highly dependent on a constant supply of oxygen and nutrients through the blood flow; it represents only 2% of the body weight, yet consumes 20% of the total body oxygen supply. Blood is supplied through an intricate network of vessels that projects from large surface, carotid, and basilar arteries deep into the brain tissue where a dense capillary mesh provides for virtually every neuron. Cerebral blood flow through this network is tightly regulated by the brain tissue energy demand.

The neurovascular (NV) unit, broadly defined as a segment of brain vasculature, displays specific anatomic features distinct from those observed in peripheral vascular beds. The NV unit is composed of functionally integrated cellular elements, including brain endothelial cells, astrocytes, pericytes, and smooth muscle cells, and acellular elements that form the basement membrane (Fig. 1). It integrates three principal functionalities: (a) the regulation of cerebral blood flow, (b) the blood-brain barrier (BBB), and (c) the neuroimmune interface.



Figure 1 Schematic diagram of the NV unit cellular anatomy (*left*) and the major transport systems/routes (*right*) involved in trafficking of solutes, nutrients, ions, peptides, neurotransmitters, and drugs across the cerebral endothelial cells.

Various neuronal groups project to cerebral microvessels to affect the spatial and temporal regulation of brain perfusion in response to brain activation, a phenomenon known as NV coupling. Functional brain imaging techniques, such as positron emission tomography and functional nuclear magnetic resonance, are based on the detection of regional increases in cerebral blood flow, glucose, and oxygen consumption that are associated with regional increases in neuronal activity. In the context of brain injury and disease, including cerebrovascular and neurodegenerative diseases, cellular and molecular effectors of NV coupling are damaged or modified resulting in various degrees of NV "uncoupling."

The BBB is a dynamic physical and functional barrier between the systemic circulation and the central nervous system (CNS) exerted by the components of the NV unit. The BBB functionality of the NV unit has been attributed to specific features of cerebral endothelial cells. Current understanding underscores the importance of multicellular interplay in the regulation of endothelial BBB phenotype. BBB facilitates the uptake of nutrients, such as amino acids, glucose, and nucleosides into the brain, restricts brain access to circulating drugs, neurotoxins and neurotransmitters, and actively extrudes metabolic products and toxins from the brain (1–6). The BBB influx and efflux activities are tightly regulated and are crucial for maintaining CNS homeostasis and microenvironment. Breaches of the BBB are ultimately responsible for the development of life-threatening

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brain edema accompanying the growth of brain tumors, ischemic stroke, and trauma (7).

The NV unit also participates in the neuroimmune axis by protecting the brain from circulating infectious agents and by regulating the access of immune and inflammatory cells into the brain. Moreover, brain repair after injury or disease is critically dependent on both adequate blood supply and intact NV coupling. The "restoration" of blood supply to deprived brain areas (e.g., stroke, neural grafts, and implants) occurs by NV remodeling that involves angiogenesis and vasculogenesis. Therefore, the NV unit is tightly integrated in brain physiology, pathology, and repair.

1.1. Cellular Anatomy of the Neurovascular Unit

Cerebromicrovascular endothelial cells (CEC) have unique structural and functional properties that distinguish them from peripheral endothelial cells (1,2): (a) They are joined by both tight junctions and adherens junctions which give rise to a tight intercellular seal that results in high transendothelial electrical resistance ($1500-2000 \Omega cm^2$) and restricted paracellular permeability. (b) CEC lack fenestrations and have a reduced number of pinocytic and endocytic vesicles. (c) CEC are enriched in mitochondria and highly active metabolic enzymes (such as γ -glutamyl transpeptidase, alkaline phosphatase, and GSH *S*-transferase). (d) CEC express various polarized transporters and receptors that mediate trafficking of nutrients and solutes across the BBB. (e) CEC are endowed by a negatively charged, heavily glycosylated layer with the sialic acid residues of acidic glycoproteins displayed on the luminal side, and the proteoglycans, acid glycoproteins and heparin sulphate-bearing glycosaminoglycan-rich basement membrane on the abluminal side (8).

Pericytes are perivascular cells with multifunctional activities. Pericytes extend long cytoplasmic processes over the surface of endothelial cells where, at points of contact, they communicate via gap junctions, tight junctions, and adhesion plaques (9,10). Interactions between pericytes and endothelial cells are important for maturation, remodeling, and maintenance of the vascular system via the secretion of growth factors or via the modulation of the extracellular matrix. There is also evidence that pericytes are involved in transport across the BBB and regulation of vascular permeability (9,10). An important role for pericytes in pathology has been indicated in hypertension, diabetic retinopathy, Alzheimer's disease, multiple sclerosis, and CNS tumor formation (9,10).

Astrocyte end-feet envelop brain capillaries; more than 99% surface of the cerebral capillary basement membrane is covered by astrocytic end-feet. Interactions between astrocytes and endothelial cells have been implicated in the regulation of phenotypic properties of CEC (1,11,12). Astrocytic mediators have been shown to affect CEC tight junctions, proliferation and angiogenesis, CEC production of anti-coagulant factors, and BBB expression

of transporter proteins (1,11,12). Astrocyte–endothelial proximity and interactions are also important in the regulation of inflammatory responses in the brain (11-13).

In addition to perivascular astrocytes and metabolites produced locally by active neurons, direct neuronal "innervation" of the NV unit has been implicated in the coupling of neural activity and blood flow (14). Projecting neuronal inputs as well as cortical interneurons transduce and translate neuronal signals into integrated microvascular responses (14).

Current appreciation of the BBB as a dynamic multicellular unit that undergoes complex regulation and remodeling in response to physiological and pathological stimuli has evolved from a more traditional concept of the predominantly physical "brick-wall" barrier. Whereas the physical barrier is still principally viewed as a function of tight junctions (addressed in Chapter 3) and to a lesser degree the basement membrane, the functional restrictive barrier is achieved through a variety of transporters selectively enriched in CEC (Fig. 1). The penetration of some blood-borne compounds through the BBB is also restricted by selective enzymatic degradation at the endothelial border (2,3).

2. TRANSPORT SYSTEMS AT THE BLOOD-BRAIN BARRIER

The functional basis of the BBB phenotype is epitomized in various polarized transport systems expressed by the CEC. These transport systems include solute carriers (SLC) (Table 1) and non-SLC transporter systems. The SLC transporters are composed of 43 families with 319 members in human and animals, while the non-SLC transporters include ATP-binding cassette (ABC) transporters, ion pumps (ATPase), ion channels, and water channels (15–17). Understanding both the distribution and structure– function relationship of these transport systems is essential for developing preventative and therapeutic strategies for neurological diseases. The following sections of this chapter will describe the classification, molecular composition, and functional properties of the transport systems expressed at the BBB.

For the purpose of clarity, we have adopted the widely accepted international nomenclature stating that (a) the molecules are abbreviated in uppercase, (b) the nucleic acid sequence encoding for the molecule is abbreviated in uppercase and in italics, and finally (c) rodent transporters are identified by a first capital letter followed by lower case letters (i.e., Oatp2/Slco1a4 for the rodent molecule and *Oatp2/Slco1a4* for the rodent gene).

2.1. Transporters for Nutrients

The uptake of nutrients from blood into the brain is facilitated by SLC transporter families. These transporters are involved in the distribution of glucose, amino acids, nucleosides, fatty acids, minerals, and vitamins in various human tissues, including brain.

Superfamily
Transporter
(SLC)
Carrier
Solute
Table 1

	A lias /Other	Total	Expre	ssion ^b	
Symbol	names	n otal members ^a	Brain	CEC	HUGO solute carrier family series/Functions
SLC1	System X-AG/	7	+	+	High-affinity glutamate and neutral amino acid
	ASC				transporters
SLC2	GLUT	14	+	+	Facilitative glucose/fructose (GLUT) transporters
SLC3	HATs	2	ND°	ND	Heteromeric amino acid transporters (HATs)
SLC4	AE/NBC	10	+	ND	Anion exchanger or sodium bicarbonate
					co-transporters
SLC5	SGLT	11	+	+	Sodium glucose co-transporters
SLC6	GAT/NET etc.,	16	+	+	Sodium- and chloride-dependent neurotransmitter
					transporters
SLC7	$CAT/LAT/y^+$	13	+	+	Cationic amino acid transporter/glycoprotein-
	system				associated amino acid transporters
SLC8	NCX	ę	+	ND	Sodium/calcium exchangers
SLC9	NHE	6	+	+	Sodium/hydrogen exchangers
SLC10		5	+	ND	Sodium bile salt co-transporters/orphan transporters
SLC11	NRAMP/DMT	2	+	Ι	Proton-coupled divalent metal ion transporters
SLC12	NKCC/KCC/	6	+	ND	Electroneutral cation-Cl co-transporters
	NCC				
SLC13	NaC/NaS	5	+	ND	Na ⁺ -sulfate/carboxylate co-transporters
SLC14	UT	2	+	ND	Urea transporters
SLC15	PEPT	4	+	+	Proton oligopeptide co-transporters
SLC16	MCT	14	+	+	Monocarboxylic acid transporters
SLC17	NPT/VGLU	8	+	ND	Vesicular glutamate transporters

(Continued)

	dammer (Arre) minne	minding imino		-	
	Alias/Other	Total	Express	ion ^b	
Symbol	names	members ^a	Brain	CEC	HUGO solute carrier family series/Functions
SLC18	VMAT/VAChT	3	+	+	Vesicular amine transporters
SLC19	RFT/ThTr1, 2	ę	Ubiquitous		Folate/thiamine transporters
SLC20	PiT-1,2	2	+	ND	Type-III Na ⁺ -phosphate co-transporters
SLC21/SCLO	OATP	20	+	+	Organic anion transporters
SLC22	OCT/OAT	18	+	+	Organic anion/cation/Zwitterion transporters
SLC23	SVCT	4	+	ND	Na ⁺ -dependent ascorbic acid transporters
SLC24	NCKX	5	+	ND	$Na^+/(Ca^{2+}-K^+)$ exchangers
SLC25		29	+	+	Mitochondrial carriers
SLC26	I	11	+	+	Multifunctional anion exchangers
SLC27	FATP1-6	9	+	ND	Fatty acid transport proteins
SLC28	CNTs	ę	+	+	Na ⁺ -coupled nucleoside transporters
SLC29	ENT	4	Ubiquitous		Facilitative nucleoside transporters
SLC30	ZNT	6	Ubiquitous		Zinc efflux transporters

 Table 1
 Solute Carrier (SLC) Transporter Superfamily (Continued)

Copper transporters	Vesicular inhibitory amino acid transporter	Acetyl-CoA transporter	Type-II Na ⁺ -phosphate co-transporters	Nucleotide-sugar transporters	Proton-coupled amino acid transporters	Sugar-phosphate/phosphate exchangers	Sodium-coupled neutral amino acid (system N/A)	transporters	Metal ion transporters	Basolateral iron transporter	MgtE-like magnesium transporters	Rh ammonium transporters (pending)	Na ⁺ -independent, system-L-like amino acid	transporters	
ND	+	+	QN		QN	QN	;+			Ŋ	QN	ŊŊ	+		
+	+	+	+	Ubiquitous	+	ND	+		Widespread	ND	+	+	+		
0	-	1	б	23	4	4	9		14	-	б	б	З		
hCtr	VIAAT	ACATN	NaPi-II	UGT etc.,	PAT1-4	SPX	SNAT1-6		hZIP	MTP1		RhAG/BG/CG	LAT		
SLC31	SLC32	SLC33	SLC34	SLC35	SLC36	SLC37	SLC38		SLC39	SLC40	SLC41	SLC42	SLC43		

^aThere is currently a total of 319 members in 43 families of SLC series among these over 225 members are identified from human genome. ^bSome member(s) of the family are expressed in brain or cerebral endothelial cells (CEC) at the mRNA, protein, or functional levels. ^cND: Not determined or unknown below the table.

2.1.1. Glucose Transporters

Glucose transporters in brain cells are essential for providing a sufficient supply of glucose, the preferred energy substrate of the brain. Two types of glucose transporters (GLUT), Na⁺-independent (facilitative) and Na⁺-dependent (secondary active), are classified into SLC2/GLUT and SLC5/SGLT families, respectively. The GLUT are expressed in all human cells and participate in glucose utilization, storage, and sensing.

2.1.1.1. SLC2/GLUT family: This family of transporters is comprised of 14 members in human, including GLUT1~12/SLC2A1~12, H⁺myo-inositol cotransporter (HMIT)/SLC2A13, and GLUT14/SLC2A14 (5,18,19). These are simple carriers catalyzing facilitated diffusion of glucose and related hexoses across plasma membranes along the electrochemical gradients. A common structural feature of these transporters is the presence of 12 transmembrane (TM) domains with intracellular N- and C-terminal ends and a unique N-linked oligosaccharide side-chain present either in the first or fifth extracellular loop. Signature sequences are present in all transporters (18). Based on sequence analyses, these transporters are classified into three subfamilies: class I (GLUT1-4), class II (GLUT6, 8, 10, and 12), and class III (GLUT5, 7, 9, 11, and HMIT). With the exception of GLUT7, 12, and 14, all the transporters are expressed in the brain (5,18). GLUT1, 4, and 5 are expressed in CEC (5,20), the 45 kDa GLUT1 isoform, GLUT2-6, 8, and HMIT are expressed in neurons, and GLUT1, GLUT5, and HMIT are expressed in glial cells (5,18,20-23).

The 55 kDa isoform of *GLUT1* is highly expressed at the BBB and displays a K_m of ~3 mM for glucose. Its distribution on CEC is asymmetrical with a higher density on the abluminal side (5:1, abluminal:luminal). Since the abluminal surface of brain capillaries is covered by astrocytic end-feet that express the 45 kDa isoform of *GLUT1*, astrocytes are also an important site of glucose uptake in the NV unit. In astrocytes, glucose is converted to lactate, which is delivered to neurons through the glial-specific monocarboxylic acid transporter-1 (MCT1) and neuron-specific MCT2. Lactate is then converted to pyruvate, which enters the tricarboxylic acid cycle to generate ATP. Glucose can also be taken up directly by neurons that express the neuron-specific *GLUT3*. The BBB GLUT1 is also involved in the transport of oxidized vitamin C (dehydroascorbic acid) into the brain where it is reduced to ascorbic acid (24,25).

GLUT4 is an insulin-sensitive glucose transporter with a K_m of ~5 mM. In contrast to *GLUT1* and *GLUT3*, which are distributed throughout the brain, in situ hybridization showed a discrete *GLUT4* mRNA expression in Purkinje cells in the cerebellum, in the vestibular nucleus, in the medulla oblongata, and in ependymal cells along the cerebral ventricles (22). Co-localization of GLUT4 and GLUT1 with the tight junction protein ZO-1 in vascular structures within the rat ventromedial hypothalamus has

also been demonstrated by immunohistochemistry (23). The vascular GLUT4 is thought to participate in brain sensing of blood glucose concentrations, whereas the neuronal GLUT4 is thought to rapidly provide additional glucose to neurons under conditions of high-energy demand (23).

GLUT5 is expressed in human microglial cells and CEC (5,20). Human GLUT5 transports fructose with a high $K_{\rm m}$ of ~6 mM and has a very low glucose transport activity (18). As fructose is not used as an energy source in the brain, the role of GLUT5 in CEC and microglia remains unclear. Recent studies suggested that reactive microglia stain for GLUT5 more intensely than resting microglia (26,27).

2.1.1.2. SLC5/SGLT family: The sodium/glucose cotransporter (SGLT) family is classified as SLC5 and has more than 220 members in bacterial and animal cells (28). Eleven members of the family (SLC5A1–11) have been identified in human. Nine of these have been functionally characterized, including six Na⁺/substrate cotransporters for glucose, myo-inositol, and iodide [SLC5A1/SGLT1, SLC5A2/SGLT2, SLC5A3/SMIT (Na⁺-dependent myo-inositol transporter), SLC5A6/SMVT (Na⁺-dependent multivitamin transport), SLC5A10/SGLT6, and SLC5A11/AIT (apical iodide transporter)], one Na⁺/Cl⁻/choline cotransporter (SLC5A7/CHT), one anion transporter (SLC5A5/NIS, e.g., Na⁺/I⁻ symporter), and one glucose-activated ion channel (SLC5A4/SGLT3) (28). *SLC5A3, SLC5A4, SLC5A6-8*, and *SLC5A10* are expressed in the brain and spinal cord (28), however none has been detected in brain vasculature. In contrast, SGLT1 and 2 are found in bovine cortical vessels and cultured bovine CEC (29) and likely participate in glucose transport across the BBB (30,31).

2.1.2. Amino Acid Transport Systems

Both Na⁺-independent and Na⁺-dependent amino acid transport systems have been implicated in amino acid transport across the BBB based on physiological and functional studies using different assay systems and inhibitors (5,32). Two Na⁺-independent systems have been identified in CEC: system L for large neutral amino acids and system y⁺ for cationic amino acids (5). Na⁺-dependent systems in CEC include systems A (all neutral amino acids), B⁰⁺, ASC (alanine, serine, and cysteine), N, X⁻_{AG} (anionic amino acids, e.g., L-glutamate), and the β -amino acid system (Table 2). Many genes encoding amino acid transporters have been cloned, characterized, and recently re-classified into different SLC families (Table 1: SLC1, 3, 6, 7, 17, 18, 32, 36, 38, and 43) according to amino acid identity of the encoded proteins (15). These transporters will be described as three groups: neutral, cationic, and anionic amino acid transporters.

2.1.2.1. Neutral amino acid transporters:

System L: System L is represented at the BBB as a Na^+ -independent transport system for large neutral amino acids (e.g., leucine) (33). Two genes

Transmort	Ganas or protains /	A mino orid	Tonio denendaria /		Exp	ression ^a
systems	SLC symbols	selectivity	Transport properties	$K_m \ \mu { m M}$	Brain	CEC
L	LAT1/SLC7A5	Large neutral	Na ⁺ -indep., 4F2hc/SLC3A2-associated	30-300	+	+
L	LAT2/SLC7A8	Large neutral	Na ⁺ -indep., 4F2hc/SLC3A2-associated	30-300	+	+
L-like	LAT3/SLC43A1	Neutral	Na ⁺ -indep., unknown protein-associated	6.58-1885	÷	ND ^b
L-like	mXAT1	ND	ON CIN	ŊŊ	ND	QN
L-like	hXAT2/mXAT2	ND	ND	ŊŊ	ND	ŊŊ
ASC	ASCT1/SLC1A4	Small neutral	Na ⁺ -dependent	9-464	+	+
ASC	ASCT2/SLC1A5	Small neutral	Na ⁺ -dependent	9-464	+	+
asc	Asc-1/SLC7A10	Small neutral	Na ⁺ -indep., 4F2hc/SLC3A2-associated	9-23	+	QN
asc	Asc-2/Slc7a12	Small neutral	Na ⁺ -indep., unknown protein-associated	2.9	÷	QN
asc?	AGT1/SIC7a13	Asp, Glu	Na ⁺ -indep., unknown protein-associated	20.1-25.5	ND	ŊŊ
p^{0+}	b ⁰⁺ AT/SLC7A9	Neutral/	Na ⁺ -indep., rBAT/SLČ3A1-associated	88	+	Ŋ
		cationic				
${f B}^{0+}$	атв ⁰⁺ /SI Сба1/	cystine Neutral/	No+/Cldenendent	73 _140		
a		louual/	ina / ci -ucpetituciti	0+1-07		
V	ATA1/SLC38A1	cautoinc Neutral/N-Me	Na^+ -dependent	> 200	+	+
V	ATA7/SIC38A7	group Neutral/N_Me	Na+.denendent	/ 200	4	4
L		ALLAL ALLAL	ina -ucputation	/ 200	F	F
A	ATA3/SLC38A4	group Neutral/N-Me	Na^+ -dependent	> 200	+	+
¢	ΡΔΤΙ /ςΙ C36Δ1	group Small nautral	H ⁺ -denendant	7_8 5mM	-	
		GABA D-		TATTIC:0-7	_	2
		Ala, Pro, Ser,				
		MeAIB				
ż	PAT2/SLC36A2	Small neutral	H ⁺ -dependent	$100 \sim 600$	+	QN
~	PAT3-4/	ND	ND	ND	ND	ND

Table 2Amino Acid Transport Systems

																											(pənı
	QZ	Q	Q	QZ	Ŋ		Ŋ		QN	Q	~∙		¢.	¢-•	+	+	+	Q	Q	qz		Q	g	Ð	+	g	(Contin
Ubiqui- tous	+	+	+	+	ND		+		+	+	+		+	¢.	+	+	+	+	+	+		+	+	+	+	+	
70-250	2150-5200	38-380	40-910	QN	340		6-10		150-1600	150 - 1600	Gu etal	2000	Gu etal., 2001	Gu etal 2003	\sim 14	\sim 14	\sim 14	18-97	18-97	40-92		17	17	1-20	629	1-20	
Na ⁺ -indep., CAT-associated transport	ND ND	NA ⁺ (neutral); Na ⁺ -indep. (cationic), 4F2hc/	SLC3A2-associated	NA ⁺ (neutral); Na ⁺ -indep. (cationic), 4F2hc/	DLCJA2-associated	Na^{T}/H^{T} -dependent	Na^+/H^+ -dependent	Na^+/H^+ -dependent		Na^+/H^+ -dependent high affinity transporter	Na^+/H^+ -dependent low-affinity transporter	$Na^+/H^+/K^+$ -dependent	$Na^+/H^+/K^+$ -dependent	$Na^+/H^+/K^+$ -dependent	$Na^+/H^+/K^+$ -dependent	$Na^+/H^+/K^+$ -dependent	Na ⁺ -indep., 4F2hc/SLC3A2-associated		Na^+/Cl^- -dependent								
Cationic	Cationic	Cationic	Cationic	ND	Neutral/	cationic	Neutral/	cauomic	Gln, Asn, His	Gln, Asn, His	His, Glu		Glutamine	Alanine	Glu. Asp	Glu, Asp	Glu, Asp	Glu, Asp	Glu, Asp	Cystine/	glutamate	Glycine	Glycine	GABA	GABA	GABA	
SLC36A3-4 CAT-1/SLC7A1	CAT-2A/SLC7A2	CAT-2B/SLC7A2	CAT-3/SLC7A3	CAT-4/SLC7A4	y+LAT1/SLC7A7		y+LAT2/SLC7A6		SN1/SLC38A3	SN2/SLC38A5	mHAT (mouse)		mNAT2 (mouse)	mNAT3 (mouse)	EAAT3/SLC1A1	EAAT2/SLC1A2	EAAT1/SLC1A3	EAAT4/SLC1A6	EAAT5/SLC1A7	xCT/SLC7A11		GLYT1/SLC6A9	GLYT2/SLC6A5	GAT1/SLC6A1	GAT2/SLC6A13	GAT3/SLC6A11	
\mathbf{y}^+	\mathbf{y}^+	\mathbf{y}^+	y +	y +	$\mathbf{y}^+\mathbf{L}$		$\mathbf{y}^+\mathbf{L}$;	Z	Z	Z		Z	Z	X^{-AG}	$\mathbf{X}^{-}_{\mathbf{AG}}$	$\mathbf{X}^{-}_{\mathbf{AG}}$	X^{-AG}	${\rm X}^{-}_{ m AG}$	$\mathbf{x}^{-}\mathbf{c}$		Gly	Gly	Beta-like	Beta-like	Beta-like	

Tranenort	Genes or proteins /	A mino acid	Tonic devendancy/		Expr	ession ^a
systems	SLC symbols	selectivity	Transport properties	$K_m \ \mu \mathrm{M}$	Brain	CEC
Beta-like	BGT1/SLC6A12	Betaine, GABA	Na^+/CI^- -dependent	679	+	+
Beta-like	TAUT/SLC6A6	Taurine	Na ⁺ /Cl ⁻ -dependent	3-13	+	ŊŊ
Beta or Gly?	VIAAT/SLC32A1	GABA, glycine	H ⁺ -dependent, vesicular inhibitory amino acids	¢.	+	ND
÷ č	vglut1-3/ SLC17A6-8	Glu	H ⁺ -dependent, vesicular glutamate	$\sim 2 \text{mM}$	+	-/ND
ć:	VMAT1-2/ SLC18A1-A2	5-HT, dopa, Histamine	H ⁺ -dependent, vesicular amines	~∙	+	
ć	VAChT/ SLC18A3	Acetylcholine	H ⁺ -dependent, vesicular amines	~•	+	
c-•	PAT1-2/ SLC36A1-2	Gly, Ala, Pro, GABA, D- Ala, MeAIB	H^+ -dependent	¢.	+	QN
÷	NET1/SLC6A2	Norepinephr- ine, dopa	Na^+/Cl^- -dependent	~•	+	QN
~	DAT/SLC6A3	Dopamine	Na^+/CI^- -dependent	¢	+	ŊŊ
ż	SERT/SLC6A4	Serotonin	$Na^+/CI^-/K^+$ -dependent	÷	+	QN
÷	PROT/SLC6A7	L-proline	Na ⁺ /Cl ⁻ -dependent	ż	+	QN
÷.	CT1/SLC6A8	Creatine	Na^+/CI^- -dependent	¢	Ubiqui-	
ć	CT2/SLC6A10	Creatine	Ionic independent	~	ND	ŊŊ
^a The expressi ^b ND: not de	on was determined at m termined or unknown.	RNA or/and protei	ı levels (not by physiological means).			

 Table 2
 Amino Acid Transport Systems (Continued)

Abbreviations: Ala, alanine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; N-Me group, N-Methy-lated group; Pro, praline; Ser, serine; XAT: 'X' amino acid transporter; MeAIB, alpha(methyl)aminoisobutyric acid; GABA, gamma-aminobutyric acid.

encoding system L (*LAT1/SLC7A5* and *LAT2/SLC7A8*), expressed in both brain tissue and CEC, have been recently cloned and characterized (34,35). The protein product of each gene forms a heterodimeric functional transporter complex with a type-II membrane glycoprotein 4F2hc (CD98) encoded by *SLC3A2* (e.g., LAT1/4F2hc or LAT2/4F2hc) (5,33). LAT1/4F2hc transports large neutral L-amino acids, triiodothyronine (T3) and thyroxine (T4), and L-dopa (36), while LAT2/4F2hc transports large and small neutral L-amino acids across the BBB (33).

Two other genes partially encoding system L functions, $y^+LAT1/SLC7A7$ and $y^+LAT2/SLC7A6$, have been identified as system y^+L transporters (Table 2) (33). Although y^+LAT2 transport activity was detected in cultured neurons (37), its cellular distribution in the brain remains unclear. The expression of both genes has been detected in human placental microvascular endothelial cells. y^+LAT1 and 2 form heterodimeric complexes with 4F2hc/SLC3A2 (e.g., $y^+LAT1/4F2hc$, $y^+LAT2/4F2hc$) and transport large neutral L-amino acids in a Na⁺-dependent fashion as well as cationic amino acids in a Na⁺-independent manner (33).

System ASC: System ASC is a Na⁺-dependent transport system for small neutral amino acids (alanine-serine-cysteine) (5,38). Two genes encoding system ASC function, *SLC1A4/ASCT1* and *SLC1A5/ASCT2*, have been cloned and characterized (Table 2) (38). One study showed that *ASCT1* was transiently expressed in cerebral capillary endothelial, neuroepithelial, and neuronal cells of mouse embryonic and neonatal brains, with the expression restricted to astroglial cells after birth (39). Another study detected both *ASCT1* and *ASCT2* mRNA in cultured mouse CEC cells (40). Immunohistochemical analyses localized ASCT2 to the abluminal side of the mouse BBB. Both ASCT1 and ASCT2 transport L-alanine, L-serine, and L-cysteine, while ASCT2 additionally transports L-threonine and L-glutamine (40).

System ASC transports small neutral amino acids in a Na⁺-independent manner (5). Two genes encoding the functional *Asc-1/SLC7A10* and *Asc-2/Slc7a12* have been identified (Table 2) (33). *Asc-1* is expressed in the brain (41) and its gene product forms a functional complex with 4F2hc that transports small neutral L- and D-amino acids (33). Asc-2 and a close homologue of Asc-2, Slc7a13/AGT1 (aspartate/glutamate transporter 1), form complexes with unknown protein(s) (42,43). Their distribution and functional roles are still unknown (44).

System A: System A is a Na⁺-dependent transport system for small neutral amino acids (proline, alanine, glycine, methionine, and glutamine) at the abluminal side of the BBB. Recently, three genes, all expressed in CEC, have been identified as system A isoforms, *amino acid transporter* A1 (ATA1)/SLC38A1, ATA2/SLC38A2, and ATA3/SLC38A4 (45). The expression of ATA2 and ATA3 mRNA is 93-fold and 2140-fold higher, respectively, than that of *ATA1* in mouse CEC (44,46). ATA2 is also involved in efflux transport of L-proline at the BBB (46).

Systems b^{0+} and B^{0+} : An amino acid transport system with a broad specificity was initially described in mouse blastocysts and named system b^{0+} (47). The symbols "⁰⁺" or "^{0,+}" indicate that the system is able to accept amino acids with a zero or +1 charge (47). System b^{0+} and system B^{0+} transport both neutral and cationic amino acids in a Na⁺-independent and a Na⁺-dependent manner, respectively. Physiological studies indicated that the system B^{0+} is present in porcine CEC, while system b^{0+} is expressed in peripheral endothelial cells (5). A gene encoding the system B^{0+} function has been identified as $ATB^{0+}/SLC6A14$. SLC6A14 is a Na⁺/Cl⁻-dependent transporter with a broad specificity for neutral amino acids and a high affinity for both cationic and neutral amino acids (5,48). Two genes encoding system b^{0+} function, *SLC7A9* and *SLC3A1*, are expressed in the brain (33,49). Their products form a heterodimeric transporter complex (SLC3A1/SLC7A9) that carries large neutral amino acids and cationic amino acids (33,36,49,50).

SLC36 family: SLC36 family genes encode four proton/amino acid transporters (PAT1-4/SLC36A1-4) driven by a proton gradient (51). Both PAT1 and 2 mediate 1:1 symport of protons and small neutral amino acids (such as glycine, alanine, and proline) generated by extracellular or intracellular proteolysis. Their mRNAs are widely expressed in mammalian tissues, including the brain. The encoded proteins have been localized to neurons (51–53).

System N: System N is defined as a Na⁺-dependent transport system for amino acids characterized by the presence of nitrogen in the side chain (L-glutamine, L-histidine, and L-asparagine). Functional studies demonstrated the presence of system N at the luminal side of the blood-brain and blood-cerebrospinal fluid (CSF) barriers (55–59). At least two genes encoding system N function, SLC38A3/SNAT3 and SLC38A5/SNAT5, have been identified in the brain (45). A close homologue of SLC38A3, SLC38A6, was also detected in the brain, but its function remains unknown (45).

2.1.2.2. Cationic amino acid transporters: Different transport systems mediate cationic amino acid transport, including Na⁺-independent systems y^+ , y^+L , and b^{0+} , and Na⁺-dependent systems B^{0+} and N (Table 2). Systems y^+L , b^{0+} , B^{0+} , and N have been described in Sec. 2.1.2.1. System y^+ is the major Na⁺-independent system for cationic amino acid transport expressed at the BBB. Four genes encoding system y^+ function, *CAT-1/SLC7A1*, *CAT-2/SLC7A2*, *CAT3/SLC7A3*, and *CAT4/SLC7A4*, are expressed in the brain (36,54,60). CAT1 protein, expressed in CEC, is an arginine transporter that forms a complex with the endothelial nitric oxide synthase (eNOS) (61,62). CAT1, 2, and 3 transport cationic L-amino acids, whereas functional properties of CAT4 have not been studied (63).

2.1.2.3. Anionic amino acid transporters: System X_{AG}^- has been defined as the major carrier system for anionic amino acids (5). Negatively charged amino acids (aspartate and glutamate) are major excitatory neuro-transmitters in the brain. Four transporters encoding system X_{AG}^- function, EAAT1/SLC1A3, EAAT2/SLC1A2, EAAT3/SLC1A1, and EAAT4/ SLC1A6 (Table 2), have been detected in neuronal and/or glial cells (38). These transporters maintain low glutamate concentrations in brain extracellular fluid. Both aspartic and glutamic acid are transported across the BBB in a Na⁺/H⁺/K⁺-dependent manner. Molecular and physiological studies confirmed the expression of *EAAT1*, 2, and 3 at the abluminal surface of bovine CEC (57). The detailed review of their transport mechanisms has recently been published (38).

The principal inhibitory neurotransmitter in the CNS, γ -aminobutyric acid (GABA), is synthesized from glutamic acid through glutamate decarboxylase. GABA is transported by Na⁺/Cl⁻-dependent β -amino acid system transporters (Table 2). GAT-1/SLC6A1 and GAT-3/SLC6A11 are mainly present in neuronal and glial cells, while GAT-2/SLC6A13 and BGT-1/SLC6A12 are found in the BBB, where they co-localize with multi-drug resistance-1 P-glycoprotein (MDR-1 P-gp) (64). Some members of SLC22 family are also involved in neurotransmitter transport. Other transporters for neurotransmitters are listed in Table 2.

2.1.3. Nucleoside Transport Systems

Brain cells are incapable of *de novo* nucleoside synthesis and depend on nucleoside supply from the circulation. Purine and pyrimidine nucleosides and their metabolic products are precursors of DNA and RNA synthesis. The purine nucleoside, adenosine, is a signaling molecule that modulates neuronal and cerebral vascular functions by interacting with receptors on brain cells and CEC (65). Furthermore, a number of nucleoside analogs used to treat viral infections (HIV, hepatitis C), leukemia, tumors, and cardiac diseases (3,4), are substrates of nucleoside transporters (NT). Nucleoside transport is mediated by both low- and high-affinity systems. Traditional classification of nucleoside transport systems is based on the transport mechanisms (e: equilibrative; c: concentrative), the sensitivity to nitrobenzylmercapto-purine riboside (NBMPR) (s: sensitive; I: insensitive), and the substrates (3.4). The high-affinity system is active, concentrative, and Na⁺-dependent, whereas the low-affinity system is equilibrative and Na⁺-independent (66.67). A recent study using rat immortalized CEC (RBE4 cells) suggested that nucleoside transport at the BBB is mediated through both equilibrative (ei and es transporters) and concentrative [cit (ci subtype inhibited by thymidine) and cif (ci subtype inhibited by formvcin-B)] transport systems (68). Currently, these NT are classified within the solute carrier superfamily as the concentrative NT family SLC28 and the equilibrative NT family SLC29 (66,67).

2.1.3.1. SLC28 family: The SLC28 family encodes Na⁺-dependent concentrative nucleoside transporters (CNT). Three members of SLC28 have been cloned and characterized: SLC28A1/CNT1, SLC28A2/CNT2, and SLC28A3/CNT3 (67–74). CNT2 was cloned from a rat BBB cDNA library as an adenosine transporter (70,71). Both CNT1 and CNT2 are expressed in an immortalized mouse CEC line (69,72), whereas CNT3 is expressed in the brain (73). Physiological data suggest luminal localization of the concentrative systems.

CNT1 transports a wide range of substrates, including natural pyrimidine and purine (adenosine) nucleosides, antiviral nucleoside analogs (AZT/ zidovudine, 3TC/lamvudine, and ddC/zalcitabine), cytidine analogs for chemotherapy (cytarabine/AraC and gemicitabine/dFdC), and a metabolite (5'-deoxy-5-flurouridine) of fluoropyrimidine capecitabine (67). In contrast, CNT2 has a limited substrate spectrum, which includes natural purine nucleosides, uridine, and antiviral agents [2',3'-dideoxyinosine (ddI) and ribavirin]. CNT3 transports natural purine, pyrimidine nucleosides, and anti-cancer and anti-viral nucleoside analogs (67).

2.1.3.2. SLC29 family: The SLC29 family encodes Na⁺-independent equilibrative nucleoside transporters (ENT). Physiological studies have defined two types of transport systems based on their sensitivity to NBMPR. es-type (equilibrative and sensitive to NBMPR) and ei-type (equilibrative and insensitive to NBMPR). Four genes of SLC29 family have been identified: SLC29A1/ENT1 (es-type), SLC29A2/ENT2 (ei-type), SLC29A3/ ENT3, and SLC29A4/ENT4, all of which are widely expressed in various tissues (66). RT-PCR analyses confirmed that both ENT1 and ENT2 are expressed in mouse and rat CEC (69,72,75,76). ENT1 transports purine and pyrimidine nucleosides with $K_{\rm m}$ values ranging from 50 μ M (adenosine) to 680 µM (cytidine), poorly transports antiviral nucleoside analogs ddC and ddI and is unable to transport uracil and AZT. ENT2 transports purine and pyrimidine nucleosides with lower affinity with the exception of inosine. In addition, ENT2 is capable of transporting antiviral agents (AZT, ddC, and ddI) and purine and pyrimidine nucleobases, except cytosine. NBMPR inhibits ENT1-, but not ENT2-mediated transport (66). ENT3 and ENT4 have been recently identified but little is known about their function and expression profile (77).

2.1.4. Other Nutrient Transporters

A number of other transporters involved in nutrient trafficking, such as acetyl-CoA (SLC33) (78), minerals or metals (SLC11, 30, 31, 39, 40, and 41) (15,79–83), folate and thiamine (SLC19) (84), and carboxylate and sulfate (SLC13) (85) (Table 1) are expressed in the brain or at the BBB. Several SLC families that are likely important in maintaining CNS homeostasis encode transporters for urea (SLC14) (86), ammonium (SLC42) (15), and

bile acids (SLC10) (87) (Table 1), but little is known regarding their expression and function at the BBB.

2.2. Transport Systems for Peptides and Proteins

Peptides are important in the regulation of neurotransmission, neuromodulation, neuroendocrine activity, cerebral blood flow, CSF secretion, and modulation of BBB permeability to nutrients. Peptides are also implicated in the pathogenesis of brain disorders, such as Alzheimer's disease, depression, and stroke. Several transport pathways are involved in peptide and protein passage across the BBB, including oligopeptide transporters (SLC15), adsorptive- and receptor-mediated endocytosis (AME and RME) and transcytosis (3).

2.2.1. SLC15 Family

The SLC15 family (Table 1) encodes four proton oligopeptide transporters (PEPT) that utilize a proton-gradient force for uphill transport of short chain peptides and peptido-mimetics (B-lactam antibiotics, angiotensinconverting enzyme inhibitors, antiviral drug valacyclovir, and anticancer drug bestatin) (88,89) into intestinal and renal epithelial cells as well as across the BBB and blood-CSF barrier (90-92). All SLC15 family genes are expressed in the brain (90). The prototype transporters of the family, PEPT1/SLC15A1 and PEPT2/SLC15A2, are expressed in the BBB and choroids plexus (93-95) and mediate the uptake of essentially any possible di- and tripeptide regardless of the substrate's net charge. Recent studies suggest that tetrapeptides (e.g., opioid peptide) as well as various drugs and pro-drugs with peptide-like structures are also carried by these transporters (91). Two recently identified members of the family, PHT1/ SLC15A4 and PHT2/SLC15A3, transport 5-aminolevulinic acid, free histidine, and certain other di- and tripeptides (89.96). Studies in mice lacking *PEPT2* (*PepT2^{-/-}*) indicated that PEPT2 is the principal peptide and peptido-mimetic transporter at the blood-CSF barrier (94).

2.2.2. Endocytosis

Endocytosis is a vesicular transport pathway used for internalization of extracellular fluid, particles of <500 nm, and membrane molecules or ligands (3). Although the CEC exhibit a low number of endocytotic vesicles, several endocytotic pathways involving delivery of peptides and proteins across the BBB have been described.

Adsorptive-mediated endocytosis: Adsorptive-mediated endocytosis (AME) and transcytosis are triggered by electrostatic interactions between the positively charged moiety of the substrate and the negatively charged cell membrane and are typically used for the transport of larger peptides across the BBB. AME has a low affinity but a high capacity for peptide transport

compared to RME. Peptides transported across the BBB via the AME pathway include cationized albumin (97,98) and IgG (99), histone (100), a dynorphine analog E-2078 (101), an ACTH analog (ebiratide) (102), and the arginine-vasopressin fragment 4–9 (103). Studies demonstrated that AME transport efficiency depends on cationic charge and lipophilicity of the peptides, but not peptide size (104).

Receptor-mediated endocytosis: The RME and transcytosis have been extensively investigated as a pathway for therapeutic delivery. Certain receptors, such as those for transferrin and low-density lipoprotein (LDL), are selectively enriched on the luminal surface of the BBB (2,3). Upon activation by ligand binding, these receptors are internalized and either transported via the early endosome to the lysosomes or transcytosed across the endothelial layer where the ligands are externalized and the receptors shuttled back to the luminal surface (3). Internalization occurs via clathrin-coated vesicles (coated pits). Clathrin adaptor-protein 2 (AP-2) complex and dynamin are main structural components of the coated pits. The AP-2 adaptor complex acts as a linchpin molecule that simultaneously binds to both clathrin and the receptors and plays a role in the recognition of internalization motifs of extracellular receptors (3,105). Several other adaptors with similar functions have been identified (105,106). Uptake of particles or ligands mediated by RME is saturable as this process is dependent on the number of available receptors on the cell surface. In contrast to peptide transporters that carry small peptides, the RME pathway is used for transport of large molecules across the BBB. Several receptors expressed at the CEC have been shown to undergo RME, including transferrin receptor (3), insulin growth factor receptor (3), low-density lipoprotein receptor (LDLR) -related proteins (LRP) (107,108), scavenger receptor (SR) (109,110), and receptor for advanced glycation end products (RAGE) (111,112). The LRP, SR, and RAGE have been implicated in the transcytosis of β -amyloid peptides and lipoproteins across the BBB (107-112).

Endocytosis also occurs via non-coated invaginations, caveolae. Caveolae are not associated with an electron-dense cytoplasmic coat and can therefore be distinguished from clathrin-coated invaginations (RME) by electron microscopy (3). Caveolae are coated with scaffolds of three caveolin proteins (caveolin-1, 2, and 3). Caveolins-1 and -2 have been detected in human CEC (113). Numerous receptors and signaling molecules are associated or interact with caveolae, including MDR-1 P-gp, receptors for insulin-like growth factors, NOS, protein kinase C, etc. (113,114). Several in vitro studies have shown that caveolae participate in trafficking of various molecules across the BBB, but their importance for BBB transport capacity in vivo remains controversial (3).

In addition to peptide and protein transport pathways described above, members of the Oatp/OATP family are involved in the transport of opioid peptides across the BBB and the blood–CSF barrier (115).

2.3. Blood-Brain Barrier and Brain Ion Balance

Various ion transporters regulate brain ion balance, which is critical for CNS homeostasis, neuronal excitability, and brain cell microenvironment. These transporters are categorized into two superfamilies, the organic anion transporter polypeptide (Oatp for rodent and OATP for human)/SLC21/SLCO family and the SLC22 family. The SLC22 family is further classified into organic anion transporters (OATs), organic cation transporters (OCTs), and zwitterions/cation transporters (OCTNs) (116,117). Many members of the OATP/SLCO/SLC21 and SLC22 families are expressed in the brain and participate in ion influx and efflux at the BBB.

2.3.1. OATP/SLCO/SLC21 Family

The flux of negatively charged compounds across the BBB is limited by the negative charge of CEC plasma membranes. OATPs are Na⁺-independent multispecific organic anion transport proteins that mediate TM transport of a wide range of endogenous and exogenous amphipathic organic anion compound, including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides, many drugs, and xenobiotics (118). Since the SLC21 classification does not permit an unequivocal and species-independent identification of genes and gene products, all OATPs/Oatps are now classified within the OATP/SLCO superfamily (118). Among 52 members of this superfamily, 36 members have been identified in human, rat, and mouse. Their proteins share structural similarity with 12 predicated-TM domains and contain a signature of the superfamily [D-X-RW-(I,V) -GAWW-X-G-(F,L) -L] (118).

Several members of the OATP/SLCO family are expressed in the brain (118). *OATP-A/SLCO1A2*, *Oatp2/Slco1a4*, and *OATP-F/SLCO1C1* (*Oatp14/Slco1c1*) have been detected in the BBB (118), while *Oatp1/Slco1a1*, *Oatp2/Slco1a4*, *Oatp3/Slco1a5*, and *OATP-F/SLCO1C1* (*Oatp14/Slco1c1*) are found in the choroid plexus (CP) (118). In addition, *SLCO2A1/Slco2a1*, *SLCO3A1/Slco3a1*, and *SLCO4A1/Slco4a1* are ubiquitously expressed (118).

Oatp2 is localized on both the luminal and abluminal surface of rat CEC and on the abluminal side of the CP (4,119). Oatp2 transports 17 β -estradiol-D-17 β -glucuronide (E-17 β -G) into the brain and extrudes digoxin from the brain (119). Other substrates of Oatp2 include bile acids, taurocholate, cholate, and oubain (3,4).

Human OATP-A shares 73% amino acid identity with rat Oatp2 and is highly expressed in brain microvessels and capillaries but not in astrocytes or neurons (119). OATP-A transports organic compounds including sulfated and glucuronidated steroids [dehydroepiandrosterone sulfate (DHEAS), estrone-3-sulfate, E-17 β -G, T3, and T4], drugs (fexofenadine), cationic compounds (ADP-ajmalinium and rocuronium), and neuroactive opioid peptides {deltorphin II and [D-Pen(2),D-Pen(5)] enkephalin} (3,115,119,120).
Rat Oatp14/Slco1c1 was identified and cloned as a BBB-specific anion transporter 1 (BSAT1) (121) enriched in cerebral capillaries compared to whole brain. Oatp14 predominantly localizes to the luminal surface of CECs and mediates unidirectional transport of T3, E-17 β -G, cerivastatin, and troglitazone sulfate, and bidirectional transport of T4 in transfected human embryonic kidney 293 cells (122). *OATP-F/SLCO1C1*, the human homologue of rat *Oatp14*, is also highly expressed in the brain (123). OATP-F shares 47–48% amino acid identity with OATP-A, OATP-C, and OATP-8. OATP-F mediates high affinity transport of T4 and T3 and may be important in the distribution of thyroid hormones within the brain (123).

2.3.2. SLC22 Transporter Family

SLC22 family is composed of at least 18 members (SLC22A1–SLC22A18) that are poly-specific for multiple substrates (116). The conserved structural features of these transporters include 12 predicated α -helical TM domains and one large extracellular loop between TM domains 1 and 2. These transporters act in three different ways: (a) as uniporters mediating facilitated diffusion in either direction (OCTs), (b) as anion exchangers (OAT1, OAT3, and URAT1), and (c) as Na⁺/L-carnitine cotransporter (OCTN2). They perform homeostatic functions in the brain and heart and participate in the absorption and/or excretion of drugs, xenobiotics, toxins, and endogenous compounds in intestine, liver, and kidney. The endogenous substrates of SLC22 family include monoamine neurotransmitters, choline, L-carnitine, α -ketoglutarate, cAMP, cGMP, prostaglandins, and urate (116).

2.3.2.1. OAT family: Five human members of this family, hOAT1 (SLC22A6), 2 (SLC22A7), 3 (SLC22A8), 4 (SLC22A11), and 5 (SLC22A10) (116), are grouped into three classes based on their energy needs: (a) Na⁺-dependent OATs, (b) Na⁺-independent facilitators or exchangers, and (c) active OATs that require ATP. Na⁺-dependent OATs have a narrow substrate spectrum, whereas Na⁺-independent OATs and active OATs have broad substrate specificities. *OATs* are mainly expressed in kidney and liver (4,124); *OAT1* and 3 are also expressed in the brain and the BBB (125,126). OAT3 is responsible for brain-to-blood efflux of homovanillic acid, neurotransmitter metabolites, indoxyl sulfate (uremic toxin), and E-17β-G at the abluminal membrane of CEC (126–129). In vitro and in vivo studies have demonstrated that both OAT1 and 3 are also involved in the transport of anti-HIV drugs across the BBB (126,128,129).

2.3.2.2. Organic cation transporters: There are 10 OCTs in the SLC22 family, three of which (OCT1/SLC22A1, OCT2/SLC22A2, and CAT3/SLC22A3) have been well characterized. They transport organic cations including weak bases, monoamine neurotransmitters, choline, co-enzymes, drugs, and xenobiotics by an electrogenic Na⁺-independent and direction-reversible transport process (116). The energy for the transport

is supplied by an electrochemical gradient of the transported organic cation. OCT2 and 3 are expressed in the rat CP, in human neurons, and in the rat and mouse BBB (130–132). The substrate spectra of OCT1, 2, and 3 overlap such that the same substrates are transported with different affinities by different transporters (116). In vitro studies showed that organic cation uptake mediated by human OCT1 (hOCT1) and hOCT2 is inhibited by some non-steroidal anti-inflammatory drugs (NSAIDs) (133). The substrates of hOCT2 include TEA (tetraethylammoniun), MPP (1-methyl-4-phenylpyridinium), choline, dopamine, histamine, norepinephrine, serotonin, amantadine, cimetidine, and memantine (116). hOCT2 participates in the regulation of interstitial and intracellular concentrations of monoamine neurotransmitters and cationic drugs in the brain (116). Choline, a precursor of the neurotransmitter acetylcholine, enters the brain by both saturable and non-saturable transport at the BBB (134,135). The saturable process is Na^+/H^+ -independent and is driven by the membrane potential as an energy source (135). OCT2 localizes to the apical membrane of CP and mediates choline transport across the ventricular membrane of the CP (136).

2.3.2.3. Zwitterions/organic cation transporters: A new family of H⁺gradient dependent organic cation/carnitine transporters has been identified as OCTNs (116). Zwitterions (hybrid ions) have both a positive and a negative charge in each of the organic ion molecules (137). Two genes encoding OCTN, *OCTN1/SLC22A4* and *OCTN2/SLC22A5*, have been cloned from human and mouse (116,121,138–140). Both *OCTN1* and 2 are expressed in the brain (121,138,139). OCTN2 is involved in the transport of L-carnitine, acetyl-L-carnitine, and β -lactam antibiotics from the circulation into the brain (139–142).

2.3.3. Other Solute Carriers for lons

A number of other SLC families encode carriers for ion exchange or ion transport across cellular membranes. Many of them are expressed in the brain or at the BBB (Table 1), including Na⁺/Ca²⁺ exchangers (SLC8) (143), Na⁺/H⁺-exchangers (SLC9) (144), electroneutral cation-Cl-cotransporters (SLC12) (145), the type-III Na⁺-phosphate cotransporters (SLC20) (146), Na⁺/(Ca²⁺-K⁺) exchangers (SLC24) (147), multifunctional anion exchangers (SLC26) (148), the type-II Na⁺-phosphate cotransporters (SLC34) (149), and the sugar-phosphate/phosphate exchangers (SLC37) (150).

2.4. Drug Efflux Systems

The multidrug resistance (MDR) phenotype of the BBB results from the expression of drug efflux transporters in CECs (3,151–155). In addition to restricting drug entry into the brain, efflux transporters are important in detoxifying harmful metabolites in the CNS and in reducing CNS side effects of drugs that have pharmacological targets in peripheral tissues. The most prominent efflux system at the BBB is the ABC transporter superfamily (16,17,155). ABC transporters are ATP-dependent TM proteins with 49 members in human classified into seven subfamilies, including ABCA (12 members), ABCB (11 members), ABCC (13 members), ABCD (4 members), ABCE (1 member), ABCF (3 members), and ABCG (5 members) subfamilies (16,17). Substrates of these transporters include metabolites, peptides, lipids, cholesterol, drugs, and xenobiotics (16,17,155). At least 19 ABC transporters are expressed in the human brain; 10 of these are also expressed in human CEC (Table 3, unpublished data).

2.4.1. ABCB1/MDR-1 P-Glycoprotein

MDR was first observed in cell lines selected by anti-cancer drugs (157–159). This phenotype is frequently associated with the overexpression of a 170 kD membrane protein encoded by *mdr-1* gene, known as P-glycoprotein (MDR-1 P-gp). ABCB1/MDR-1 P-gp acts as an ATP-dependent drug efflux pump with a broad substrate spectrum, resulting in resistance to a variety of structurally and functionally unrelated cytotoxic agents in MDR cells and tumors in vivo (157–160). In 1992, several groups reported that MDR-1 P-gp is expressed at the luminal surface of porcine, murine and bovine CEC cultures and functions as a BBB drug efflux pump (161–163). Mice lacking *mdr1*-type P-gp (*mdr1a^{-/-}* or *mdr1a/b^{-/-}* mice) displayed severe functional BBB deficits to neurotoxins (ivermectin) (151–155). Extensive in vitro and in vivo studies have provided solid evidence that MDR-1 P-gp is a major drug efflux pump at the BBB as well as a formidable opponent for drug delivery into the CNS (164–168).

It is estimated that about 50% of drug candidates may be substrates of MDR-1 P-gp (169). These substrates include analgesics (asimadoline and morphine), anti-epileptic drugs, anthracyclines (daunorobicin, doxorubin, and epirubcin), anthracenes (bisantrene and mitoxantrone), vinca alkaloids (vinblastine, vincristine, etc.), camptothecin derivatives (CPT-11 and topote-can), epipodophyllotoxins (etoposide and teniposide), tubulin polymerizing drugs (colchicines, paclitaxel, etc.), antibiotics (actinomycin D and erythromycin), HIV-1 protease inhibitors (ritonavir, saquinavir, and indinavir), digoxin, calcium channel blockers, immunosuppressive agents (cyclosporine A and FK506), corticoids, pesticides, and fluorophores (calcein-AM, rhodamine 123, Hoechst 33342/33258, etc.) (157,158). A number of inhibitors or chemosensitizers have been identified and developed for MDR-1 P-gp, including PSC833, LY335979, cyclosporine A, verapamil, quinidine, GF120198, reversin 121 and 125, xenova (XR5944 and XR9576), and OC144–093 (155).

In vitro studies have demonstrated that MDR-1 P-gp also transports lipids, cholesterol, and peptides (such as β -amyloid1-40) (16,17,170), but its role in lipid and peptide transport across the BBB remains elusive. Other members of the ABCB subfamily (*ABCB4*, *B6*, *B7*, *B8*, and *B9*) are also expressed in the human brain (Table 3) and CECs (*ABCB9*) (171) but their functional properties have not been characterized.

Symbol	Brain	CEC ^a	Function/Disease
ABCA1	+	+	Cholesterol efflux from cells onto HDL ^a ; Tangier disease; FHDLD ^a
ABCA2	+	ND^{a}	Transport of steroids, lipids and related molecules? drug resistance?
ABCA5	+	+	Cholesterol/sterol transport?
ABCB1	+	+	Multidrug resistance/BBB; transport of drugs, lipid, sterol, peptides
ABCB4	+	ND	Phosphatidycholine and cholesterol transport; Cholestasis; ICP ^a
ABCB6	+	ND	Ion transport
ABCB7	+	ND	Fe/S cluster transport (sideroblastic anemia and ataxia)
ABCB8	+	ND	?
ABCB9	+	+	Associated with lysosomal markers (function?)
ABCC1	+	+	Multidrug resistance/BBB: transport of glutathione- conjugated drugs
ABCC2	+	+	Organic anion efflux (D-J syndrome ^a), drug resistance
ABCC4	+	?	Nucleoside transport, resistance to nucleoside analogues
ABCC5	+	+	Nucleoside transport, resistance to nucleoside analogues
ABCC8	+	?	Sulfornylurea receptor; FPHHI ^a
ABCF1	+	ND	?
ABCF2	+	ND	?
ABCG1	+	+	Cholesterol transport
ABCG2	+	+	Toxin efflux; drug resistance; sterol transport
ABCG4	+	+	Cholesterol transport

 Table 3
 ABC Transporters Expressed in Human Brain Tissues and CEC

^aCEC, cerebromicrovascular endothelial cells; HDL, high-density lipoprotein; FHDLD, familial hypoapoproteinemia; FPHHI, familial persistent hyperinsulinemic hypoglycemia of infancy; ICP, intrahepatic cholestasis of pregnancy; D-J syndrome, Dubin-Johnson syndrome; ND, not determined.

2.4.2. ABCC/MRP Family Proteins

The MRP/ABCC family has 13 members, from which several are involved in drug transport (155,172) and are expressed in the brain (Table 3).

ABCC1/MRP-1: ABCC1/multidrug resistance-associated protein-1 (MRP-1) was first identified in Pgp-negative human lung cancer cell lines selected by the anti-cancer drug doxorubicin (173,174). MRP-1 is a 190 kDa-membrane organic anion/GS-X pump (glutathione-X conjugate pump) that acts as an ATP-dependent efflux transporter for substances conjugated or co-transported with glutathione or glucuronide (17,175). It is estimated that about 20% of all drug candidates may be substrates of

MRP-1, including anti-cancer drugs (vinca alkaloids, anthracyclines, epipodophyllotoxins, anthracenes, methotrexate, and camptothecin derivatives), heavy metal oxyanions, (oxidized) glutathione, cysteinyl leukotrienes, activated aflatoxin B1, steroid hormones, and bile salts (155). The substrate spectrum of MRP-1 overlaps with that of MDR-1 P-gp.

MRP-1 is expressed in CEC and isolated vessels from human, bovine, rat, and mouse brain (176-180). The level of MRP-1 expression is higher than that of MDR-1 in both cultured CEC and brain microvessels captured by laser capture microdissection (LCM) from sections of normal human brain and glioblastoma multiforme (176,178). Unlike MDR-1 P-gp, the polarization of MRP-1 at the BBB is still controversial. Drug transport assays using in vitro BBB models demonstrated energy-dependent luminal efflux activity for glutathione-conjugated compounds (179,181–183); a recent report indicated that MRP-1 is predominantly localized on the luminal side of cultured bovine CEC (184). However, in polarized epithelial cells (kidney and liver), MRP-1, 3, and 5 are routed to the basolateral membrane (4,155), and MRP-1 is also expressed at the basolateral side of the choroids plexus epithelia (183,185). $Mrp-1^{-/-}$ mice demonstrated a deficiency in LTC4-mediated inflammatory reactions and increased sensitivity to the cancer drugs etoposide and vincristine. However, changes in BBB function were minimal compared to wild-type animals (151,152,184), suggesting that other MRP family members compensate the deficiency of *mrp-1* in knockout animals.

A variety of inhibitors have been described for MRP-1, including indomethacin, probenecid, fluorescein, leukotriene C4 (LTC4) analog MK571, LY402913, *S*-decylglutathione, sulfinpyrazone, and benzbromarone (3,186).

ABCC2/MRP-2: ABCC2/MRP-2 is a multispecific anion transporter found in liver, kidney, and small intestine (136). Cellular overexpression of MRP-2 confers drug resistance to vinblastine, etopside, doxorubicin, cisplatin, and other drugs (155,187). In contrast to MRP-1, MRP-2 is localized to the apical membrane of polarized epithelia (kidney and liver) (155,188,189). Some studies reported a low level of *MRP-2* expression at the luminal surface of CEC (190–192). Interestingly, *MRP-2* is over-expressed in CEC isolated from drug-resistant epilepsy patients (190,193); corroborative studies in *mrp-2* deficient rats demonstrated that mrp-2 restricts the activity of the antiepileptic drug phenytoin in the brain (190,193,194).

Other MRP proteins: Among several other members of ABCC/MRP family that can transport certain drugs in cells (3,155), only *ABCC4/MRP-4* and *ABCC5/MRP-5* are expressed in the brain or in CECs (Table 3). Over-expression of *MRP-5* or *MRP-4* confers resistance to nucleoside analogs in transfected cells (195,196). *MRP-5* is expressed in cultured human CECs (176) and is upregulated in the epileptic brain (193).

2.4.3. ABCG2

ABCG2/BCRP was first identified in Pgp- and MRP-1-negative breast cancer-resistant cell lines selected for high resistance to mitoxantrone and alternatively named breast cancer resistance protein (BCRP) or mitoxantrone resistance protein (MXR) (156,186,197). Compared to MDR-1 P-gp and MRP-1, ABCG2 is a "half-transporter" with one nucleotide-binding domain and forms homodimers in cancer cells (155,186). Recent studies have shown that *ABCG2* is also highly expressed in human brain tissue and CEC (198–200). Eisenblatter and Galla (201,202) identified a porcine homologue of *ABCG2* mRNA overexpressed in hydrocortisone-treated CEC cultures. Drug transport assays using an in vitro BBB model suggested functional polarization of ABCG2 on the luminal surface of CEC (176). Mice lacking *Abcg2* gene (*Abcg2^{-/-}* mice) displayed sensitivity to the dietary chlorophyll-breakdown product and a novel type of protoporphyria (203,204), however, the BBB function has not been examined in these animals.

The substrate spectrum of ABCG2 overlaps with that of MDR-1 P-gp or MRP-1 (186) and includes anti-cancer drugs (mitoxantrone, doxorubicin, topotecan, methotrexate, etc.), phototoxins (pheophorbide-a and protoporphyrin IX), prazocin, antiviral agents (zidovudine and iamivudine), fluorescent dyes (i.e., Lysotracker, rhodamine 123, fluorescein diacetate, and Hoechst 33462 or 33342), and possibly sterols (hydrocortisone, estradiol 17- β , and estrone) (176,197). Several inhibitors of ABCG2, such as Fumitremorgin C and its analogs, GF120918, flavopiridal, and tryprostatin A, have been described (197).

The expression of three other members of ABCG subfamily, ABCG1, Abcg3 and ABCG4, has been detected by RT-PCR in human brain tissue, CEC or rodents (205) (Zhang and Stanimirovic, unpublished data) (Table 3). ABCG1 is mainly expressed in brain, spleen, lung and placenta tissues and transports lipids and cholesterol (16,17,206), whereas ABCG4 was cloned from a brain cDNA library (207,208). Their functions in the brain remain unknown.

2.5. Monocarboxylic Acid Transport System (Solute Carrier Family 16)

Lactic acid, ketone bodies, and other monocarboxylate compounds are abundant in the brain and their distribution is regulated by proton-coupled monocarboxylate transporters (MCT) (SLC16A) expressed at the BBB. There are 14 members in the MCT/SLC16A family (SLC16A1–SLC16A14) (209), many of which are expressed in the brain or the CP including *SLC16A1/MCT1*, *SLC16A2/MCT8*, *SLC16A4/MCT5*, *SLC16A6/MCT7*, *SLC16A7/MCT2*, *SLC16A8/MCT3 A9/MCT9*, *SLC16A11/MCT11*, and *SLC16A14/MCT14* (209–211). Only four members of the family, MCT1-4, have been experimentally demonstrated to engage in a proton-driven transport of monocarboxylates (209). *MCT1* is expressed on both luminal and abluminal sides of CECs and in ependymocytes, astrocytic end-feet, pericytes, and the CP (210). MCT1 mediates uptake of ketone bodies and lactic acid from the blood and efflux of lactic acid from the brain into the circulation (212). The extrusion of probenecid, mercaptopurine, aluminum citrate, and AIT-082 from the brain is inhibited by monocarboxylic acids (MCA) (213). Although MCT2 expression has been detected in immortalized mouse CEC (214), its role in the BBB transport is not known.

3. TRANSPORT SYSTEMS AND THERAPEUTIC DELIVERY ACROSS THE BBB

The delivery of therapeutics across the BBB remains one of the most perplexing challenges in developing treatments for neurological diseases. Current brain drug delivery practices employ invasive and non-invasive approaches. Invasive approaches include neurosurgical procedures, such as cisternal, intracerebroventricular and intracerebral injections, cell and tissue grafting. Non-invasive strategies include pharmacological and physiological methods to facilitate transport of drugs via intercellular or transcellular routes (215,216). Various BBB transport systems have often been exploited to design rational drug delivery strategies. Although detailed description of these approaches is beyond the scope of this chapter, three principal strategies will be briefly mentioned.

Nutrient analogs or mimetics: Drugs are designed as analogs or mimetics of known natural substrates of the BBB transport systems. Examples of such compounds include nucleoside analogs, neurotransmitters (amino acids), sterols (E-17 β -G), hormones (T3, T4, etc.), L-dopa, MCA moiety-containing drugs (HMG-CoA reductase inhibitors), etc.

Inhibitors of drug efflux pumps: A number of inhibitors for drug efflux transporters, such as MDR-1 P-gp, MRP, and ABCG2, have been developed. These inhibitors, co-administered with the drug substrate for a given efflux pump, increase substrate delivery to the brain in animal models. This approach is particularly important for the management of brain tumors, where MDR phenotype results from the overexpression of efflux transporters in both tumor vasculature and parenchyma. Since efflux pumps share substrate specificity for many drugs, inhibitors with pleiotropic effects on several drug efflux pumps may be advantageous.

Chimeric peptides: The principal strategy currently being developed to deliver macromolecules across the BBB is the chimeric peptide strategy (215,216). This approach takes the advantage of various receptors, transporters or pumps selectively expressed on brain capillary endothelium that mediate transcytosis of proteins essential for normal brain function across the BBB, including transferrin, insulin growth factor, and LDL (3,215,216). Macromolecule delivery to the brain is achieved by coupling peptides, proteins, and nucleic acids to agonist/antibody "vectors" that bind to these transporters or receptors, and undergo a receptor-mediated

transcytosis. Proof of principle for this approach has been obtained using an anti-transferrin receptor antibody (OX-26) chemically coupled to peptides, such as endorphin, vasoactive intestinal peptide, and brain-derived neuro-trophic factor, or oligonucleotides and plasmid DNA (215,216).

4. CONCLUSION

The presence of different transport systems at the BBB is essential for maintaining the CNS homeostasis. The integrity and functionality of the BBB are affected in disease states, such as stroke, multiple sclerosis, Alzheimer's disease, brain tumors, AIDS, hypertension, and seizures. Changes in the expression and function of BBB transporters may contribute to the development and progression of these diseases. A comprehensive understanding of BBB transport systems will be pivotal in developing strategies for modulating BBB permeability and delivering therapeutics for the treatment of neurological diseases.

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Drug Delivery Through the BBB: Liposomes, Nanoparticles, and Other Non-Viral Vectors

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1. INTRODUCTION

Many classical treatments of central nervous system (CNS) disease do not access the brain parenchyma owing to the presence of the blood-brain barrier (BBB), which separates the blood compartment from the extracellular fluid compartment of the CNS. The same problem exists in medical imaging for the delivery of diagnostic agents to the CNS (1). The BBB is made up of endothelial cells that form very occlusive tight junctions (2). It is widely accepted that only compounds that are unionized at physiological pH, lipophilic, and of low molecular weight can cross the BBB (3). However, the physico-chemical properties of most drugs (size, solubility, charge, etc.) mean that most of them cannot cross this natural barrier. Furthermore, low pinocytic vesicular traffic and efficient efflux mechanisms in the BBB contribute to failure of treatment (4–6). More than 98% of all potential drugs targeted to the brain do not cross the BBB (7). Consequently, various strategies, reviewed in Temsamani et al. (8) have been explored for drug delivery across the BBB. These include BBB disruption, hyperosmotic shock, administration of vasoactive substances, direct intraventricular drug administration, microparticle implantation, drug modification, receptor-mediated

transcytosis, and peptide-vector strategies (9–16). The present article focuses on non-viral strategies that imply colloidal drug delivery systems (DDSs).

A colloidal DDS is a structure composed of biocompatible components such as lipids (phospholipids, triglycerides, etc.), sugars, and/or polymers that form particles, which enable drug encapsulation. Their use allows drug distribution to be governed by the properties of the delivery system. Polymer nanoparticles, microparticles, or liposomes are the most frequently employed delivery systems.

The reasons why a colloidal DDS is one of the strategies explored to target the CNS are detailed in the first part of this chapter. The various in vitro and in vivo models developed to evaluate the transport of/from colloidal DDSs are described in the second part. Finally, the third part focuses on the necessary developments and various mechanisms allowing colloidal DDSs to cross the BBB. This part also focuses on the results obtained over the past 5 years in the field of CNS targeting with colloidal DDSs.

2. RATIONALE FOR THE USE OF COLLOIDAL DDS TO TARGET THE CNS

The ideal vector for *trans*-BBB delivery should be (a) highly efficient in delivering the drug in a target-specific manner, (b) stable in vitro as well as in vivo, (c) protect the drug from degradation, (d) control the rate of drug release, (e) be non-toxic and non-immunogenic, and (f) have the propensity to allow preparation in large quantities (17). Viral vectors have been used, particularly for gene therapy, for brain diseases. However, they do not cross the BBB, and when intracerebral injection into the brain has been used, inflammation and demyelinization have been observed due to a pre-existing immunity to viruses in humans and primates (17–19). Colloidal DDSs, like liposomes, are low or non-immunogenic, non-mutagenic, and present a high loading capacity (17).

2.1. Encapsulation and Controlled Drug Release

Liposomes and nanoparticles are the main colloidal DDSs used to target the brain. With their vesicular structure, liposomes and nanocapsules allow drugs to be encapsulated in their hydrophilic or lipophilic core. Drugs can also be encapsulated inside the polymer or lipid matrix of nanospheres. Numerous studies have demonstrated the ability of colloidal DDSs to encapsulate drugs, regardless of their physico-chemical properties (hydrophilic, lipophilic, anionic, cationic, etc.).

In some cases, the encapsulated drug can be protected from degradation by the colloidal DDS; very sensitive molecules, such as proteins or peptides can therefore be administered (20). Amongst the different molecules encapsulated in colloidal DDSs to treat CNS pathologies, camptothecin, a plant antitumoral alkaloid, is a good example with regards to the protection provided by colloidal vectors. In the drug structure, lactone functionality appears to play an important role in the biological activity of the process. However, camptothecin lactone opens rapidly and completely and generates the carboxylate form in human plasma. Due to the poor water solubility of the lactone form and its unstable biological pH, and due to the low biological activity and severe toxicity of the carboxylate form, camptothecin has been encapsulated in solid lipid nanoparticles. Promising results showed an AUC/dose ratio for the camptothecin-loaded solid lipid nanoparticles being much higher than for a camptothecin control solution, especially in brain (21).

When encapsulated, sustained drug release from the lipid or macromolecular structure is generally observed (21). This characteristic is very interesting for the optimization of drug pharmacokinetics and for decreased numbers of administrations.

2.2. Colloidal DDS Biodistribution: How to Target the CNS?

In addition to their ability to encapsulate, protect the drug, and control its release, the colloidal structures can mask the physico-chemical drug properties and modify its biodistribution. This effect is very important when a drug has unwanted affinity for a specific organ. In the case of doxorubicin, after systemic administration, the drug concentrates in the heart exhibiting very important cardiac toxicity. To overcome this toxicity, doxorubicin has been encapsulated in liposomes with positive results (22). The commercial products such as Myocet[®] and Doxil[®] are used to treat some types of cancers, including breast and ovarian cancers and Kaposi's sarcoma. In the same way, due to its chemical properties, tubocurarine, a hydrophilic drug (quaternary ammonium salt), is not able to penetrate the BBB. It was nevertheless used with success as a marker of the distribution of nanoparticles in the CNS after its encapsulation in nanoparticles (23). As a consequence, the systemic toxicity of a drug on non-targeted organs was decreased and the efficiency of the molecule was increased.

In the drug-targeting field, the biodistribution of the drug should be controlled by the distribution of the colloidal DDS. But the CNS is not the preferred organ of distribution of classical intravenously administered colloidal particles. Particles are essentially found in the liver and spleen, endocytosed by macrophages concentrated in these organs (24) or in tumoral tissues due to the enhanced permeability and retention (EPR) effect (25– 27). Usually, no therapeutic response can be observed in the CNS. However, the colloidal DDS can be surface-modified to target the CNS. Two ways have been explored. The first one consists in decreasing the macrophage uptake that increases the circulation time of the DDS systems. A more important proportion of nanoparticles reaches the CNS and come in contact with the BBB. Stealth liposomes or nanoparticles are formulated in that way. In general, they are surface-modified with hydrophilic polymers such as poly(ethylene glycol), to prevent DDS opsonization and macrophage capture. PEGylated poly (hexadecyl cyanoacrylate) nanoparticles made from PEGylated amphiphilic copolymer penetrate into the brain to a larger extent than non-PEGylated PHDCA (28,29). Nevertheless, long circulating poloxamine 908-coated poly(hexadecylcyanoacrylate) particles (poloxamine 908 is an hydrophilic polymer) fail to increase in concentration in the brain (28). The same negative results were observed with PEG-liposomes (30). These results were attributed to the incapacity of the surface-modified structure to interact with endothelial cells of the BBB. Therefore, a compromise between a reduced interaction with the macrophages and increased interactions with endothelial cells needs to be found (28).

The second way of modification is again based on the alteration of the vector's surface. It generally takes advantage of specific receptors present on the BBB. Colloidal DDSs have been shown be able to carry some proteins, such as antibodies that bind to a specific receptor on the endothelial cell. These are called immunoliposomes or immunonanoparticles and will be detailed in the following. Huwyler et al. (30) described a coupling procedure that allowed conjugation of a thiolated antibody to maleimide-grafted liposomes sterically stabilized with PEG and used both modifications to target the BBB.

Therefore, the classical properties of colloidal DDSs, their versatility in size, composition, structure, and their modularity make them good candidates for CNS-targeted delivery of drugs.

3. METHODS AND MODELS USED TO INVESTIGATE DRUG DELIVERY THROUGH THE BBB USING COLLOIDAL DDS

The BBB models aim to quantify the ability of a drug to cross the BBB and to estimate the ability of novel delivery tool to transport a drug from the blood to the CNS. Toxicity and the molecular mechanisms involved in the transport can also be investigated with BBB models.

Under in vitro conditions, the permeability of the BBB is often evaluated using labeled drugs or components of the DDS itself (fluorescence or radioactivity). Under in vivo conditions, quantification is generally based on measuring the biodistribution of the labeled drug (or particles) within the CNS after administration (22,28). The regression of a lesion, such as a tumor, or a pharmacological effect can also be used to evaluate the efficiency of the transport system (23,31,32).

3.1. Various Criteria Used to Estimate the Quality of a Model

The quality of the model used is related to its ability to mimic physiological or pathological conditions. For the BBB, the main parameters to be considered are its composition, structure, and properties.

The first criterion is based on the study of the integrity of tight junction. On the one hand, the level of integrity corresponds to electric resistance estimates of the in vitro cellular layer, which should be close to the estimated in vivo value of 1500–2000 Ω cm⁻² (2); this high value correlates with the low permeability of the barrier and is partly due to the presence of numerous tight junctions (2). On the other hand, after exposure to nanoparticles, Western blot analyses were used to detect a potential decrease in the expression of claudin-1 and ZO-1 the essential proteins involved in tight junctions (33).

The second criterion to be taken into account is the presence of transport systems, receptors (LDL, transferrin), and markers specific to the BBB (34–36). The main carriers at the BBB are glucose transporters, γ -glutamyl transpeptidase, and P-glycoprotein. P-glycoprotein is responsible for an active efflux transport, which leads to the extrusion of many drugs from the CNS parenchyma to the systemic circulation.

The physical integrity of the BBB is assessed with $[{}^{3}H]$ -or $[{}^{14}C]$ -sucrose or inulin, which are known to diffuse very slowly across the BBB. Under in vitro conditions, BBB permeability is evaluated by measuring the flux of these paracellular markers added to the luminal side of a cell monolayer (37). Under in vivo conditions, co-perfusion of these hydrophilic vascular space markers is carried out in the presence of the studied compounds (38); these molecules are used as tracers that allow a calculation of the vascular volume (39,40).

Recently, imaging of cerebral blood flow has been used in vivo to verify that aggregation of nanoparticles does not occur and that occlusion of the cerebral vessels is not a complication of nanoparticle injections (33). It has been performed by the administration of high concentrations of various nanoparticles made of emulsifying wax and Brij[®] 78 (40 µg ml⁻¹) for a total nanoparticle concentration of 200 µg in the presence of [H³]-diazepam. The brain permeation of the [H³]-diazepam was evaluated in the presence or absence of the nanoparticles (33). No significant alteration in cerebral perfusion flow was seen at high nanoparticle concentrations. Furthermore, no significant regional differences in cerebral perfusion flow were observed when nanoparticles were present (33).

The possible interaction of the vector with the natural passive permeation of essential nutrients can also be studied in BBB models. Numerous components of colloidal DDS can interact with the transport of cationic choline via the choline transport protein. Labeled choline has been used and its transport assessed in the presence of colloidal DDS, in vitro and in vivo (33).

3.2. In Vitro Models of BBB

In 1996, Tamaï and Tsuji (10) divided in vitro models of BBB into two categories: isolated brain capillaries and primary cultured brain capillary endothelial cells. This classification is still used today with recent developments in the field.

Isolated brain capillaries were usually obtained by mechanical homogenization of cerebral gray matter. However, this model has been virtually abandoned because of the mechanical damage, the decrease in ATP levels and the difficulty to assign the direction of transport. Primary culture of brain capillary endothelial cells (BCEC) is now preferred over brain capillary isolation. Human, bovine or murine BBB endothelial cells can now be kept in primary culture and cultivated as confluent monolayers. Radioactive or labeled DDS particles to be tested are incubated with the cell cultures. Particle uptake by BBB endothelial cells can be quantified after different incubation times (41).

As cells kept in primary cultures have a short survival time in vitro, immortalized cells (42) such as rat brain endothelial cells (41) have been developed. Quantification of particles is carried using the same method as previously described for BCEC cultures. However, it has been postulated that the development of mature tight junctions in immortalized BBB endothelial cells is not complete (10).

To overcome these problems, the culture of bovine brain capillary endothelial cells, either with rat astrocytes or in presence of an astrocyteconditioned medium, has been developed. Endothelial cells are grown on one side of a membrane and astrocytes are grown on the bottom of a dish (43). This organization allows for humoral interchange without direct cell contact (43). In such a model, a large increase in P-gp expression was apparent in endothelial cells cultured with astrocytes compared to endothelial cells cultured alone (43). Furthermore, the P-gp is not only expressed but also functionally active in this co-culture system. Using this system, the in vivo and the in vitro values of drug transport across the BBB strongly correlated for numerous compounds (imipramine, nicotine, caffeine, morphine, phenytoin, etc.) with different physico-chemical properties (43).

3.3. In Vivo Models of BBB

The in vivo models of the BBB use a carotid artery bolus to quantify the trans-endothelial transport. It consists of a rapid bolus injection via the common carotid artery, followed by animal decapitation. The main limitation of this brain uptake index (BUI) method relates to the fact that the test substance is available for brain uptake only during a single capillary transit that lasts approximately 1 section (44).

An in situ brain perfusion technique was developed in the rat by Takasato et al. (45). In their model, perfusion in the internal carotid artery by a retrogradely cannulation of the external-carotid artery was done following the ligation of the occipital artery, the superior thyroid artery, the pterygopalatine artery and the common carotid artery. The perfusion was done for a very short time with a suspension of the colloidal DDS. Using this model, several authors studied inulin and sucrose diffusion, as well as the expression of BBB marker (33,39,45–47).

Intravenous bolus injection or intravenous infusion allows for an even more sensitive measurement of brain uptake for slow penetrating substances. However, peripheral catabolism of the substance tested can occur during the course of the experiment (12). Bickel et al. (12) compared these three techniques and listed the various parameters to be considered when making a choice for a delivery model. The list includes, the pharmacokinetic characteristics of the method, the measured parameters, the saturable transport, the sensitivity and the technical difficulty of the methods (12).

4. THE CONTRIBUTION OF COLLOIDAL DDS TO CNS TARGETED-DRUG DELIVERY

The CNS neural and glial cells require essential nutrients, such as glucose, to function efficiently. Physiological mechanisms of nutrients transport across the BBB have been described (7,10,12). While the first strategy of DDS-based drug delivery to the CNS was using a direct particle implantation within the brain, the second strategy is now taking advantage of the existing physiological mechanisms that allow molecule passage from the blood compartment to the CNS. A new and emerging strategy is based on the existing physiological drug efflux mechanisms that "pump" undesired substances from the CNS to the blood. Consequently, inhibition of efflux mechanisms by use of DDS could decrease the elimination of specific chemotherapies from the CNS compartment. These strategies are detailed below and Table 1.

4.1. Implantation of DDS in the Brain

The first approach to overcome the BBB using DDSs is to use DDS drug delivery into the CNS by local stereotactic implantation. Implantable polymers are extensively studied, particularly for cancer-based chemotherapy of the CNS (16). Polyanhydride and poly(lactide-co-glycolide) polymers are the most widely investigated family of polymers in this field due to their biocompatibility with the CNS (16,48). Microparticles $>1 \mu m$ are formulated and stereotactically implanted directly into the CNS parenchyma. The advantage of this method is a sustained local exposure to high amounts of drug while avoiding significant systemic effects (16). Currently, the most developed applications are in neurology and neuro-oncology for the treatment of malignant gliomas (11). Implantation can be performed directly in the vicinity of the tumor or in the resection wall after its removal (11). Nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), 5-fluorouracil, idoxuridine and BCNU have all been encapsulated in microspheres (11,49,50). Phase I/II clinical trials have been carried out in

	vivo Applications	U CUIIUIUAI D					
References	Encapsulated drug	Vectors	Studied samples	Results (in brain)			Proposed mechanism
21	Camptothecin	NJS		$AUC (ng hr^{-1} g^{-1})$		MRT (hr)	C57BL mice No mechanism pronosed
			Camtothecin solution	189		5.2	
			Camptothecin- SLN (1.3 mg kg ⁻¹)	1958		5.9	
			Camptothecin- SLN (3.3 mg kg ⁻¹)	5143		28.0	
15	FudR	SLN)	AUC (ug hr g ⁻¹)	MRT (hr)	TE	Kunming mice
				0			Decrease of SPM capture
			FudR solution	31.6	12.3	11.8	Adsorption to capillary walls
			DO-FudR	168.5	20.9		Endocytosis by EC
			DO-FudR-SLN	347.1	31.1	29.8	Release within EC and delivery to brain
							TITN IN AT AT AT AT

Table 1 In Vivo Applications of Colloidal DDSs in the Brain Delivery Field

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		Sprague Dawley rats positively charged liposomes		Sprague Dawley ratsOX26: receptor- mediated endocytosis
Sprague Dawley rats OX26: receptor- mediated endocytosis			Complete reduction 0/6 5/6	
% ID g ⁻¹ tissue	0.008 0.008 0.030	Effect on catatonia	No reduction 6/6 0/6	Gene expression
	Solution of daunomycin PEG-liposomes PEG-OX26- linosomes	Solution of dopamine	Dopamine- liposomes	PEG-OX26- Liposome
Liposomes/ OX26/ PEG		Liposomes		Liposomes
Daunomycin		Dopamine (100 mg kg ⁻¹)		Plasmide
30		68		09

(Continued)

Table 1 Ir	1 Vivo Applications 6	of Colloidal I	DDSs in the Brain D	elivery Field (Continued)	
References	Encapsulated drug	Vectors	Studied samples	Results (in brain)	Proposed mechanism
22	Doxorubicin	Polymeric nano-	Solution of doxorubicin	Doxorubicin concentra-	Wistar rats endocytosis by EC
		particles	Doxo-Np	110ns <0.1 μg g ⁻¹ <0.1 μg g ⁻¹	
			Doxo-polys80-Np Solution of doxorubicin+ polys80	$>6\mu g~g^{-1}$ < 0.1 $\mu g~g^{-1}$	
69	Doxorubicin	Polymeric nano- particles		Survival rate	Rats bearing glioblastoma BBB disruption by glioma Ps and Np enhance delivery through the
			Untreated Blank	17 days 20 days	000
			nanoparticles- polys80 Doxorubicin in	22 days	
			Doxorubicine in saline⊥nolvs80	>180 days	
			Doxo-Np Doxo-Np Doxo-polys80	50 days >180 days	

		NMRI mice Adsorption on np Doceius Aiffraism	(gradient) (gradient) Phagocytosis by EC Degradation products: absorption enhancers	Swiss mice CNS penetration Non-specific BBB permeabilization Toxicity of the carrier CNS penetration	(Continued)
Mice Specific mechanisms of delivery Not a simple disruption of the BBB					
Antinocicep- tive effect in % of the MPE (45 min IV injection)	0.75 9.30 52.00	Analgesic activity and		I	
	Empty PBCA Dalargin solution Dalargin+ polys80-PBCA	Np dextran Np polys85		Dalargin- PBCA Np PS Np PBCA Np+Dalargin +polysorbate	
Polymeric nanopar- ticles		Polymeric nano-		Polymeric nano- particles	
Dalargin		Dalargin Kyotorphin Amirintalina		Dalargin	
38		32		37	

Table 1 II	n Vivo Applications	of Colloidal I	DDSs in the Brain De	slivery Field (Continued)	
References	Encapsulated drug	Vectors	Studied samples	Results (in brain)	Proposed mechanism
62	Dalargin Loperamide	Polymeric nanopar- ticles		Antinocicep- tive effect	C57BL mice
			Dalargin-PBCA np PBCA+polys80+ Dalargin		Apo B and E involved in the transport
			Loperamide		Capture by the EC after absorption of
					apo B and E Receptor-mediated endocytosis Diffusion of the drug
					in the brain Release within the EC
23	Tubocurarine	Polymeric nanopar- ticles		EEG	of transcytosis
			Solution of tubocurarin	No modification	Endocytosis

Polys80 triggers a complex series of cell-surface events leading to the internalization of the np		
No modification	No modification Epileptiform spikes 100% increase of lifespan of glioma mice (ED ₅₀ of 18–38 days with liposomes)	
Tubocurarin-Np	Tubocurarin + polys 80 Polys80- tubocurarine-Np Saline	liposomes
	Liposomes	
	Plasmide encoding antisense mRNA to EGFR	
	61	



Drug Delivery Through the BBB

patients to assess the potential of this mode of implantation (51,52). The main limitation of this approach is the poor diffusion of the drug inside the CNS parenchyma.

4.2. Blood to Brain Transport

4.2.1. Increase of Lipophilic Properties

4.2.1.1. Chemical modification of the drug: Optimal properties for drugs penetrating through the BBB by passive diffusion are defined by their lipophilicity (log *D* at pH 7.4), hydrogen-bonding capacity, and molecular size. Log *D* values for brain uptake should be in the range of 1–4. The upper limit of the molecular weight for brain penetration seems to be 450 Da, and the polar surface area should be below 90 Å² (3). Benzodiazepines, neuroleptics and tricyclic antidepressive agents all have these properties.

When the physico-chemical properties of a drug do not allow it to cross the BBB, some chemical modifications can be made. Prodrugs, a combination of a chemical moiety of the parent drug, can be synthesized, thus generally increasing the lipophilicity of the initial drug. The prodrug should however be cleaved into the active substance and the chemical moiety after passing the BBB.

Numerous chemical strategies have been used to increase drug lipophilicity including the addition of methyl groups to barbiturates. Dihydropyridine, adamantine and fatty acyl carriers, such as *N*-docohexanoyl have also been used as lipid carriers of drugs. In Scherrmann's study (14), specific modifications such as parachloro halogenation, methylation, and glycosylation have been employed to enhance the lipophilicity of peptides and/or to enhance membrane permeability and metabolic stability. Multiple parameters should be considered in results analysis.(1) This strategy is not specific to the BBB and the permeability across all cell membranes are enhanced leading to a higher uptake of drug into peripheral tissues (44). Since high doses had to be administered, significant peripheral side effects have been observed. (2) Even when optimal chemical properties are met, some drugs remain CNS-inactive due to the influence of other mechanisms that affect the final drug distribution.

4.2.1.2. Encapsulation in SLN: Even after modification, "lipidization" is not always efficient for brain targeting. Solid Lipid Nanoparticles (SLN) have been formulated to target the CNS. These are mainly composed of lecithin, Pluronic[®] F68 or poloxamer 188, and glycerol tristearate or stearic acid. The SLN are an alternative to polymer nanoparticles or liposomes (53,54); they have very low levels of toxicity and the ability for controlled drug release, and drug targeting. They allow encapsulation of lipophilic or hydrophilic products. Camptothecin and FudR, two antitumoral drugs, were encapsulated in SLN, leading to their distribution within the brain

and greatly enhanced anti-tumor effect, when compared to their intravenous administration. Wang et al. (15) encapsulated the lipophilic molecule 3', 5'-dioctanoyl-5-fluoro-2'-deoxyuridin (DO-FudR) in solid lipid nanoparticles and showed the brain AUC of DO-FudR (168.5 µg h g⁻¹) to be greater than the AUC of the solution of the FudR (31.6 µg h g⁻¹). Furthermore, when both solutions were compared, SLN improved drug penetration through the BBB with an AUC of 347.1 µg h g⁻¹. Until now, penetration mechanisms have not been detailed in the literature; however, adsorption through the capillary walls due to the lipophilic composition of the carrier is possible. The loaded SLN may be endocytosed by endothelial cells, release of drugs within these cells may occur with subsequent delivery of drug to the brain (15). Further experiments are still needed in order to gain more insight into the actual mechanism involved in SLN-based drug delivery across the BBB.

4.3. Tight Junction Disruption

Tight junction disruption is thought to be involved in various CNS diseases, possibly leading to the opening of the BBB (2,55). For example, tumor vasculature is abnormal and leaky and gadolinium-DTPA enhancement can be detected, indicating a change in permeability (1). It has also been shown that a number of mediators involved in pain response, including cytokines, chemokines, cellular adhesion molecules, and kinins play a role in altering the cytoarchitecture and permeability of the BBB (55). Such alterations of the BBB may be beneficial resulting in an improved therapeutic outcome (55).

To increase the ease with which colloids pass through the BBB, some groups have induced BBB disruption by intravenously injecting hyperosmotic solutions of mannitol or arabinose prior to the injection of colloidal DDSs; this leads to an immediate increase of BBB permeability and to an artificial and transient BBB opening (56). In this context, the passage mechanism corresponds to paracellular brain entry due to increased leakiness of the BBB (57).

The use of cyclodextrins, cyclic oligosaccharides of 6 to 8 units of Dglucopyranose, is another way to open the BBB. These are known to alter cellular membrane integrity leading to the rupture of tight junctions. This mechanism has been used to explain why the presence of γ -cyclodextrins or hydroxypropyl- γ -cyclodextrins increased the passage of doxorubicin (an antitumoral molecule) through an in vitro model of the BBB (58).

Although reversible, this mode of delivery is particularly invasive and can lead to unwanted neurological side effects due to BBB opening in healthy regions of the brain. Furthermore, even after the mannitol-induced opening of the BBB, no treatment benefit was observed. These results were attributed to the finding that while mannitol may increase the passage of
hydrophilic substances across the BBB, it does not necessarily improve the delivery of drug to the specific tumor site (16).

4.4. Carrier-Mediated Transport

Essential compounds, including amino acids, monocarboxylic acids, sugars, and nucleosides, have been shown to cross the BBB via specific carriermediated transporters (55). Carrier-mediated transport at the BBB involves a substrate-transporter interaction at the level of the BBB. This delivery route is a saturable process. Glucose peptide conjugates and niosomes bearing glucose ligands have been developed as the glucose transporters GLUT-1 is present on BBB endothelial cells (59).

4.5. Receptor-Mediated Endocytosis

Receptor-mediated transport is known as a natural and efficient system that allows the blood to brain passage of various molecules via endosomal uptake mechanisms (10). Blood-borne leptin, insulin or transferrin are known to bind to their cognate receptor located on BBB endothelial cells and be transferred into the brain's interstitial space. This active transport involves specific receptors, such as insulin, transferring, or the leptin receptors all of which are present on the luminal BBB. Different strategies have been explored to take advantage of this ability, using the receptor's ligand combined with colloidal DDS.

Due to its physiological effect leading to hypoglycemia, insulin was found to be a poor candidate for receptor mediated DDS delivery. Consequently, transferrin has mostly been studied with various colloidal DDSs. Still, its natural plasmatic concentration limits its binding efficiency due to competition between bound transferrin on the colloidal DDS and plasmatic transferrin (12). The use of OX26, a monoclonal antibody that binds to transferrin receptors, is an interesting alternative to native transferrin. On the one hand, OX26 is generally chemically conjugated to the colloidal DDS via maleimide-functionalized molecules, which are incorporated in the surface structure of the particles; on the other hand, OX26 binds to the extracellular domain of the transferrin receptor without interfering with transferrin binding (12). This leads to the formation of immunoliposomes or immunoparticles. The use of OX26 is still conceptual since this mouse monoclonal antibody is directed against the rat transferrin receptor. No specific antibody directed against the human transferrin receptor has been described.

Receptor-mediated transport is assumed to be the major mechanism responsible for the favorable results observed with loaded-liposomes (Table 1). Various classes of active drugs have been encapsulated in liposomes for CNS-targetting. Thus, the treatment of tumors (with daunomycin) or gene expression in the brain (with β -galactosidase plasmid) have been studied (30,60). PEGylated and/or OX26-immunoliposomes have shown encouraging results where an important proportion of the initial injected dose (100 mg kg⁻¹) of liposome encapsulated-daunomycin (0.03% of injected dose per gram brain tissue) was found in rat brains compared to the non-liposome solution of daunomycin (0.008% of injected dose per gram) (Table 1).

Based on the same principle, thiamine has been linked to distearoylphosphatidylethanolamine via a polyethylene glycol spacer (61): This watersoluble micronutrient is essential for normal cell function. A great number of thiamine transporters have been found at the level of the BBB. Thiamine was recently proposed as a surface ligand bound to nanoparticles for increased drug delivery of wax nanoparticles to the brain (33). This group demonstrated that the thiamine-coated nanoparticles associated with the BBB thiamine transporter had a significantly (P < 0.05) increased uptake transfer constant (between 45 and 120 sec) of $9.8 \pm 0.3 \times 10^{-3}$ ml s⁻¹ g⁻¹ compared to $7.0 \pm 0.3 \times 10^{-3}$ for uncoated nanoparticles (33).

4.6. Absorptive-Mediated Endocytosis

Enhanced contact between colloidal particles and endothelial cells is possible modification of the particle's surface charge. This mechanism is named absorptive-mediated endocytosis (10). However, membranes of other cell types are also negatively charged and therefore, specific targeting of brain endothelial cells cannot be expected with this approach. Furthermore, catatonia was observed after the administration of positively charged, dopamine-loaded liposomes, probably induced by the electrostatic interaction of the particles with negatively charged endothelial cells.

4.7. The Influence of Surfactant on the Passage of Colloidal DDSs Through the BBB

Surfactants are amphiphilic molecules frequently used in colloidal DDS formulation to increase particle's stability. Surfactant adsorption on the particles can prolong nanoparticle circulation time by decreasing macrophage uptake thereby increasing contact with the BBB. The encapsulation of dalargin or doxorubicin in polymeric nanoparticles led to an antinociceptive effect, analgesic activity, or enhanced pharmacokinetic parameters when compared to the use of a simple solution of the drug (Table 1). In 1999, Gulyaev et al. (22) showed significant transport of doxorubicin into the CNS after systemic administration of polysorbate 80-coated poly(butylcyanoacrylate) nanoparticles. Other groups had similar findings following administration of dalargin (38,62), loperamide (31) or tubocurarine encapsulated within surfactant-coated hydrophilic nanoparticles (Table 1).

In all cases, the presence of polysorbate 80 adsorbed on the particles was necessary to achieve brain targeting. Various mechanisms have been proposed to explain this finding, including (1) the fact that degradation products of the polymer could have a role in absorption, (2) the hypothesis that nanoparticles reach the brain intact and cross the BBB by endocytosis (22). However, Olivier et al. (37) proposed that polysorbate 80 could be toxic to the BBB, leading to BBB permeabilization and that increased brain delivery of the drug could be explained by the toxicity of the carrier at the level of BBB endothelial cells. Other authors also proposed that the use of selected clones of cerebral endothelial cells could explain the toxicity observed (38), (3) the fact that a specific role of polysorbate 80 could be to promote the physiological coating of the injected nanoparticles by apolipoprotein E (63). The apolipoprotein E-polysorbate 80 encoated nanoparticles may then be up-taken by the brain capillary endothelial cells via receptor-mediated endocytosis (62), (4) the notion that numerous non-ionic surfactants have the ability to inhibit drug efflux transport (see next paragraph for the mechanism) and that polysorbate 80 might have the same properties (62).

Finally, literature pertaining to the transport of peptides into the CNS demonstrates the numerous mechanisms by which transport can occur. Carrier-mediated transport, receptor-mediated endocytosis, and absorptive-mediated endocytosis have all led to positive results using peptides (10). In general, it appears that two steps are involved in CNS-directed colloidal vector transport. The first one corresponds to drug modification, which can be necessary for association with the vector. The search for suitable vectors for this drug in terms of composition (stealthiness, charge, size, etc.) is the second step of development and should be studied in parallel to the first step (12).

5. BRAIN TO BLOOD INHIBITION

P-Glycoprotein is a membrane-associated, energy-dependent efflux transporter expressed in the brain parenchyma, as well as in the blood-brain and blood-cerebrospinal fluid barriers (64). Consequently, its presence on in vitro BBB models is a quality criterion (42,43). Its function at the level of the BBB is believed to be to prevent the accumulation of potentially harmful compounds in the brain by actively removing such compounds from the brain (10.64). Drugs that are affected by such efflux processes include the vinca alkaloids (vinblastine and vincristine), anthracyclines (doxorubicin and daunorubicin), the RNA transcription inhibitor actinomycine-D and the microtubule-stabilizing drug paclitaxel (65). Therefore, for a molecule to remain in the parenchyma after crossing the BBB, inhibition of Pgp is necessary. As P-gp binds many different hydrophobic compounds, it has been shown that a large number of factors can inhibit P-gp activity, either by blocking drug binding, by interfering with ATP hydrolysis or by altering the integrity of cell membranes into which P-gp is inserted (for a review see Ref. 66). The use of such P-gp blocking compounds, such as verapamil, cyclosporin A, and reserpine, is limited by their toxicity at doses needed to block P-gp. Surfactants, including Cremophor-EL[®], Triton[®] X-100, Tween[®] 40, Tween[®] 80, and Solutol[®] HS15, have also been shown to interact with P-gp (67). Their incorporation in colloidal DDSs may lead to enhanced circulation time and to P-gp inhibition, allowing an increased efficiency of colloidal DDS delivery to the CNS. This approach could complement other strategies used.

6. CONCLUSION

The use of colloidal DDSs that specifically target the CNS, is a promising tool due to the various mechanisms described by which they are able to overcome the BBB and due to their ability to encapsulate and deliver drugs. Various mechanisms may run in parallel or may be co-operative, thus facilitating drug delivery to the brain with minimal peripheral side-effects (41). Colloidal DDS is a promising tool for pharmaceutical industry.

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8

Immune Functions of Brain Endothelial Cells

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Activated T cells, regardless of their specificity, cross the blood-brain barrier (BBB) and enter the central nervous system (CNS) under physiological conditions, a process called immune surveillance (1,2). In certain CNSdirected immune diseases such as multiple sclerosis (MS), trafficking across the CNS barrier is enhanced due to actual disruption of the barrier and/or enhanced immune-endothelial cell molecular interactions. This trafficking results in an intense perivascular leukocytic infiltration that appears to underlie the development of the initial disease lesion and, at least, to contribute to ongoing disease progression (demyelination and neuronal cell death) (3). Despite breach in the BBB, leukocyte entry does not appear to be random but rather selective. The MS lesions are predominantly comprised of T cells and activated macrophages, with occasional B cells, and plasma cells (4–6).

It is now believed that, by virtue of its selective permeability properties and immune functions, the BBB plays an active role in the regulation of immune cell and molecule entry. The presence of Gd-enhanced brain MRI lesions (7) and immunochemical studies are strong indicators that the BBB is disrupted in both MS and in its animal model experimental autoimmune encephalomyelitis (EAE), primarily in active lesions. Extravasation of immune cells occurs during or immediately following BBB damage and breakdown (8,9) and is facilitated by simultaneous increases in adhesion molecules and inflammatory mediators by brain endothelium (10,11). The BBB could also play a significant role in T-cell activation whereby antigen could be presented to blood lymphocytes by brain ECs (12). Conversely, BBB-ECs could be implicated in infiltrating immune cell suppression and the production of beneficial factors for the CNS in response to infiltrating cell challenge.

The characterization of the cellular and molecular events that occur in the course of neuro-inflammation at the level of the BBB and the immunological role of brain endothelium as the first cell that comes into contact with immune cells continues to be of considerable interest. Such mechanisms represent potentially critical checkpoints for initiation of CNS-directed disease and are susceptible to antagonism by therapeutic agents. This chapter will focus on the immune properties of BBB-ECs in health and in the course of inflammation. Specifically, we will address brain ECs and adhesion molecule expression, cytokine and chemokine production, antigen presentation, and brain ECs as a source of neurotrophins.

1. ADHESION MOLECULES AND CELL TRAFFICKING

An important early step in cell migration into the CNS is the reciprocal attachment of adhesion molecule receptors on infiltrating immune cells to corresponding ligands localized on brain endothelium. Three families of adhesion molecules are implicated in cellular transmigration at the level of the BBB: selectins, integrins, and members of the immunoglobulin super-family of adhesion molecules (13).

Selectins, single transmembrane glycoproteins with an extracellular lectin domain that bind to distinct carbohydrate moieties, are important for initial cell tethering of leukocytes on the surface of endothelial cells so as to allow rolling in the direction of blood flow. These molecules are found reciprocally on both blood-borne cells and endothelial cells. L-selectin is nearly ubiquitous on all leukocytes. Both P- and E-selectin can be induced in brain ECs by TNF α , IL-1 β , IFN γ and LPS (14–17). In the presence of inflammatory factors such as histamine, P-selectin is rapidly translocated from cytoplasmic storage vesicles, the Wiebel Palade bodies, to endothelial cell membranes (17). E-selectin is expressed at a later stage, as this molecule is synthesized de novo (18). On CNS endothelium, T-cell rolling is a process dependent on P-selectin and its ligand P-selectin glycoprotein ligand (PSGL)-1 on T cells, as recently demonstrated by intravital microscopy in mice (19). Activated T cells use P-selectin to enter the CNS very early in EAE (20). However, both P- and E-selectins do not appear to have a role

in late stage disease as the expression of both is minimal after EAE initiation (14). It would then seem that selectin expression, although important in initial T cell-EC tethering interactions, plays a minimal role in late transmigration of leukocytes into the CNS in the course of diseases such as MS. E-selectin has been identified on microvessels in MS lesions (21) but no studies to date have reported increased levels of L-selectin on lymphocytes derived from MS patients. In EAE, blockade studies or knockout models remain unconclusive as to the function of selectins in lymphocyte trafficking (22). The principal role of selectin molecules remains the trafficking of neutrophils, a cell type rarely found in, or even in the proximity of MS lesions. In the absence of subsequent firm adhesion mediated by members of the immunoglobulin superfamily of adhesion molecules and integrins, selectin cell binding becomes transient and cells quickly return into the circulation.

Integrins are found on various CNS localized cells including on neural cells (microglia, oligodendrocytes, and astrocytes), infiltrating T cells, and ECs (10,23). Firm T-cell adhesion to endothelium is mainly mediated by leukocyte ligands leukocyte adhesion molecule (LFA)-1 and very late antigen (VLA)-4, and their associated receptor pairs intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (or alternatively, the connecting segment (CS)-1 of fibronectin as discussed below), respectively, on endothelium. The interaction between these molecules eventually leads to immune cell diapedesis. The presence and the functional importance of ICAM-1 and VCAM-1 have been documented in MS and in EAE. The expression of ICAM-1 and VCAM-1 in vivo is analogous to the observations made in vitro, as observed in normal brain compared to MS lesions (24). In the normal brain, the presence of ICAM-1 is restricted to microvessel ECs; in CNS inflammatory conditions (MS brains), ICAM-1 can also be localized to astrocytes and microglia (23-26). The expression of both adhesion molecules can be modulated in a time- and dose-dependent manner with pro-inflammatory cytokines IFNy, TNFa, IL-1, and LPS (16,27-31). The cytokines IFN γ and TNF α have been found in increased levels in the CSF, blood and serum of MS patients (32-36).

We and others have demonstrated that human BBB-ECs constitutively express moderate levels of ICAM-1 and low levels, if any, of VCAM-1 (16,29,31,37). Both ICAM-1 and VCAM-1 are increased on MS tissue microvessels (21,23). Similarly, the expression of ICAM-1 is six-fold higher on brain ECs in EAE and levels of expression correlate with relapse and remission (38). Studies using HUVECs and bulk lymphocyte populations derived from MS patients have demonstrated the importance of the ICAM-1/LFA-1 and VCAM-1/VLA-4 pairs of molecules through the use of blocking antibodies. Several groups have shown that anti-ICAM antibodies block T-cell adhesion to human brain ECs (30) and have been shown to inhibit EAE (39). Several groups have demonstrated a preferential involvement of LFA-1/ICAM-1 interactions in T-cell migration (16,40,41). In our laboratory, addition of ICAM-1 blocking antibodies to transwell migration assays decreased the migration of human Th1 and Th2 cells, human monocytes, and human B cells (42–44), suggesting that the ICAM-1/LFA-1 interaction is involved in the process of immune cell transmigration but not in selective recruitment of immune cell subsets.

Some reports suggest that antigen specific cell recruitment into the CNS in EAE animals is dependent on VLA-4/VCAM-1 interactions (20,45) as VLA-4 deficient antigen-specific T cells fail to cross the BBB (46,47). The VLA-4 was shown to be up-regulated on activated T cells (48) and more recently, anti-VLA-4 antibodies were proposed to be of significant clinical importance (as will be discussed below). However, in our hands, experiments using anti-VCAM-1 blocking antibodies on HBECs demonstrated that VCAM-1 is not involved in the migration of either Th1 or Th2 lymphocytes, nor in the migration of monocytes and B cells across BBB endothelial cells (42-44). These contradictory observations can be explained and reconciled in the light of the real nature of the ligands of VLA-4. Although VCAM-1 was the first receptor described for VLA-4 and is still considered to be the major binding partner of VLA-4, VLA-4 has also been shown to bind to fibronectin, a component of the basement membrane. The fibronectin gene contains three exons that can be spliced. The connecting segment (CS)-1 is an alternative splice format of fibronectin and a subset of these molecular variants. The CS-1 has been shown to bind to VLA-4 (49) and blocking the CS-1 fragment has also previously been shown to affect lymphocyte adhesion (50).

The expression of CS-1 has now been demonstrated in human aortic endothelial cells (51) and in HUVECs (52). The CS-1 expression by HUVECs is regulated with pro-inflammatory cytokine IL-1B (52). Normal synovium ECs contain little CS-1 fibronectin suggesting that the cytokine milieu present in chronic inflammatory conditions may be very important for the expression of this fragment on ECs. Thus far, CS-1 expression has not been reported in CNS endothelium. However, Yednock et al. (53) demonstrated that EAE induction could be efficiently blocked using an anti-VLA-4 antibody and we have demonstrated that the addition of anti-VLA-4 blocking antibody to transwell migration experiments significantly decreased monocyte and B-cell migration (43,44). Furthermore, using the anti-VLA-4 blocking antibody, Alter et al. demonstrate decreased B cell migration across fibronectin coated transwells in the absence of HBECs which confirms that VLA-4 does in fact bind with fibronectin. Blocking VLA-4 is of particular interest given that an anti-VLA-4 antibody (Antegren) has recently been proposed as a therapy for MS. Treatment with this antibody results in fewer inflammatory brain lesions and relapses during the course of therapy (54). Our in vitro assay indicates that anti-VLA-4 antibody blocks the migration of multiple cell types and is implicated in the MS

disease process. We thus postulate that the antimigratory effect of VLA-4 blockade is mediated by the inability of VLA-4 to bind to CS-1 rather than by blocking VLA-4/VCAM-1 interaction. This would also explain the relative failure of anti-VCAM-1 antibodies or molecules to inhibit (1) the in vitro migration of leukocytes subsets in human and (2) the development or the severity of EAE.

To summarize, LFA-1/ICAM-1 and VLA-4/and its ligands seem to have more prominent roles at the site of inflammation as compared to selectins and are, for the most part, responsible for the adhesive properties of leukocytes participating in CNS-directed inflammation.

2. SOLUBLE ADHESION MOLECULES

Adhesion molecules also exist in soluble forms, even in healthy humans (55). Increased levels of sICAM-1 and sVCAM-1 have been identified in the serum and CSF of MS patients and correlated with disease activity and relapse (56–60). Although still controversial, positive correlations of adhesion molecules have been made with MRI markers of disease activity (61,62) and the area of Gd-enhancing lesions (63,64). The cellular source of these soluble adhesion molecules however, remains unclear. Lymphocytes, monocytes, and astrocytes could potentially be a source of these molecules. In vitro, production of soluble adhesion molecules has been demonstrated using activated ECs (65). The finding that ECs could be source of soluble adhesion molecules was confirmed using human brainderived BBB-ECs for both sICAM-1 (66) and sVCAM-1 (67). As HBECs shed their adhesion molecules, the resulting decreased levels of ICAM-1 may limit the amount of immune cells that can adhere to and infiltrate across CNS endothelium. Soluble adhesion molecules may also be a beneficial down-regulatory mechanism against aggressive auto-immunity such that the released molecules (sICAM or sVCAM) may bind to and block cognate receptors on immune cells, thus limiting subsequent immune cell adhesion to CNS endothelium. Soluble VCAM-1 may bind to VLA-4 on immune cells limiting subsequent interaction with VCAM-1 or even the CS-1 fragment on ECs. IFN β , currently used as a therapy for relapsing MS, has been linked to increased serum levels of sVCAM-1 and sICAM-1 found in patients (68,69). Increased sVCAM-1 serum levels correlate with decreased MRI lesions (69,70). The levels of soluble forms of selectins in MS remain unclear.

3. CYTOKINE AND CHEMOKINE PRODUCTION BY BBB-ECs

Cytokines are classified as either pro- or anti-inflammatory. Numerous proinflammatory cytokines have been shown to be elevated in the blood, serum, and CSF of MS patients during the course of disease. Pro-inflammatory cytokines also predominate at sites of tissue damage following CNS injury or inflammation in EAE mice. Endogenous CNS cells (microglia and astrocytes) are considered to be the dominant source of pro-inflammatory cytokines TNF α , IL-1, IL-6, and IL-12 within the CNS (71–73). Perivascular microglia also secrete the anti-inflammatory cytokines IL-10 and TGF β (74). CNS-infiltrating immune cells are additional sources of inflammatory factors; perivascular immune cells also produce IFN γ at the site of lesion.

BBB-ECs comprise a potentially important source of cytokines. Brain microvascular ECs produce and secrete the pro-inflammatory cytokines IL- $1\alpha/\beta$, IL-6, and GM-CSF in response to inflammatory challenge, soluble or cell mediated (35,75–79). TNF α production has also been reported in human brain ECs following TGF β , LPS or IL-1 α stimulation (37,76,80) although generally, ECs have been considered as a target and not as a source of TNF α .

Furthermore, brain endothelium is a potential source of anti-inflammatory cytokines such as IFN β and TGF β (76). Although the notion of BBB-EC production of the anti-inflammatory cytokine IFN β in response to inflammatory challenge, such as T-cell infiltration, is appealing, human brain derived EC cultures used in our laboratories do not produce IFN β (37) unless they are infected with adenoviruses or retroviruses (personal unpublished observations). IFN β production by BBB-ECs is thus unlikely to happen in vivo in humans. We do believe however, that in experiments in which adeno- or retroviruses are used as vectors to carry DNA or RNA signals within ECs, levels of IFN β should be monitored and controlled to ensure that an unspecific effect of viral transfection does not occur.

Chemokines constitute a large group of small (8–10 kDa) cytokines secreted within a target tissue and are responsible for the selective recruitment and activation of immune cells in vivo and in vitro. The current paradigm of chemokines and their role in leukocyte extravasation into lymphoid organs (81) can now be extended into CNS endothelium vascular beds. Chemokines are classified into four subfamilies based on differences in the arrangement of the first two cysteine (C) residues. More than 40 chemokines have been identified; the selectivity of these is reviewed in Chapter 14. Chemokines relevant to the human BBB, especially in the context of neuroinflammation, include CCL2/MCP-1 (monocyte chemoattractant protein-1), CCL3/MIP-1 α/β (macrophage inflammatory protein-1), CCL5/RANTES (regulated upon activation, T-cell expressed and secreted), CXCL8/IL-8, and CXCL10/IP-10 (IFN-inducible protein-10) (82). CC-chemokines attract lymphocytes and monocytes/macrophages, as well as basophils, eosinophils, dendritic cells and NK cells. CXCL8/IL-8, a chemokine included in a subgroup of chemokines containing an ELR motif at the N-terminal (glutamic acid-leucine-arginine), is primarily chemoattractant

for neutrophils, whereas CXCL10/IP-10, a CXC chemokine lacking the ELR motif, is a potent chemoattractant for activated T cells. Both CCL2/MCP-1 and CXCL8/IL-8 are expressed by BBB-ECs in vitro under non-inflammatory conditions (83,84). We and others have also shown that in vitro human BBB-EC stimulation with pro-inflammatory cytokines, such as IFN γ or TNF α , results in the up-regulation of CCL2/MCP-1 and CXCL8/IL-8, as well as the additional production of CCL5/RANTES and CXCL10/IP-10 by such cells.

Chemokines bind and signal via G protein-coupled receptors that can be stimulated by more than one chemokine. Studies in animal models of neuroinflammation, namely EAE, demonstrate that the regulation of chemokines and their receptors is not straightforward. Numerous studies have identified the importance of CCL2 in neuroinflammatory disease (85.86). This chemokine regulates migration of multiple cell types with potential to contribute to tissue injury or repair. The CCL2 immunoreactivity can be detected in the brain (87) in reactive astrocytes and macrophages in and around inflammatory plaques in MS and EAE (88-92) and in brain ECs (93) at onset of inflammation and prior to clinical expression of EAE. CCL2 has convincingly been shown to play a critical role in the induction of EAE as the presence of anti-CCL2 antibodies induce a less severe EAE course (92). The in vitro actions of CCL2/MCP-1 and CCR2 have been shown to be promiscuous; chemokines other then CCL2 are agonists for CCR2 and CCL2 may act through receptors other than CCR2. In addition to CCL2/MCP-1, other chemokines are also up-regulated in EAE and in MS inflammatory plaques (94). These include CCL5/RANTES, CXCL10/IP-10 and CCL3/MIP-1a, all reported to be produced by brain ECs. Additionally, all major cell types within the brain can synthesize chemokines including microglia, astrocytes, and macrophages (89,95).

The CCL2/MCP-1 levels seem to be decreased in the CSF of MS patients (96). Lower CCL2/MCP-1 CSF levels can be postulated to result from CCL2/MCP-1 transcytosis across ECs from the abluminal to the luminal side of vessels. The CCL2/MCP-1 binding sites have recently been identified on brain microvessels suggesting that CCL2/MCP-1, produced by ECs or by CNS-endogenous cells (microglia and astrocytes), can be transported to the luminal surface of ECs and presented to circulating immune cells (97) (Chapter 14 for details). Transcytosis and chemokine immobilization on EC membranes has previously been demonstrated with the chemokine CXCL8/IL-8 using electron microscopy in peripheral ECs (98,99). This group suggested that chemokine immobilization is required to promote chemoattraction. CCL2/MCP-1 presentation at the level of brain endothelium is significant as this may indeed be the chemokine that directs the cell type infiltrate into the CNS in the course of neuroinflammation. Accordingly, CCL2/ MCP-1 appears to have an essential role in monocyte entry into the CNS in EAE (86,100,101). Anti-CCL2 can inhibit monocyte migration across HUVECs (102) and human brain derived BBB-ECs (43). We further demonstrate that CCL2/MCP-1 is involved in directing human T-cell subset migration (42) as well as the migration of MS patient derived lymphocytes (82) in our in vitro migration assay across human brain derived BBB-ECs. Human B-cell migration across BBB-ECs is also partially controlled by CCL2/MCP-1 (44).

Differential chemokine receptor expression by different leukocytes and the pattern of chemokine production by HBECs, as well as the capacity of HBECs to present endogenously produced chemokines, is most likely the driving force for a given infiltrate present within the CNS.

4. ANTIGEN PRESENTATION AT THE BBB

Entry into the CNS can occur as a result of activated T-cell binding to upregulated EC ligands. This, as mentioned previously, is a consequence of inflammatory mediators acting on the BBB, or subsequent to the recognition of antigens presented on blood vessel walls. Brain antigen-specific T cells are amongst the first to enter the CNS in EAE (1). Ongoing studies aim to define the antigen presenting capacity of BBB-ECs.

Naïve CD4⁺ T-cell activation and proliferation requires at least two signals: the first signal results from engagement of T-cell receptor/ CD3 molecules with a specific antigen bound to an MHC class II molecule expressed on the surface of an antigen presenting cell (APC). The second signal comes from co-stimulatory interactions between surface receptors on T cells and their cognate ligands on APCs such as CD28-B7, or CD40-CD40L. Without this second activation (co-stimulation), T cells become unresponsive or anergic (103). Expression of MHC class II and co-stimulatory molecules is generally restricted to professional APCs. However, under certain inflammatory conditions, these molecules can be induced on a variety of cell types. In EAE, perivascular macrophages and microglia within the CNS express high levels of MHC class II and co-stimulatory molecules and are usually considered to be the two cell types involved antigen presentation (104,105). In human, perivascular microglia also constitutively express MHC class II and B7 molecules in vitro (106) and in vivo.

Brain endothelial cells have been described as poor or nonprofessional antigen presenting cells. Several groups have independently shown that human and animal BBB-ECs do not constitutively express MHC class II or B7 molecules under resting conditions (107,108). However, initial studies performed in the 1980s also indicate that under inflammatory conditions, brain ECs have the capacity to process and to present MBP antigen to T cells, as well as to sustain T-cell proliferation of pre-sensitized leukocytes (108,109). This is a significant observation as cerebral microvessels, mainly due to their location and the surface area that they cover, are an important interface between cells of the immune system and the CNS. Reports about the expression of MHC class II on human brain microvessels within MS lesions remain conflicting. Dore-Duffy and colleagues have demonstrated significant levels of MHC class II on brain endothelium in MS lesions (21,110), whereas Bo et al. (111) localized MHC class II expression to macrophages and microglia, and showed an absence of this molecule on endothelial cells.

We and others have demonstrated that human brain ECs, when stimulated with IFN γ express significant levels of MHC class II, B7.1, and B7.2 molecules, as compared to human microglia (112–114). However, in the same study, we also demonstrate that these activated cells are unable to sustain T-cell proliferation, unless exogenous IL-2 is added (112). Similar observations have been made in HUVECs and in brain ECs from rodents (115–117). Taken together, these observations confirm that human and animal brain derived ECs can express MHC and co-stimulatory molecules but cannot act as professional APCs. We postulate that, because of the very large surface area of ECs, the appropriate molecular antigen presenting machinery is dispersed on the cell surface and does not allow the optimal T-cell signaling to achieve full stimulation.

Increasing attention has recently been given to the role of $CD4^+CD25^+$ regulatory (suppressor) T cells in neuroinflammation (118). Induction of these cells occurs following T-cell interaction with non-professional APCs, as best demonstrated using immature dendritic cells (119,120). In autoimmune disease, such regulatory T cells have been demonstrated to exhibit an inhibitory activity of autoreactive T cells (118,120–125). A defect of these cells is reported to be present in MS patients (126). As brain-ECs are poor APCs, they cause T-cell unresponsivness subsequent to T cell:EC interaction (112). We have also identified brain ECs as suppressors of infiltrating leukocytes. In accordance with this concept, we have shown that transmigrated monocytes down-regulate their antigen presenting capacity and cytokine and chemokine production compared to their non-migrated counterparts (127). We thus believe that the immune function of brain ECs as regards antigen presentation is anti-inflammatory and down-regulates the pro-inflammatory environment by generating CD4⁺CD25⁺ regulatory T cells.

5. NEUROTROPHIN PRODUCTION BY HBECs

An ongoing debate in MS relates to the relative contribution of immune mediated vs. neurodegenerative mechanisms in the disease pathogenesis. Recent analysis of early active lesions of MS, using up-dated immunohistochemical and molecular biologic techniques, have emphasized the case-to-case heterogeneity with regard to the extent of oligodendrocyte loss or injury, the extent of axonal injury, and the presence of ongoing remyelination (4). Over time, there is continued tissue loss of myelin, Recently, a number of studies have proposed that autoimmune inflammation can have a neuroprotective role in the CNS (131–134). MBP reactive T cells limit secondary degeneration after optic nerve injury (131) and surprisingly, transplantation of activated macrophages can promote lesion repair (135). Although the factors involved in this immune mediated neuroprotection remain unknown, possible explanations include production of neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) by migrating immune cells. In the field of MS, neurotrophins are known for their dual functions: (1) to provide trophic support and prevent neuronal cell death, as well as to enhance oligodendrocyte survival, proliferation, and support remyelination (136,137), and (2) to act as immunomodulators. More recently, a pre-form of NGF (i.e., pro-NGF) was also shown to induce oligodendrocyte death, a finding that still needs to be confirmed.

Studies in animals demonstrate that systemic administration of NGF reduces the severity of EAE in nonhuman primates (138) and changes the T-cell infiltrate from Th1 to Th2. The EAE induced by injection of MBP specific T cells can be prevented by co-injection with NGF secreting MBP-specific T cells (139). Within the CNS, NGF could exert functional effects on both neuronal and glial populations. Furthermore, NGF can down-regulate MHC class II, B7.1, and CD40 expression on rat microglia in culture (140,141). In EAE, NGF was shown to regulate monocyte migration across the BBB (139). In the marmoset monkey (non-human primate) EAE model, NGF was also shown to decrease the production of pro-inflammatory cytokine IFN γ by infiltrating T cells (138). Therefore, NGF seems to be capable of suppressing new CNS lesion formation at numerous levels.

The identified sources of neurotrophins within the CNS include microglia, astrocytes and neurons. Although initially characterized in specific neuronal populations, neurotrophins have now been shown to be produced by both glial and non-neural cells, including cells of the immune system such as T cells, B cells, and monocytes (142–144). Production of NGF by glial cells is induced by pro-inflammatory cytokines, including IL-1 and TNF α (145– 147). NGF mRNA expression by mouse astrocytes in vitro can also be induced by IFN β (148). We have recently identified human brain microvascular ECs as an additional potential source of NGF within the CNS. The NGF production has recently been demonstrated in rat brain capillaries (149,150) and has also been shown in human dermal microvascular ECs and in HUVECs (151,152). Additionally, HUVECs are also reported to secrete BDNF (153,154). Whether HBECs produce pro-NGF or the mature NGF remains to be established.

Immune Functions of Brain Endothelial Cells

NGF production by human brain derived BBB-ECs is dependent on cell-cell contact between T cells and endothelial cells and is augmented when lymphocytes are exposed in vitro or in vivo to IFN β (170). This effect is not mediated through ICAM-1 nor VCAM-1, and NGF production by BBB-ECs is not regulated by IFN γ , TNF α , or IL-1 as shown for ECs derived from rat brain (149). The precise signals that modulate NGF secretion are not yet defined but our study provides evidence that lymphocyte contact with BBB-ECs is a potent stimulator of NGF release. Interestingly, NGF induction by lymphocytes derived from MS patients inversely correlated with EDSS, the clinical measure of disability in MS, with total T2 lesions volume, and with brain atrophy, all of which are magnetic resonance-based measures of the extent of tissue injury and loss.

As many therapeutic drugs, including IFN β , are prevented from entering the CNS due to the presence of the BBB, the effect of IFN β on the secretion of NGF by BBB-ECs could represent a potentially important mechanism of IFN β action and is a good example of the immunomodulatory role of brain ECs. This then suggests that HBECs, the gatekeepers of the CNS, in addition to having a barrier function for immune cell entry, respond to noxious inflammatory cell infiltration by the production of NGF.

NGF secretion may also act in an autocrine fashion on HBECs. There are currently no reports of whether HBECs express NGF receptors but the receptors p75^{NGFR} and trkA are present on HUVECs and rat brain capillary ECs (149,150,152). These cells proliferate in response to NGF stimulation. It is therefore possible that in addition to promoting protection and survival of neurons and oligodendrocytes, and down-regulating CNS-localized immune responses, NGF may have an autocrine function in HBECs to counteract infiltrating cell induced damage at the level of the BBB. We, however, currently have no data to support this assumption.

6. THERAPEUTICS

Currently approved therapies for the treatment of MS include GA and IFN β . While the goals of both are to reduce inflammation in the periphery and within the CNS, the mechanisms by which they do so are distinct.

GA is a random copolymer of alanine, glutamic acid, tyrosine, and lysine, originally designed to simulate the structure of MBP. One possible mechanism of action of GA involves Th2 cell mediated bystander suppression via the secretion of anti-inflammatory cytokines. Several reports have indicated that GA induces a shift in peripheral T helper cells from Th1 to Th2 responses although the precise mechanism of how this occurs remains unclear (155). A Th2 bias has been demonstrated in EAE but also in MS patients undergoing therapy with GA (156–158). Injection of GAreactive Th2 biased cells prior to immunization can suppress EAE (159). GA-reactive Th2 cells have been shown to readily traffic into the CNS of EAE-affected animals (160). With regards to ECs, GA does not seem to directly affect brain ECs as it does not alter cultured EC adhesion molecule (161) and chemokine expression by HBECs (unpublished data). GA treatment of HBECs does however have an effect on T-helper cell subset migration by a yet unidentified mechanism (171).

IFN β is the other currently approved therapy in MS. Unlike GA, IFN β is not thought to cause a Th2 shift in the immune system but rather to affect several components in the process required for migration of inflammatory cells thereby excluding leukocytes from the CNS. IFNB decreases the production of MMP-9 by T cells, which corresponds to decreased T-cell migration (162,163). In fact, animals treated with IFNB show a reduced number of inflammatory cell infiltrates in the CNS (164). IFNB can also affect antigen presentation and can be considered as anti-proliferative (165,166). With regards to ECs, IFN β has been shown to decrease adhesion molecule expression. In collaboration with Dr. Calabresi, we have shown that IFNB facilitates the conversion of VCAM-1 in HBECs into its soluble form (67). IFNβ may also directly or indirectly stabilize the BBB. IFNβ treated patients show rapid resolution of Gd-enhancing activity on serial MRI scans (167). Recently, Kraus et al. (168) demonstrated a direct stabilizing effect of IFNβ on bovine brain ECs as assessed by permeability and TJ molecule expression. IFNB has also been shown to interfere with matrix metalloproteinase activity and, as discussed above, IFNB can also potentiate neurotrophin NGF production by HBECs.

7. IMMUNE PROPERTIES OF HBECS VS. NON-CNS DERIVED ECS

In humans, ECs most frequently used for the study of migration are HUVECs, on occasion grown with astrocytes derived conditioned media for the induction of BBB characteristics. However, important differences exist between ECs derived from different organs with respect to their immune functions. In our laboratories, we compared HUVECs to HBECs in terms of permeability and control of cellular migration (42). HUVECs grown in the presence of glial-cell derived media proved to be less permeable to large molecular weight tracers as compared to non-supplemented HUVEC cultures. However, the permeability of supplemented HUVECs was comparable to that observed in non-supplemented HBECs, suggesting that HBECs are functionally different cells. Migration studies further demonstrate that, under identical culture conditions, the rate of T-cell migration is much higher across HUVECs as compared to HBECs, suggesting once again that HBECs are a more stringent barrier, not only to molecules but also to cells. This is also applies to monocyte transmigration where

we demonstrate a 10% migration rate across HBECs over 48 hours (43) compared to the reported 40-50% across HUVECs over a 2 hour period (169). Thus we strongly believe that HBECs represent a less permissive barrier to cell migration than HUVECs.

Other groups have also identified important differences between HBECs and HUVECs. Using cDNA microarray analysis, Kallman et al. (153) showed that primary HBECs are characterized by a distinct gene expression pattern that differs from HUVECs with at least 35 gene transcripts only detected in HBECs. Gene products exclusively expressed or expressed in significantly increased levels by HBECs compared to HUVECs consist of factors with growth-supporting properties [vascular endothelial growth factor (VEGF)] and those involved in neuroprotection (BDNF). Differences related to molecules specifically involved in migration, as reported by Kallmann et al. (153), include the secretion of CCL2/MCP-1 in significantly higher levels by HBECs vs. HUVECs. Furthermore, in contrast to HUVECs, which constitutively express high levels of VCAM-1 and ICAM-1, we and others have shown that HBECs express moderate levels of ICAM-1 and no VCAM-1 under basal conditions (16,29,37). The differential expression of chemokine CCL2/MCP-1 and adhesion molecules has important implications for immune cell transmigration across the BBB. Therefore, in order to study the mechanisms implicated in immune cell infiltration as would occur in the pathogenesis of MS, it is especially relevant to use endothelial cells derived from the organ of interest.

8. EFFECT OF ASTROCYTE-DERIVED MEDIA ON IMMUNE FUNCTIONS OF HBECs

Numerous adhesion and transmigration studies of leukocytes across ECs make use of astrocyte-conditioned media for the generation of an in vitro model of the BBB. It is therefore of interest to consider the effect of glial factors on HBEC immune properties. The addition of astrocyte-conditioned media to HBECs does not seem to alter any of the above-discussed immune properties of HBECs. We have reported that glial factors do not influence HBEC MHC/B7 expression (37). We further demonstrated that glial factors moderately up-regulate ICAM-1 on HBECs and have no effect on VCAM-1 expression or the pattern of HBEC chemokine production (37). The ICAM-1 induction by pro-inflammatory cytokines IFN γ and/or TNF α is much higher compared to the levels observed after culture with astroglial factors. Astrocytederived factors are however important in barrier integrity.

9. CONCLUSION

In summary, considerable changes occur at the level of the BBB throughout inflammatory neurological diseases resulting in enhanced BBB permeability

and augmented cellular trafficking. Immune cell extravasation into target organs, including into the CNS, has been characterized as a highly regulated and well-ordered multi-step process. Steps involved have generally been well documented with reports of minor variations depending on the vascular site and leukocyte subset involved. Our data suggest that the BBB actively participates in the process of neuroinflammation. Ideally located between circulating T cells and extravascular sites of antigen exposure, ECs are capable of selecting infiltrating cells through the expression of specific inflammatory cytokines, adhesion molecules and chemokines. ECs may also have the ability to secrete neuroprotective molecules (i.e., NGF) in response to inflammatory cell challenge.

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9

T-Cell Migration Across the Blood–Brain Barrier in CNS Inflammatory Diseases

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T-cell migration into the central nervous system (CNS) is a critical element in inflammatory and autoimmune diseases of the nervous tissue. Although several components involved in this process have been identified, we still lack a complete understanding of the initiation of CNS immune reactivity and T-cell recruitment into the CNS. Elucidating the pathways that promote T-cell migration and recruitment into the CNS is therefore necessary in order to gain an understanding of CNS immune reactivity and to design novel immune therapies that interfere with inflammatory and autoimmune diseases of the CNS.

Naïve T cells mature in the thymus and, if unchallenged, recirculate between blood and lymph nodes. These naïve, antigen-specific T cells predominantly react to particular antigens presented by antigen presenting cells (APCs) in the secondary lymphoid organs. As a result of antigen recognition, T cells become activated, polarize into T helper (Th) 1, Th2 or other effector T cells, and acquire effector functions such as cytokine production and cytotoxic mediator expression that will be executed in the tissue of antigen origin. In parallel with the activation of effector functions, adhesion molecules and chemokine receptors are up-regulated on the surface of activated T cells. Increased adhesion molecule and chemokine receptor levels allow T cells to change their migratory paths and home to non-immune tissues, such as the CNS. In order to access the CNS, T cells must migrate
out from the blood across a specialized endothelial cell barrier, the bloodbrain barrier (BBB), which governs entry into the nervous tissue. Although this is the major migratory pathway for immune cells, other mechanisms have also been suggested for T-cell entry into the brain and spinal cord. Upon their migration into the CNS, T cells induce molecular changes in the nervous tissue and initiate an inflammatory reaction. In the following chapter, we will assess the current understanding of T-cell activation against antigens derived from the nervous tissue, and review the mediators, cell surface adhesion molecules, and chemokine receptors that guide T-cell migration across the BBB and influence T-cell survival and initiation of immune responses in the CNS during autoimmune and inflammatory diseases.

1. T-CELL ACTIVATION AGAINST ANTIGENS DERIVED FROM NERVOUS TISSUE

1.1. Do T Cells Need to Be Activated Prior to Their Migration into CNS?

In our current model of T-cell migration into the CNS, we postulate that T cells must be activated in order to home to nervous tissue (1-3). However, it has been suggested that non-stimulated T cells are also capable of migration into the brain (4), and lymphocytes that are a part of the normal naïve recirculating T-cell pool have been shown to be in the cerebrospinal fluid (CSF) (5). This controversy may arise from the existence of multiple pathways and mechanisms that could mediate T-cell migration into CNS. At least three major pathways have been suggested to be involved in this process, (a) migration from blood to CSF across the choroid plexus of the ventricles, (b) migration from blood to the subarachnoid space across the arachnoid epithelium forming the middle layer of the meninges, and (c) through the perivascular space across the BBB (reviewed by Ransohoff et al. (6). T cells could be directed to these different migratory pathways according to their subtypes, antigen specificity, or activation stage. For example, it was proposed that naïve T-cell migration could be mediated through the CSF rather then via the BBB, while activated pro-inflammatory T cells prefer to migrate through the BBB. Th1 or Th2 cell polarization is another possible regulator of T-cell migration across the BBB. Human allogeneic or MBP-reactive Th2-polarized lymphocytes have been shown to migrate more avidly than Th1-polarized lymphocytes across an in vitro endothelial cell layer (7).

Although antigen specificity may also contribute to T-cell migration into the CNS, this factor is likely to be more important in retention of T cells inside the CNS (discussed in more details in paragraph 3) than in their migration across the BBB (8). In addition to T-cell characteristics that may influence migration, the presence of an anti-inflammatory environment in the CNS has long been suggested to influence entry, as well as retention, of T cells in the nervous tissue. The anti-inflammatory environment of the CNS involves the combination of a relative lack of co-stimulatory molecules, histocompatibility antigens, and APCs, with an abundance of anti-inflammatory mediators (notably transforming growth factor-beta $(TGF-\beta)$) in the nervous tissue. These elements all contribute to the immune privileged nature of the CNS. In an immunologically privileged environment, naïve T-cell entry into the CNS might lead to immunogenic tolerance induction in situ due to the absence of professional APCs in the noninflamed nervous system (4). Survival of naïve T cells in the CNS should require local activation. Although the possibility of naïve T-cell activation in the CNS cannot be excluded, the probability of such event is very low. Even already differentiated Th1 cells may require re-stimulation within the CNS in order to initiate inflammation (9), and disease severity in experimental autoimmune encephalomyelitis (EAE) has been demonstrated to depend on the reactivation of infiltrating cells (10).

Based on this information we could conclude that efficient initiation of T-cell mediated inflammation in the CNS requires T-cell activation in the periphery and activated T-cell migration across the BBB. In order to understand this process further, we will first focus on the process of T-cell activation against nervous tissue antigens (Secs. 1.2–1.4), then analyze the mechanism of activated T-cell migration across the BBB (Sec. 2) and finally evaluate molecular changes generated by activated T cells in CNS and contributing to the initiation of immunity in this tissue.

1.2. Mechanisms of T-Cell Activation Against CNS Derived Antigens

1.2.1. Antigen Drainage from the CNS to Peripheral Immune Tissues

If activation is required for the migration and retention of T cells in the CNS, the question to focus on is the mechanism and tissue site of this process. Naïve T cells most likely traffic through secondary lymphoid organs, including peripheral and mesenteric lymph nodes, Peyer's patches and spleen, in which they may encounter antigen in a context that induces both their activation and clonal expansion (reviewed in Ref. 11). If only CNS antigen-specific T cells can be retained in the CNS, and if T cells are activated primarily in the secondary lymphoid tissues, then CNS antigens must be presented in secondary lymphoid tissues. This raises the question of how CNS originated antigens can reach the secondary lymphoid organs. It was demonstrated that antigens from other peripheral tissues are efficiently delivered by dendritic cells to secondary lymphoid organs through afferent lymphoid vessels. This mechanism has not seemed applicable to CNS due to the prevailing theory that the CNS lacks lymphoid drainage and that nervous tissue contains only a limited number of dendritic cells. In this context, it was proposed that the main possible autoantigens in the CNS autoimmune disease multiple sclerosis (MS) (i.e., myelin basic protein, (MBP) and Golli-MBP—the developmentally early precursor of MBP) are expressed in lymphoid tissues, and that cells of the immune system become exposed to CNS antigens (12–14). However, Golli-MBP antigen expression is mostly localized in primary lymphoid tissues indicating the importance of this process in inducing tolerance against antigens of the CNS rather than activating nervous tissue antigen-specific T cells. Subsequently, multiple mechanisms for protein or antigen drainage from the CNS to the periphery, specifically into secondary lymphoid organs, such as the cervical lymph nodes were suggested. These drainage pathways were discovered by tracking intracerebrally infused soluble protein antigens, viral pathogens, or different dyes such as India ink through the body. In 1950, Cairns (15) suggested that about 90% of an intracerebral inoculum of a phage label spills over to the blood almost immediately following intracerebral injection. Later, a series of studies by Cserr and colleagues and by other groups confirmed that large molecular weight tracers, such as dextrans and horse radish peroxidase injected into brain parenchyma can spread along perivascular spaces and are observed in the surroundings of large vessels at the surface of the brain. It was suggested that tracers also leak across the leptomeningeal sheath surrounding the subarachnoid perivascular space into the CSF. Other channels for flow were also suggested in CNS, including the sub-ependymal zone of the ventricular ependyma, subpial space, tissue spaces within arachnoid trabeculae and the arachnoid, and spaces between fiber tracts in the white matter (reviewed in Refs. 16, 17).

The significance of such drainage pathways from brain to periphery was suggested to be important in initiation of immunity in the CNS. This view was not widely appreciated until the drainage of larger proteins, such as ovalbumin (OVA) into peripheral immune tissues was demonstrated (18). It was suggested that drainage of MBP and other CNS antigens might be regulated similarly and that this drainage is important in initiation of CNS autoimmunity. It was also demonstrated that OVA drainage via the flow of cerebral extracellular fluids from brain to blood across the arachnoid villi to lymph along certain cranial nerves (primarily olfactory) and spinal nerve root ganglia is important in peripheral activation of immune cells (19). These drainage pathways are facilitated by the cerebral extracellular fluid or brain interstitial fluid that provides a directional flow for delivery of CNS-derived antigen to peripheral lymphoid organs (19–26). Through the efflux of extracellular fluids, brain-derived protein antigens drain more rapidly into peripheral lymph nodes after administration (27,28).

Cervical lymph nodes and their afferent lymphatic vessels are the primary sites of drainage for brain-derived protein antigens (27–29). It was shown that in the cervical lymph nodes, brain-derived protein antigens

could elicit antigen-specific humoral immune responses, including generation of antibody-secreting plasma B cells and the appearance of specific antibodies in the blood (25,26,30).

However, it was also demonstrated that a significant portion of protein antigen could be retained in the CNS following intracerebral microinjection (19,31). The retention of soluble protein antigen in the brain, or at least the detectable amount of it, is time limited, restricted up to about 3–7 days (32). By contrast, following intracerebral administration, Mycobacterium bovis strain Bacillus Calmette-Guerin (BCG) is internalized by cells of the mononuclear phagocyte lineage through phagocytosis and is retained in the brain parenchyma for up to 1 year (33). Viral pathogens are also detectable in CNS for a longer period of time. These studies confirm that proteins originating either from the brain's own components or microinjected into the brain can drain to the peripheral lymphoid tissue and might induce specific immunity. Although this drainage might not be sufficient to induce peripheral immune responses, other factors are probably involved in this process.

1.2.2. Antigen Delivery from CNS to Peripheral Immune System by APCs: Reversed Migration of APCs from CNS to Periphery

Recently, it was proposed that rapid access of tissue-derived antigens to secondary lymphoid organs was not sufficient to induce optimal T-cell activation and that local APCs that arrive from the tissue of antigen origin are also critical in this process (34). This might partly explain why, despite the drainage of CNS antigen and the presence of nervous tissue antigens in secondary lymphoid tissues, immune reactivity against CNS antigen, and initiation of MS or EAE, the experimental animal model of MS, only occurs at low frequency. Several types of cells, such as astrocytes, microglia, endothelial cells, perivascular macrophages, and dendritic cells have been proposed as APCs in the CNS (35–41). However, only bone marrow-derived perivascular macrophages and dendritic cells are potent migratory cells capable of trafficking to peripheral lymphoid tissue. Some in vivo data support the importance of these cells in initiation of CNS autoimmunity, since bonemarrow-derived elements are necessary for the development of EAE (42).

We have demonstrated that dendritic cells can accumulate in the CNS in response to intracerebrally injected antigen and can migrate out of the CNS and induce systemic immune responses in the secondary lymphoid organs. One consequence of this migration could be the homing of antigen-specific T cells to the brain. This study indicates that brain-derived dendritic cells may behave similarly to skin (34,43)—and Peyer's patch (44)—derived dendritic cells in that they pick up antigen from peripheral tissues, migrate to draining lymph nodes, and induce a second wave of antigen presentation necessary for induction of immunity against tissue-localized antigen (34).

Our data further indicate that despite the dampened immune response at this immunologically privileged site, when an immune response is initiated in the CNS, it follows the pattern previously described for antigens derived from other organs. This conclusion was based on the observation that intracerebrally injected antigen-loaded GFP-expressing dendritic cells accumulate in cervical lymph nodes and induce the accumulation of antigenspecific (H-2K^b-SIINFEKL tetramer⁺) activated CD8⁺ T cells in the brain (45). Reverse transmigration of dendritic cells through an endothelial cell monolayer has been previously demonstrated (46). However, reverse migration of dendritic cells through the BBB has not been shown. Our data are in agreement with results from other laboratories showing that intrathecally injected dendritic cells migrate to cervical lymph nodes and CNS-derived antigens can be found in dendritic cells in cervical lymph nodes of monkeys with EAE (47,48). T-cell activation results from a primary response to antigen presenting dendritic cells in secondary lymphoid organs and not from the cross-presentation of antigens by APCs in peripheral tissue. Thus, brain-derived dendritic cells might be important in initiation of immunity against brain antigens.

The question remains whether dendritic cells are resident cells in the CNS or whether they are recruited under inflammatory conditions. It was originally suggested that dendritic cells cannot be detected in the brain parenchyma under steady state conditions but appear in infections, EAE, and ischemia (49–54). However, there is evidence supporting the concept of resident dendritic cells in the CNS. It was demonstrated that the choroid plexus contains dendritic cells that produce IL-10 under normal conditions (54–56) and that dendritic cells are normally present in the CSF (57,58). One additional possibility for dendritic cell appearance in the CNS is that these cells can develop from resident microglia cells. In vitro, microglia can differentiate into dendritic cells in the presence of the growth factor granulocytemacrophage colony-stimulating factor (GM-CSF) (49-51,59). EAE cannot be induced in GM-CSF deficient mice, which underscores the importance of this process in vivo (60). Several cells in the CNS, including endothelial cells of the BBB, can produce GM-CSF that could provide a source for this mediator under inflammatory conditions (61). The importance of dendritic cells in MS was also suggested and dendritic cells are detected in MS lesions (62).

Obviously, the migratory pathways of dendritic cells in the CNS and the signals that can mediate their migration need further study. The suggested role of the chemokine receptor CCR7 in migration to and or from the brain was based on the observation that CCR7 is necessary for the development of lymphoid organs and that CCR7 ligands, CCL19 and CCL21, are also expressed at the BBB. In addition, CCR7⁺ dendritic cells can be found in MS lesions suggesting that this could be a mechanism for recruiting dendritic cells to the CNS (63–65). It is also possible that migration of monocytes across an inflamed BBB would lead to the development of dendritic cells in the CNS. In support of this idea, it was observed that when monocytes migrate across the endothelium in an in vitro system in the ablumenal-to-lumenal direction, they differentiate into dendritic cells (43,63). The possibility of such an event in vivo in the CNS has to be further studied. Finally, it is important to note that cellular migration in the CNS follows specific pathways. It was shown that stem cells migrate along the external capsule (66) and that injected antigen and dendritic cells, as well as endogenous dendritic cells, are also distributed along this pattern (45,67). These cells, along with other proteins, inflammatory mediators or infectious agents, might migrate along preferential routes of interstitial fluid flow in the brain (17). The importance of bulk flow of interstitial fluid in initiation of CNS immunity has to be further studied.

1.3. Activation of T Cells in the Secondary Lymphoid Organs Against CNS Derived Antigens

The previous paragraphs described how CNS antigens access the periphery via at least two major processes: either by antigenic drainage or via delivery by APCs. However, we also have to examine whether CNS antigen presentation in the secondary lymphoid organs can induce antigen-specific T-cell activation and preferential homing to the nervous system. This is a fundamental question in the initiation of primary immune responses, as well as for the reactivation of antigen-experienced T cells in lymph nodes. Our laboratory showed that antigen-specific T cells can be activated in the periphery and accumulate in the brain in response to injection of soluble ovalbumin (OVA) protein antigen into the brain parenchyma (67). T-cell accumulation in the brain peaks 7 days following intracerebral OVA injection, although direct drainage of OVA to cervical lymph nodes was detected within hours following antigen delivery (67). This delayed T-cell accumulation in the injection site further suggests that migration of APCs from the CNS is important in this process. Others have also shown that T cells can be activated by intracerebrally injected dendritic cells that migrate from the brain to secondary lymphoid organs (48). Brain-emigrant, dendritic cellmediated antigen presentation is necessary and sufficient to induce infiltration of leukocyte functional antigen LFA-1^{high} T-cell populations to the brain (45). The importance of peripheral T-cell activation by CNS APCs, particularly dendritic cells, is further supported by several reports. It has been shown that injected CNS antigen-loaded dendritic cells can elicit CNS directed autoimmunity when injected into naïve mice (68,69). In addition, in cervical lymph nodes from monkeys with EAE, CNS antigens are present in dendritic cells (47). Dendritic cells have also been shown to cross present CNS tumor antigens and induce cytotoxic T-lymphocyte accumulation in an implanted brain tumor (70). Some reports indicate that CNS APCs might have a dual

function, either mediating tolerance or activation of antigen-specific cells. The conditions that regulate these functions need to be further studied. For example, in chronic EAE, APCs prepared from brain induce unresponsiveness in T cells (71) indicating that dendritic cells could be part of the immune privilege factors in CNS inflammatory disease. The studies summarized above show clear evidence that prior peripheral T-cell activation is important in initiating CNS immunity and T-cell migration across the BBB.

2. MIGRATION OF T CELLS ACROSS THE BBB IN CNS INFLAMMATORY DISEASES

2.1. The BBB in Health

One of the most important CNS features involved in regulation of activated T-cell migration into the nervous tissue is the BBB. In the BBB, a network of adherent junctions and tight junctions (TJs) form a complex barrier between capillary endothelial cells. These complex junctional structures are formed by transmembrane adhesive proteins, which promote homophilic adhesion among the cells and create zipper-like structures along the cell borders. Inside the cells, junctional adhesive proteins are linked to the actin cytoskeleton and this interaction stabilizes adhesion. Several endothelial functions are regulated by junctions, including growth and apoptosis, and recent results indicate that these structures have a central role in stabilizing the endothelium in the resting condition that corresponds to its physiological state. The mechanism for BBB formation is not yet fully understood; however, astrocytes and astrocyte-conditioned media have been shown to confer barrier-like properties, such as TJ molecule expression and high electrical resistance, on endothelial cells in culture (72,73), while the absence of this stimulation has been associated with lower resistance and increased permeability of brain derived endothelial cell monolayers (74). Detailed investigation has provided data to suggest astrocyte release of angiopoietin-1 may be a key player in BBB development (75). In contrast, angiogenic agents, such as vascular endothelial growth factor (VEGF), are antagonistic to barrier maintenance and development suggesting that TJ formation occurs after vascularization (75). In cases of injury, TJ expression is lost and VEGF is up-regulated. After vascular growth is complete, TJ expression is recovered (76).

It is well accepted that TJs play an essential role in restricting permeability of the BBB (77). At least 15 peripheral and three integral proteins (occludin, JAMs, and claudins) have been identified in the formation of TJs (reviewed in Refs. 78, 79) (Fig. 1). Homo-dimeric association of transmembrane molecules occludin, claudins, and junction-associated molecules (JAM) across the endothelial junction allows the formation of a tight barrier between endothelial cells. Several intracellular components are associated



Figure 1 Schematic representation of surface adhesion proteins, transmembrane tight junction adhesion proteins, and intracellular tight junction associated proteins in blood–brain barrier endothelial cells.

with these junction-spanning proteins, providing signaling components and attachment to the actin cytoskeleton. These include the zonula occludens (ZOs), cingulin, and AF-6 (80–82).

One of the earliest TJ molecules identified, occludin, is a transmembrane protein with two large extracellular loops and an intracellular tail at both the N and C terminus. This protein interacts with other occludin molecules on adjacent endothelial cells. Although a major component of the TJ, occludin is not necessary for barrier formation or function. Occludin deficient mice are small, have some histological abnormalities in testis, salivary glands, and bones, and calcium deposits are seen in the brains. However, no gross abnormalities in TJs are observed (83). Additionally, other TJ proteins, such as ZO-1, can associate with TJs in the absence of occludin (84). Disruption of occludin in brain microvessels, however, may reflect TJ damage in brain. Immunohistological staining of occludin is disrupted in MS, both in lesions and normal appearing white matter (85).

Other transmembrane proteins with two extracellular loops like occludin, the so-called claudins, have also been shown to be major components of the TJ. Claudins can interact as homo and heterodimers across the junction between two cells and, together with occludin, provide much of the protein barrier in the TJ. Heterodimers may be mildly mismatched, leading to aqueous pores in the junction (82,86). It was suggested that claudins function like occludin molecules, but they are not homologous. Interestingly, claudins might play an important role in regulating permeability at the BBB. Unlike occludin-deficient mice, claudin-5 deficient animals, while not viable after a few hours after birth, show TJ abnormalities although the BBB appears normal on gross examination. Claudin-5 knockouts showed unusually high permeability of the BBB to small (<800 Da) proteins. The BBB is still impermeable to large molecules, however, and neither bleeding nor edema was associated with claudin-5 deficiency (87).

As claudin-3 is also found at the BBB, it is likely that functional redundancy is able to rescue some claudin-5 function. Claudin molecule expression has also been analyzed in EAE. Claudin-3, but not claudin-5, immunoreactivity is lost in this neuroinflammatory disease (81). This is particularly interesting because the chemokine MCP-1, which has been shown to be up-regulated in EAE, causes loss of claudin-5 expression at the BBB (88).

Junctional adhesion molecules (JAMs) are members of the immunoglobulin superfamily. The three transmembrane JAMs A, B, and C have two extracellular immunoglobulin domains and an intracellular tail that binds other TJ proteins, including ZO-1, cingulin, and occludin (89,90). Unlike occludin and claudin, although associated with TJs, JAMs are not sufficient for TJ formation (91). Like claudin, JAMs can form homo- and heterodimers across the endothelial junction. JAMs, while also found on T cells, can interact with integrins on the lymphocyte surface and are likely to be involved in migration of T cells across the TJ via both homo and heterodimeric interactions (92). Antibodies to JAMs can inhibit recruitment across endothelium (93), although in infectious systems, they can mediate complement cytotoxicity of the endothelial cells and increase permeability (94).

Like JAM, platelet endothelial cell adhesion molecule (PECAM), also known as CD31, is a transmembrane immunoglobulin superfamily integrin (95). In addition to endothelial cells (96), platelets and leukocytes express PECAM (95,97) and antigen activation leads to increased PECAM expression on T cells (98). Homodimeric interactions between endothelial cells and leukocytes are important for the migration of T cells across the BBB, which will be described later in this chapter. In vivo, PECAM deficient mice develop early onset EAE due to increased vascular permeability of the BBB in these mice (99).

The Zonula occludens (ZO) proteins are intracellular molecules of the membrane associated guanylate kinase homologs (MAGUKs) family. There are at least three members of this ZO family, ZO-1, ZO-2, and ZO-3, although ZO-3 has not been associated with the formation of endothelial

TJs (100). ZO-1 is a key structure molecule of TJs and interacts with many TJ associated proteins including ZO-2, occludin, claudin, cingulin, and JAMs (101). ZO-1 is largely responsible for bridging transmembrane molecules with the cytoskeleton binding F-actin in an ATP sensitive interaction (102). Interestingly, to date, no knockout animal has been generated for ZO-1, although the prediction for such a deficiency would be the malformation of TJs. However, it was also suggested that under normal conditions ZO-2 molecules bind to most ZO-1 binding proteins (91) and could possibly compensate for absent ZO-1 molecules. ZO-1 expression is regulated under inflammatory conditions and immunohistochemical staining of ZO-1 is disrupted in MS, both in areas of ongoing damage and in uninflamed white matter (85,103). However, the regulation and role of this molecule in inflammatory diseases is currently confusing since supernatant from both pro- and anti-inflammatory helper T cells are equally capable of dysregulating ZO-1 expression and disrupting ZO-1 and ZO-2 integrity of brain endothelial cell monolayers in vitro (7).

Another important intracellular TJ protein is cingulin. Cingulin also helps to connect transmembrane proteins with the dynamic cytoskeleton. Cingulin is a coiled-coil protein with a globular head that binds ZO-1, ZO-2, AF6, and JAMs, and a tail that interacts with cytoskeletal myosin (101–104). The role of cingulin in inflammatory diseases of the CNS is not well understood at this time.

Several molecules may play roles in the signal transduction process of TJ mediated events. Of these molecules, AF6 is likely responsive to signaling in endothelial cells. This Ras effector binds both Ras and ZO-1 in TJs (105). Disruptions in Ras and AF6 disturb cellular junctions (106). AF6 deficiency is lethal in mice due to placental developmental failure. Examination of embryos revealed the absence of neuroectoderm TJs, although ZO-1 localization appears not to be affected (105).

The above description of the molecules present in the TJ illustrates that this is a complex structure made up of transmembrane junctional spanning molecules involved both in maintaining barrier integrity and in facilitating cellular migration. Intracellular proteins provide connections between membrane proteins and the cytoskeleton. They respond to intracellular signals to adjust TJ structure and behavior. The critical role of TJ disruption in CNS inflammatory diseases provides a therapeutically attractive target that needs to be further considered.

2.2. The BBB in Disease

Expression of adhesion molecules and arrangement of TJ proteins provide the protein structures that control entry of lymphocytes into the brain. The next important factors to consider when discussing T-cell migration into the CNS are the chemical signals, cytokines, which lead to changes in structural proteins resulting in changes in lymphocyte migration across the BBB.

The earliest cytokines implicated in modifying BBB properties were interleukin (IL)-1 and IL-6. Both cytokines are found in the brain and are expressed in response to inflammation in the CNS. These cytokines could either be produced by resident brain cells or infiltrating white blood cells. For example, IL-1 is produced by astrocytes and microglia under inflammatory conditions, such as tumor necrosis factor (TNF) - α (described below) or bacterial lipopolysaccharide (LPS) treatment, and in EAE (107-109). In vitro treatment of brain endothelial cells with IL-1 or LPS lead to the secretion of IL-6 by endothelial cells (110,111). IL-1 also increases leukocyte adhesion to, and migration across, monolayers of mouse brain endothelial cells and decreases BBB integrity as measured by a drop in transendothelial electrical resistance (TEER) across an in vitro BBB model comprised of rat endothelial cells and astrocytes grown on opposing sides of a permeable membrane (112-114). IL-6 treatment of the in vitro BBB also leads to decreased TEER measurements (114). In accord with this, IL-6 deficient mice are resistant to EAE. However, the mechanism of this resistance remains controversial. It was suggested that this effect is primarily due to a deficiency in proper autoreactive T-cell development, as opposed to problems with the inflammatory response at the BBB (115,116). Others have suggested that the resistance of IL-6-deficient mice to EAE might be mainly due to enhancement of Th2 responses (117). However, it was also proposed that the failure to induce EAE in IL- $6^{-/-}$ mice is not due to the absence of priming, since lymphocytes of immunized IL- $6^{-/-}$ mice proliferate in response to MOG peptide and produce pro-inflammatory cytokines including IL-2 and interferon (IFN- γ) (118). The same investigators demonstrated a striking difference between MOG-immunized wild-type and $IL-6^{-/-}$ mice in the expression of endothelial VCAM-1 and ICAM-1 molecules. These molecules are dramatically up-regulated in the CNS in wild-type but not in IL- $6^{-/-}$ mice. They suggested that the absence of VCAM-1 on endothelial cells of the BBB in $IL-6^{-/-}$ mice is responsible for their resistance to EAE (118). The fact that superantigens can overcome EAE resistance in a myelin oligodendrocyte protein (MOG)₃₅₋₅₅ peptide induced disease model in mice points to a transient IL-6 independent but TNFR1 dependent proinflammatory pathway in EAE pathogenesis, and further suggests a crucial function for IL-6 in disease perpetuation (119).

Additional proinflammatory cytokines, such as IFN- γ and TNF- α may also play a critical role in CNS inflammatory diseases and T-cell migration across the BBB. These proinflammatory cytokines are most commonly associated with activated Th1 cells (120,121), although there is some evidence that astrocytes can also secrete TNF- α (122). T cells secreting IFN- γ and TNF- α can induce an inflammatory reaction in each other, in the endothelium, and in the CNS parenchyma. Both cytokines can increase

the adhesion of leukocytes to brain endothelial cells (113) and in the presence of IL-1, can increase the number of leukocytes migrating across an in vitro BBB (112). IFN- γ was shown to decrease TEER, increase permeability, and decrease occludin expression in human umbilical vein endothelial cell (HUVEC) layers (123). Human brain endothelium was shown to increase MHC class II and change cellular morphology in the presence of IFN- γ (124). Similarly, permeability to fluorescently labeled dextran increased in both rat pial vessels and bovine brain endothelial cells after treatment with TNF- α (125,126). Lower TEER levels were found for TNF- α treated in vitro rat BBB-endothelium, as compared to untreated cells (114). Neuropeptides, such as Substance P (SP) may also possess inflammatory properties. This neuropeptide is secreted by endothelium, leukocytes, astrocytes, and neurons. Treatment of dermal endothelial cells with SP leads to an increased expression of selectin, ICAM-1, and VCAM-1 adhesion molecules (127–129). In rat brain endothelium, SP increased the expression of ICAM-1 molecules indicating the role of this neuropeptide in trans BBB migration of LFA-1 expressing T cells (130). Increased expression of MHC class II and loss of barrier integrity is also seen in rat brain endothelium treated with SP (131). Other proinflammatory cytokines, such as IL-17, produced by activated T cells (132), affect the BBB, but less directly. IL-17 promotes amplification of the inflammatory response by endothelial cells. IL-17 up-regulates the expression of inducible nitric oxide synthase (iNOS), a marker of inflammation, in vascular endothelium (133) and increases the secretion of proinflammatory chemokines MCP and lymphotoxin in HUVECs (134). HUVECs also secrete GM-CSF, IL-6, and IL-8 when exposed to IL-17. Brain lesions found in MS patients have increased IL-17 levels. The exact role of this proinflammatory cytokine on the BBB has vet to be elucidated.

In general, inflammatory responses are controlled by the release of antagonistic anti-inflammatory cytokines. Transforming growth factor $(TGF-\beta)$ is one example of these anti-inflammatory cytokines. TGF- β cytokine is secreted by neurons and glia cells, as well as by macrophages (135) and some specialized "regulatory" helper T cells (136). TGF-B treatment reduces the migration of splenocytes across an in vitro BBB in an antagonistic assay with IL-1, IFN- γ , and TNF- α . Injections of TGF- β into mice lessened the number of lymphocytes found in the CNS after both actively and passively induced EAE in vivo (137). Another anti-inflammatory cytokine that has been shown to play an important role in regulating T-cell migration across the BBB and influencing anti nervous tissue immunity is IL-10. This cytokine is produced by astrocytes and microglia in inflammation (108), and by activated regulatory T cells (76). The IL-10 mitigates the effects of IFN- γ on HUVECs, maintaining near normal TEER and occludin expression levels and minimizing the increase in permeability (123). Mice deficient in IL-10 have an early and severe form of EAE as compared

to wild-type (138,139), while mice that over-express IL-10 in activated T cells are completely resistant to disease induction (139). TGF- β and IL-10 work to control inflammation and antagonize the effects of pro-inflammatory cytokines such as IL-1, IFN- γ , and TNF- α . The balance of these, and probably other pro- and anti-inflammatory cytokines in the CNS is crucial in maintaining the immune privilege nature of the nervous tissue (briefly discussed in Sec. 3).

2.3. T-Cell Migration Across the BBB

2.3.1. T-Cell Adhesion to the BBB

Immune cell entry into the brain first requires that blood lymphocytes attach to the vessel wall. Initial tethering is followed by signal transduction, firm adhesion, and finally, transmigration of lymphocytes between two endothelial cells and passage through basement membrane to the brain parenchyma. The adhesion proteins responsible for this attachment process are found on both endothelial and T cells.

The first interaction between lymphocytes and endothelial cells is mediated by P and E selectin, glycoproteins with lectin domains that are found on the luminal surface of the vessel wall, and their ligands, carbohydrate molecules expressed on leukocytes (140). This tethering allows the T cell to slow down and begin rolling along the surface of the endothelium (141). The importance of selectins in the initial stages of trans-BBB migration has been demonstrated in multiple ways. P- and E-selectin expression on endothelial cells increases in the presence of inflammatory cytokines, but decreases after 24 hours (142). Mice injected with TNF- α have increased P- and E-selectin expression on brain microvessels. In addition, $TNF-\alpha$ induced increased rolling and adhesion of leukocytes is significantly decreased in P- and E-selectin deficient mice (143) or after P- or E-selectin antibody treatment (144). Fewer activated T cells penetrate the brains of P-selectin deficient and low P-selectin expressing strains of mice than high P-selectin expressing strains (145). Anti-P-selectin antibody treatment also eliminates rolling and reduces adhesion of leukocytes to CNS microvessel walls in mice suffering from EAE (146) and significantly reduces the migration of adoptively transferred encephalitogenic T cells into the brain 2 hours after injection (145). However, the relative importance of selectins in lymphocyte recruitment into brain may be more apparent in health and in disease initiation than in chronic disease, since the effects of selectin activity deficiency appears to decrease as inflammatory processes are established (147).

After tethering, lymphocytes and the BBB interact via chemokines and chemokine receptors. Binding of inflammatory chemokines released by the BBB to their G-protein linked receptors on the T-cell surface initiates a signal cascade that results in T-cell polarization characterized by actin and adhesion molecule redistribution (148) and cell migration (149). The uropod, a sort of cellular prehensile tail, further stabilizes cellular adherence and the projected filopodia at the moving front direct the motion of the cell (150). Receptors clustered at the leading pole of the cell detect chemokine gradients (150). A multitude of chemokines has been suggested to participate in T-cell migration across the BBB.

One of the chemokines most strongly linked to lymphocyte migration into the CNS is monocyte chemoattractant protein (MCP), which interacts with CCR2 receptors on activated monocytes, T cells, and endothelial cells (151). MCP RNA is expressed in murine astrocytes during EAE (152) and MCP RNA and protein have been found in astrocyte cell culture after stimulation by IL-1, TNF- α , or IFN- γ (153,154). Brain endothelial cells are also able to produce this chemokine after IL-1, TNF- α , or IFN- γ stimulation of rat brain endothelial cell lines (155). Interestingly, resting, non-activated human brain endothelial cells were found also to express this chemokine (156). Lymphocytes collected from patients suffering from MS do not efficiently cross monolayers of human brain endothelial cells in the presence of anti-MCP antibodies. In addition to effects on lymphocytes (148), MCP treatment of cultured brain endothelial cells and isolated brain microvessels leads to the loss of the TJ protein ZO-1 localization to cell junctions (88). The role of CCR2/MCP in vivo has been investigated using CCR2 and MCP knockout mice and by injecting neutralizing antibodies. CCR2 deficient mice are nearly resistant to EAE, and MCP deficient animals suffer only a mild disease (157,158). Treatment of mice with neutralizing antibodies against MCP during remission also reduces the severity of EAE symptoms in mice (136). This in vivo evidence underscores the role of MCP in lymphocyte migration into the CNS.

IFN- γ -inducible protein-10 (IP-10) and monokine induced by IFN- γ (MIG) both interact with a common receptor, CXCR3, and are important in T-cell migration through the BBB (159). Production of both MIG and IP-10 is detectable in brain endothelial cells stimulated with inflammatory cytokines, TNF- α , and IFN- γ (156,160). Astrocytes produce IP-10 in both EAE and MS (159,161). The number of CXCR3 expressing T cells is increased in blood and CSF of patients suffering from MS (156,159,162). The function of IP-10 and MIG in T-cell migration across the BBB is yet uncertain. It has been reported that anti-MIG antibody treatment decreases the number of T cells which cross a monolaver of brain endothelial cells (160), and anti-IP-10 antibodies are protective in murine EAE induced by the adoptive transfer of activated encephalitogenic T cells. However, treatment of rats with anti-IP-10 antibodies prior to or at the time of active EAE induction results in worse EAE symptoms, an observation that was also made in the IP-10 knockout mouse (163,164). Anti-IP-10 antibody treatment of active murine EAE has no effect (164). These conflicting results in vivo may not reflect the effect of IP-10 deficiency in lymphocyte penetration of the BBB, but rather disturbances in lymph node size and production of TGF- β (163,164).

Macrophage inflammatory protein (MIP) -1 and regulated upon activation, normal T cell expressed and secreted (RANTES) are a second pair of chemokines with possible roles in T-cell entry into the CNS. The receptor CCR5 is used by both chemokines, although MIP-1 can also interact through the CCR1 receptor. MIP-1 and RANTES are both produced in increased levels by brain endothelial cells treated with TNF- α and IFN- γ . MIP-1 and RANTES are also produced by invading leukocytes in EAE (152), and MIP-1 mRNA expression in microglia and macrophage in MS has also been described. Isolated brain microvessels, and microglia, as well as encephalitogenic lymphocytes, express both CCR5 and CCR1 (151,159,162,165). Like IP-10 and MIG, the role of MIP-1 and RANTES in T-cell migration in CNS autoimmune disease is not clear. The CCR1 knockout mouse strain suffers less severe EAE symptoms than their wildtype counterparts (166), and treatment of mice receiving activated encephalitogenic T cells with anti-MIP-1 antibodies at the time of transfer results also in a less severe form of EAE. The CCR5 and MIP-1 knockout mice display no differences in disease course, and an anti-RANTES antibody also has no effect. This may suggest that these chemokines have a less important role in autoimmune T-cell infiltration or may reflect compensatory changes elsewhere in the body.

CCL19 and 21 may play a role in inflammatory CNS diseases (see above discussion). These chemokines are not obvious participants in T-cell migration into the CNS. Typically thought to be primarily used by CCR7 bearing naïve T cells to home to lymph nodes, these chemokines have also been found to be expressed by brain microvessels, microglia, and infiltrating leukocytes in EAE (63). Likewise, invading encephalitogenic T cells express CCR7 and CXCR3, receptors for CCL19 and CCL21 (63,167). However, more work needs be done to clarify the role of these chemokines in BBB inflammation and infiltration.

It was shown that chemokine activation of lymphocytes leads to the activation of transmembrane integrins (168). Integrins lymphocyte function associated antigen (LFA) -1 and macrophage (Mac) -1 interact with intercellular adhesion molecule (ICAM) -1 and -2, while very late antigen (VLA) -4, among others, binds vascular adhesion molecule (VCAM) -1. These interactions promote stronger adhesion to, and migration across, endothelial junctions (127).

ICAM-1 is a 90 kDa surface transmembrane glycoprotein containing five immunoglobulin domains. Found on the surface of endothelial cells, this adhesion molecule interacts with LFA-1 on T cells (169). LFA-1 expression increases upon T-cell activation in the CNS (98). Activation of LFA-1 on the T cell is triggered by chemokines (168), and after binding to ICAM, LFA-1 activation leads to polarization and cell motility within minutes (127). ICAM expression increases on endothelial cells after incubation with TNF- α , IL-1, IFN- γ activated T-cell membranes or supernatant from Th1 cells (7,170–173). Activation of ICAM on endothelial cells following crosslinking leads to increased calcium influx and cytoskeletal rearrangement as seen by the appearance of actin stress fibers (174,175). Analysis of the ICAM protein suggests that the extracellular portion of ICAM is necessary for adhesion of lymphocytes to the endothelial cell, but the intracellular portion is necessary to allow migration across the monolayer (176). Both anti-ICAM antibody treatment and ICAM deficiency have been shown to allow less T-cell migration across primary brain endothelial cells and cell line monolayers (7,176). In vivo, ICAM/LFA-1 interactions are important in both EAE and MS. Isolated brain endothelial cells from an EAE resistant rat strain were discovered to display less ICAM on activation with IFN- γ than endothelial cells isolated from an EAE susceptible strain (177). Anti-ICAM antibody treatment in EAE attenuates disease symptoms (178). MS lesions have increased ICAM expression on endothelial cells and on some astrocytes and the infiltrating leukocytes were found to express LFA-1 (179). These data show the importance of ICAM and LFA-1 interactions on lymphocyte adherence to the BBB.

Vascular cell adhesion molecule (VCAM) -1 is also a member of the Ig superfamily. This 110 kDa transmembrane protein has seven Ig domains and interacts with VLA-4, also known as $\alpha 4\beta 1$ integrin, on the surface of lymphocytes and monocytes. VCAM-1 is expressed on astrocytes in culture and in brain tissue, and expression increases under inflammatory circumstances (180-182). More commonly, VCAM-1 has been studied on endothelial cells, both in culture (183,184) and in vessels (184). TNF- α and IL-1 up-regulate VCAM-1 on brain endothelial cells and tissue (171,185). Interestingly, the anti-inflammatory cytokine, IL-4, also up-regulates VCAM-1 production on HUVECs (186,187). In addition to inflammatory cytokines, membranes isolated from activated T cells also increase VCAM-1 expression levels on human brain endothelial cells, although not to the degree that TNF- α does (173). Interactions between VCAM-1 and VLA-4 are stimulated by chemokine induced clustering of VLA-4 on lymphocytes (188). Crosslinking of VCAM-1 by VLA-4 leads to adherence, as well as signaling, to the endothelial cell. IL-1 stimulated HUVECs treated with VCAM-1 cross-linking antibodies form actin stress fibers, monolaver gaps, and lose their electrical resistance (189). Although VCAM-1 signals to the endothelial cell, VCAM-1 and VLA-4 are not directly involved in transmigration (147,172,184,190). VCAM-1/VLA-4 interactions appear to be most important for adhesion and rolling of lymphocytes over the endothelium as shown by partial loss of these phenomenon after treatment of cells, vessels, and animals with anti-VLA-4 (146,184,191–193) or with anti-VCAM-1 antibodies (172,184,193). In CNS autoimmune inflammation, increased expression of VLA-4 on lymphocytes is observed in MS patients (194), and increased expression of VCAM-1 is observed in mice suffering from EAE as well (147,193). Low levels of VLA-4 expression on encephalitogenic T-cell lines may attenuate pathogenicity (195,196). Multiple laboratories have investigated use of anti-VLA-4 antibodies and antagonists in EAE. The conclusions remain somewhat varied, possibly due to time points and model systems chosen. Anti-VLA-4 treatment delays actively induced EAE in rats and passively induced EAE in mice (191,196), although the treatments do not lessen the peak severity of the disease. On the other hand, anti-VLA-4 treatment in the adoptive transfer rat model does prevent disease (191,192), while treatment after the onset of disease reduces symptoms in guinea pigs and mice (197,198). Anti-VLA-4 treatments are currently in clinical trials for use in MS: while the monoclonal antibody natalizumab (199) (Antegren, Tysabri[®] Biogen Inc., Cambridge, MA and Elan Corporation, PLC, Dublin, Ireland) has received approval by the U.S. Federal Drug Administration, antisense therapy (ATL 1102 Antisense Therapeutics Limited Victoria AU and Isis pharmaceuticals) is still awaiting it. The clinical applicability of anti adhesion molecule therapy in MS and other CNS inflammatory diseases needs to be further exploited.

2.3.2. T-Cell Migration Across the BBB

Once firmly attached, lymphocytes migrate to endothelial junction borders where they begin diapedesis or migration between the endothelial cells. This process is usually rapid and is followed by an equally rapid reassembly of junctions, which prevents increased permeability (200). It was proposed that on adhesion to an inflamed endothelium, T cells may transfer signals directing junction rearrangement and opening of endothelial junctions. The exact nature of these signals is only partially known today.

T-cell diapedesis across the BBB may also follow specific rules and molecular mechanisms exclusive to the BBB (Fig. 2). In T-cell diapedesis, activated T cells interact with barrier components of TJs, such as occludin and claudin, as well as with "migratory chaperone" adhesion molecules such as JAM and PECAM. Many of these interactions are homologous interactions, due to the binding nature of these molecules. Activated T-lymphocytes up-regulate occludin expression from nearly absent levels in resting T-lymphocytes (201). This opens up the possibility of occludin-mediated diapedesis of activated T cells in the CNS, but this process has not been studied in detail. PECAM is very likely to be important for diapedesis across the BBB. This molecule is constitutively contained in vesicles located just beneath the endothelium plasma membrane and is recycled continuously from this compartment along the endothelial border. When leukocytes transmigrate, PECAM in the endothelium recycles and concentrates around the migrating leukocyte, thereby establishing a homophilic interaction



Figure 2 Process of T-cell adhesion to and migration across blood-brain barrier endothelium.

with PECAM expressed on the leukocyte membrane (202,203). The induction mechanism of this vesicular reorganization is currently not known. Antibodies against PECAM block migration of monocytes across endothelial cell monolayers in vitro (200) and block antigen-specific T-cell migration into the CNS (98). EAE data have proved to be more puzzling as anti-PECAM treatment of rat recipients of encephalitogenic T cells provided no protection against the resultant EAE (204), while PECAM-deficient mice have an earlier onset EAE than wild-type counterparts (99). These in vivo data show that there is still more to learn about the role of this important protein in migration across the BBB. The JAMs, like PECAM, may also contribute to leukocyte diapedesis. JAMs are capable of homo and heterodimer interactions; in addition to other JAMs, they can bind integrins LFA-1 and VLA-4 on the lymphocyte (200). JAMs may function in diapedesis by forming a transient ring through which leukocytes can tunnel (205). In addition, CD99, which is also expressed at the membrane of leukocytes and at interendothelial contacts, is required for this process and blocking CD99 in vitro leads to the arrest of migrating monocytes as they cross-intercellular junctions (206). A general model was proposed in which proteins at endothelial junctions establish homophilic interactions with identical proteins present on leukocytes. These interactions might then direct the passage of leukocytes through the endothelial border (101,207).

2.4. Differences in Diapedesis of Different T-Cell Subtypes: Th1/Th2, CD8⁺, CD4⁺ in BBB Migration

Are there any differences between antigen-specific versus non-specific T-cell migration across the BBB?

MS and EAE are thought to be primarily mediated by T-helper cells of the Th1 phenotype. These cells can be characterized by high levels of IFN- γ , TNF- α , and GM-CSF production and the ability to efficiently activate cells of the monocyte/macrophage lineage. In peptide induced EAE models, Th1 type cells preferentially accumulate in the CNS (208). Human CSF is enriched with Th1 type cells in neuroinflammatory diseases (209). Interestingly, in spite of their crucial role in disease pathogenesis, Th1 cells are outnumbered in MS lesions by CD8⁺ cells (210) and EAE can be induced in animals by adoptively transferring myelin antigen-specific Th2 or CD8⁺ cells (211,212). These data indicate that CNS specific autoimmune disease may be initiated by T cells of different phenotypes. In both MS and EAE, however, there are important differences in the kinetics and in the extent of accumulation of T cells with different characteristics.

The recruitment of encephalitogenic Th1 cells into brain parenchyma primarily depends on the expression of chemokines (such as MCP-1, MIP-1, and RANTES) in brain and adhesion molecules (such as ICAM-1 and VCAM-1) on the BBB as described earlier. The products of activated Th1 cells up-regulate these factors in BBB endothelial cells. ICAM-1, the ligand of LFA-1, and VCAM-1, the ligand for VLA-4, are both up-regulated on BBB in response to Th1 derived cytokines (7,190). Up-regulation of these molecules therefore promotes the recruitment of Th1 cells during inflammatory processes in the CNS. On the other hand, Th2 cells with anti-inflammatory capabilities do not up-regulate adhesion molecule expression on BBB endothelial cells (7,190). Yet, several studies suggest that Th2 type cells may migrate preferentially into the brain under non-inflammatory conditions (7,190). BBB endothelial cells can preferentially activate Th2 type cells (137). Studies with in vitro brain slice cultures indicate that Th2 cells have counter-regulatory effects on Th1 cell mediated up-regulation of adhesion molecules on BBB (213). Treatment with the drug approved for MS, copaxone (glatiramer acetate), induces myelin antigen-specific Th2 cell populations (214). These cells home to the CNS and accumulate in the brain (215,216) where they may have immunoregulatory effects on Th1 mediated attack on CNS antigens. Altogether, Th2 cells promote the anti-inflammatory properties within the CNS.

CD8⁺ T-cell accumulation in brain is also very important under various conditions. As mentioned above, CD8⁺ T-cells accumulate in MS lesions and play an ill-defined role in the pathogenesis of the disease (210). $CD8^+$ T cells are also enriched in the CNS of guinea pigs with EAE (217) and show increased P-selectin mediated adherence to inflamed vessels as compared to CD4⁺ T cells when prepared from human patients with MS (218). CD8⁺ T-cell recruitment is also induced in viral infections of the CNS. Virus-specific CD8⁺ cells mediate BBB breakdown in lymphocytic choriomeningitis virus infection (219) and accumulate in high numbers in the CNS in response to influenza infection (220). CD8⁺ T cells are preferentially enriched vs. CD4⁺ cells in brain parenchyma relative to secondary lymphoid organs in response to dendritic cell injection into the brain (48). However, findings in our laboratory did not confirm these results, as both $CD4^+$ and $CD8^+$ T cells are recruited in response to dendritic cell injection into the brain and their ratio is not significantly different from that found in secondary lymphoid organs (45). T cells entering the CNS also exert a potent effect upon the recruitment and accumulation of T cells with the same or other specificities, as well as upon monocytes/macrophages through the production of cytokines and chemokines. In EAE, it has been shown that neuroantigen-specific CD8⁺ T cells can recruit neuroantigen-specific CD4⁺ cells by the secretion of IP-10 (221). In addition, blockade of IP-10 leads to a significant decrease in EAE severity in the relapsing-remitting EAE model in the SJL mouse strain (157). $CD4^+$ and $CD8^+$ T cells are simultaneously recruited into CNS in several other neuroinflammatory conditions, for instance in neuroinflammation induced by HIV and in toxoplasmosis (222,223).

Specificity strongly influences accumulation of T-cell populations in the CNS. This effect is primarily mediated through the presentation of specific antigen to T cells and their reactivation and retention at the site of antigen presentation. In an adoptive transfer model of EAE, myelin basic protein specific T-cell preferentially accumulate in the CNS as compared to T cells specific to irrelevant, non-CNS antigens (224). Early studies also showed that there is significant accumulation of non-CNS antigen-specific T cells during the course of EAE, but this requires the accumulation of specific T cells beforehand (8). These studies also suggest that T cells must be activated to enter the CNS. However, later studies show that although naïve T cells can also enter the CNS, they need to recognize their specific antigen in the brain in order to get retained (225).

Taken together, these data suggest a mechanism in which specific T-cell accumulation and activation in the brain "opens up" the way for additional T-cell populations to enter the CNS, including both activated and naïve T cells, from which specific T cells get activated and retained in the CNS. T cells not specific for CNS antigens may die by apoptosis in the brain (226) or may leave the CNS. Experiments with T cells expressing green fluorescent protein confirmed these findings: myelin basic protein specific green fluorescent protein expressing T cells "storm" the brain rapidly, whereas the accumulation of OVA (non-CNS) specific activated T cells occurs to a much lesser extent, and these cells are not retained in the CNS (227,228). The fine specificity of T cells determines their localization within the CNS: depending on the neuroantigen they are specific for, they may accumulate in either brain or in the spinal cord (229). Targeted homing of lymphocytes with various phenotypes to different anatomical locations in the CNS might also play a role in this process (230). T cells with identical specificity for MBP, primed under slightly different conditions, are capable of causing clinically different diseases. Examination of the various parts of the CNS revealed that the inflammatory infiltrate localizes to the spinal cord in animals afflicted with classical EAE, but predominates in the brainstem and cerebellum in animals, which displayed non-classical EAE. Moreover, inflammatory cells observed in non-classical EAE lesions are enriched in eosinophils, whereas inflammatory cells observed in classical EAE lesions display large numbers of either neutrophils and mononuclear cells (when classical EAE was induced with Th2 cells) or a predominance of mononuclear cells (when classical EAE was induced with Th1 cells). What guides T cells to the different anatomical locations? Adhesion/homing molecules, and in particular chemokines and chemokine receptors, may be differentially expressed on different T-cell subtype, accounting for the various lymphocyte migration patterns. The specific homing regulating processes in the CNS have yet to be clarified.

3. RETAINMENT AND SURVIVAL OF ACTIVATED T CELLS IN THE CNS AND THE INITIATION OF IMMUNITY IN THE CNS

In the previous paragraphs, we discussed the molecular mechanisms of T-cell diapedesis into the CNS. The next question that we have to consider is the mechanism of T-cell survival in the nervous tissue in an anti-inflammatory environment. It was proposed that T-cell restimulation in the CNS and adaptive immune responses participate in this process. However, it is becoming very evident that the innate immune system also plays a very important role in this process through Toll-like receptor signaling (9). Toll-like receptor mediated signaling can pre-activate local resident APCs in CNS and this pre-activation is required for a sufficient cytokine response and induction of T-cell mediated inflammatory cascade (9.231). These findings indicate that endogenous activation stages of APCs in the CNS are critical in development of autoimmune diseases such as MS. T cells that recognize their specific CNS-derived antigen in lymph nodes and enter the CNS fundamentally changed their recirculation pattern (Fig. 3). Local antigen expression also contributes to the survival and localization of T cells in the CNS (229). This was further supported by the observation that only



Figure 3 Comparison of T-cell migration patterns in the steady state (health) and in CNS inflammation (disease). In health, naïve lymphocytes circulate through the blood and the secondary lymphoid organs. They express low levels of the adhesion molecules (LFA-1, VLA-4) necessary to enter CNS tissue. The BBB also expresses low levels of the ligands for these adhesion molecules. Together these factors result in low T-cell infiltration of the brain. T cells which do infiltrate are likely to experience an absence of survival factors and die by apoptosis or encounter nonactivated APCs and become tolerized or die by apoptosis. In inflammation, however, there is an increase of T-cell migration and retention in the brain. Both T cells and BBB endothelial cells up-regulate adhesion molecules. The APCs in the brain become activated and restimulate entering T cells leading to proliferation or differentiation of the T-cell. Effector cells, which develop into memory T cells may return to the peripheral circulation. Activated T cells that enter the brain but are not restimulated die by apoptosis.

CNS antigen (MBP) specific T cells and not OVA specific T cells entered the CNS when adoptively transferred (227,228). But how do antigen-specific T cells create an inflammatory environment in the CNS upon their migration across the BBB?

It is clear that this is a highly regulated, complex process that involves multiple inflammatory mediators. Some of the roles of these mediators in T-cell migration across the BBB have already been reviewed above. However, T-cell secretion of these mediators, particularly proinflammatory cytokines in the brain parenchyma induces inflammatory molecular changes in the brain microenvironment. These molecular changes further amplify the inflammatory process in the CNS.

In general, the context of T-cell activation by antigen fundamentally determines the array of cytokines produced by T cells (reviewed in Ref. 232) (Fig. 4). Differentiation of Th1 type cells leads to production of cytokines regarded as pro-inflammatory, for instance IL-2, IFN- γ TNF- α , and GM-CSF. The Th1 products activate effector mechanisms of delayed-type hypersensitivity, up-regulate MHC expression, and lead to production of anti-microbial agents such as reactive nitrogen and oxygen intermediates and cytotoxic antibody production of the IgG2a isotype. Differentiation of helper T cells towards the Th2 direction induces the production of IL-4, IL-5, IL-10, and IL-13. These cytokines induce B cell differentiation and



Figure 4 T-cell activation and accumulation in CNS inflammation. CD4 Helper T cells differentiate into regulatory, type 1 inflammatory or type 2 anti-inflammatory effector T cells. The cells enter CNS with different kinetics and alter the CNS immune environment by secreting cytokines. CD8 cytotoxic T cells can respond to CNS derived antigens and accumulate in CNS, as in viral diseases and toxoplasmosis. T-cell specificity influences accumulation and retention of both CD4 and CD8 cells in the CNS as CNS antigen specific T cells are retained in the CNS while nonspecific T cells recirculate to the blood or apoptose.

antibody production of the IgG1 and IgE isotype and participate in the development of immediate hypersensitivity and allergy. The signature cytokine produced by Th3 cells is TGF- β , Th3 cells are the major effectors in immune responses in the gut associated lymphoid tissue by inducing the production of IgA.

Th1 cells have long been considered the effector cells of CNS inflammation, whereas Th2 type cells have been considered to be anti-inflammatory. However, this view has been challenged recently after it was shown by several groups that Th2 type cells could also mediate CNS inflammation of a different type from Th1 type cells (212,233-235). Our picture of Th1/ Th2 mediated inflammation has also been considerably altered in recent years by a large volume of data on antigen-specific regulatory T cells and their influence on CNS inflammation. In the upcoming paragraphs we would like to address the role of T-cell cytokine production in the CNS and the effect of these upon CNS inflammation.

IFN- γ is probably the most studied cytokine in CNS inflammation. Its role in the CNS is pleiotropic and controversial in several aspects. IFN- γ clearly exerts a variety of pro-inflammatory effects, including up-regulation of both MHC classes I and II mediated antigen presentation in several cell types (236-238), astrocyte proliferation and reactive gliosis (239,240), and the production of reactive nitrogen and oxygen intermediates (241,242). Together with LPS, it also induces IL-12 production in CNS cells supporting further Th1 differentiation (243). In addition, IFN- γ induces significant up-regulation of adhesion molecules on several CNS cell types (244-248). IFN- γ also has direct cytotoxic effects on oligodendrocytes (249). Considering all these pro-inflammatory effects, it was rather surprising when several investigators demonstrated that EAE is more severe in mice that lack IFN- γ and in mice treated with anti- IFN- γ antibodies (250–253). This effect is likely due to the important role in effector T-cell elimination, since it is necessary to suppress activated T-cell expansion (251). A possible indirect mechanism of this effect is reactive nitrogen-mediated T-cell killing (254). Interestingly, IFN-y treatment of human patients with MS led to opposite results and the worsening of disease (255,256). The pleiotropic effects of IFN- γ will require additional studies to clarify the qualitative and quantitative aspects of IFN- γ production in the CNS.

The TNF- α family of cytokines also has pleiotropic pro-inflammatory effects in the CNS similar to those of IFN- γ . Besides inducing up-regulation of adhesion molecules and leakiness of the BBB (discussed previously), TNF- α leads to astrocyte activation (257,258), MHC and co-stimulatory molecule up-regulation on several CNS cell types (259–261), and reactive nitrogen and oxygen intermediate production (262). Its cytotoxic effect is important on both CNS resident cells-like oligodendrocytes (263–265), and possibly on infiltrating T cells. As opposed to IFN- γ , treatment of mice with anti-TNF- α leads to amelioration of the disease (266). Both IFN- γ and

TNF- α lead to the expression and up-regulation of a series of chemokines and chemokine receptors. In this sense, these two cytokines have synergistic effects. TNF- α and IFN- γ synergize with additional cytokines in the CNS, such as IL-17 and GM-CSF. IL-17 administration exacerbates EAE severity (267) and induces pro-inflammatory cytokine and chemokine expression in endothelial cells (134). GM-CSF is very important in the activation of myeloid cell populations in the CNS. This cytokine promotes the differentiation of dendritic cell-like populations from microglia in vitro (50) and its crucial role has also been proven in vivo; mice deficient in GM-CSF do not develop EAE (60).

IL-2 is one of the most important cytokines produced after T-cell stimulation. This major autocrine T-cell growth factor also has multiple effects in the CNS. IL-2 knockout mice are significantly less susceptible to EAE induction than their wild-type counterparts (268). Functional IL-2 receptor is present on resident cells of the CNS (269), which suggests that IL-2 may directly act on these cells as well. In fact, IL-2 enhances microglia to engulf T cells dying by apoptosis (270) and induces oligodendroglial proliferation and differentiation in culture (271). However, since these latter studies were performed in vitro, it is not clear how IL-2 may affect these processes in vivo. IL-2 may also affect BBB permeability (272,273) and influence cognitive processes (274).

The major acute phase proteins IL-1 and IL-6 have divergent and partially overlapping roles in CNS inflammation. The effects of IL-1 are pro-inflammatory in most in vitro experimental systems although there are differences depending on the model used and include the following: endothelial cell activation, microglia activation and induction of reactive nitrogen intermediate production (275), astrocyte activation (276,277), induction of chemokine (278,279) Such as MIP-1 α , and the induction of fever (reviewed in Ref. 280). The effects of IL-6 are not as clear, as it seems to have important anti-inflammatory properties as well. In vitro experiments show that IL-6 promotes the differentiation and survival of neurons, probably through the induction of neurotrophic factors in astrocytes and other glial cell types (281-285). However, IL-6 production in plaques in Alzheimer's disease promotes neural degeneration (286-288). IL-6 production in MS may promote B cell activation in the CNS (289). The effects of IL-6 require further clarification: it may be an important drug target in several inflammatory CNS diseases.

The anti-inflammatory cytokines that balance the effect of IFN- γ and TNF- α are primarily IL-10 and TGF- β . IL-10 and TGF- β have essential anti-inflammatory effects on microglia, astrocytes, and BBB endothelial cells. Their functions significantly overlap and include the following: down-regulation of MHC expression and antigen presentation (290–294), suppression of pro-inflammatory cytokines (such as TNF- α and IL-12) (108,291,295,296) and chemokine (297), as well as reactive nitrogen and oxy-

gen intermediate production. TGF- β also inhibits neuronal cell death (298). Depending on the experimental system used, there are slight differences in results obtained when CNS cells were treated with these cytokines. These cytokines are also very important in the differentiation, as well as function, of regulatory T cells. CD4⁺CD25⁺ regulatory T cells can decrease EAE severity in an IL-10 dependent manner (299). Also, dendritic cells prepared from brain of mice with chronic EAE can produce large amounts of IL-10 that may lead to differentiation of regulatory T cells and the down-regulation of CNS immune responses (71). The approved treatment for MS, copaxone induces CNS homing IL-10 producing T cells (300), which also implies an inhibitory role for IL-10 in CNS immune responses.

The prototypic Th2 cytokines, IL-4, IL-5, and IL-13, are also considered to balance the effect of pro-inflammatory Th1 type cytokines. Using mostly in vitro experiments, it was shown that IL-4 downregulates MHC expression and antigen presentation on microglia (292,301) as well as down-regulating astrocyte proliferation (302,303). Both IL-4 and IL-5 induce production of the neurotrophic factor NGF (304). IL-4, similarly to IL-10, downregulates costimulatory activity on microglia (305). IL-13 was also shown to block macrophage activation in CNS as well as to induce death of activated microglia (306,307), in which it synergizes with IL-4. When comparing the effects of IL-4 and IL-10 on EAE in vivo, studies show that IL-10 is a more critical downregulatory factor in murine EAE than IL-4 (139).

It is very important to recognize, however, that the above-mentioned studies on down-regulatory effects of Th2 cytokines were performed in vitro. In vivo, interpretation of the data does not seem to be equally clear. Several studies have shown that Th2 type cells can also induce destructive inflammatory processes in the CNS. Originally, Lafaille et al. (212) used recombination activating gene (RAG) knockout mice transferred with Th2 type cells to induce clinical symptoms of EAE. More recently, Lawrence Steinman's group showed that it is possible to induce allergic reactions to CNS antigens. These allergic reactions are mediated by the prototypical mediators of immediate hypersensitivity reactions: IgE, histamine, and serotonine and can be blocked by inhibiting the effect of these mediators (233–235). It is important to contemplate all these aspects when considering Th2 type cytokines as potential drug targets, since these cytokines are major inducers of allergic reactions.

Finally, T cells themselves have been shown to produce neurotrophic factors (308). These factors may play an important role in the effect of the MS drug copaxone, since copaxone (glatiramer-acetate) induced T cells are capable of producing neurotrophic factors (309). In EAE, there seems to be a compartmentalization in the production of neurotrophic factors between T-cells specific to or not specific to CNS antigens. The interesting observation is that the non-specific cells produce neurotrophic factors (310). In vitro

neurotrophic factors also down-regulate the effects of pro-inflammatory cytokines (305). It is important to consider these beneficial effects of T cells when designing therapies aimed at modulating T-cell function.

In summary, T-cell activation is probably important in recruitment and retention of T cells in the CNS. Additional signals, such as inflammatory mediators, trauma, or stress signals are also required for long-term retention of T cells in the CNS. This is further supported by the observation that naïve TCR transgenic T cells cannot initiate EAE (311), and RAG knockout TCR transgenic mice only get EAE under non-sterile conditions (312).

4. CONCLUDING REMARKS

Undoubtedly the BBB plays a critical role in T-cell migration into the CNS and in regulating initiation of immune responses in inflammatory diseases of the nervous system. In the past several years, studies addressing the mechanism of this migration revealed a complex, highly regulated process that is influenced by multiple adhesion molecules, inflammatory cytokines, and chemokines. In this chapter, we reviewed most of these regulatory elements with particular focus upon the process of peripheral T-cell activation leading to migration and retention of activated T cells in the immunologically privileged nervous tissue. To elucidate the exact mechanism involved in this process will help in the design of novel and innovative therapies for CNS inflammatory diseases.

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Endothelial ICAM-1-Mediated Signaling and Its Role During Lymphocyte Migration to the CNS Parenchyma

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1. INTRODUCTION

The endothelial cells (ECs) that constitute the blood-brain barrier (BBB) have been described as the gatekeeper to the brain. This analogy is correct in as much as brain ECs actively regulate the passage of molecules and cells to and from the central nervous system (CNS). In the context of immune cell traffic, the level of control exerted by the cerebral vascular ECs appears complex. It has been recognized for several decades that EC surface adhesion molecules are responsible for the capture of circulating leukocytes. In recent years, however, it has been realized that the same adhesion molecules are capable of triggering outside-in signaling cascades, which are critically required for successful leukocyte diapedesis. These observations lend strong support to the proposition that ECs respond to adherent activated leukocytes and that leukocyte emigration is dependent on a close partnership with the ECs. This concept has led to the focus of attention being directed more towards ECs and their role during leukocyte extravasation.

In this review we discuss recent findings and current knowledge relating to EC signaling through adhesion molecules, in particular ICAM-1, and speculate how these may enable the leukocyte to overcome the tight BBB.

2. GENERAL PRINCIPLES GOVERNING LEUKOCYTE MIGRATION

During homeostatic immune surveillance or an inflammatory response leukocytes exit the circulation. For this they must be able to interact with and cross the single layer of ECs that line the blood vessels.

The molecular interactions involved in leukocyte trafficking across the vasculature have been studied extensively and a multistep paradigm has been proposed by Butcher (1) and Springer (2) which to this day is accepted to illustrate most of the events involved. This model is based on a series of overlapping events during which leukocytes are slowed within the blood stream, captured, and eventually emigrate through the endothelial barrier, either by a paracellular or transcellular route. Amendments have been made to this model to provide room for the proactive EC contribution in this process (3,4) (Fig. 1). The interaction of leukocytes with the endothelium is mediated by several groups of cell adhesion molecules; some that are expressed constitutively and others whose expression is induced following pro-inflammatory cytokine stimulation.

The initial transient interactions of a leukocyte with the endothelium are mediated by members of the selectin family of proteins (5). Selectins are single chain transmembrane proteins containing a lectin domain in their extracellular portion and a short cytoplasmic tail that is capable of initiating intracellular signals following receptor engagement (6-8). The three principal selectins involved in this process are leukocyte (L)-selectin, which is found on all leukocytes (except a subpopulation of memory T cells), platelet (P)-selectin, and endothelial (E)-selectin, both of which are expressed on endothelium. Carbohydrate ligands expressed on the leukocyte or EC surface engaged with selectins and tether the lymphocyte to the endothelium. Due to the shear forces of the blood flow and the transient nature of the interaction the leukocytes travel in a rolling movement along the surface of the EC. During this period they receive and send additional signals to the endothelium. In the absence of subsequent adhesive interactions, lymphocytes are released into the circulation as the selectinligand interactions are relatively weak and transitory.

During their rolling across the endothelial surface, lymphocytes receive signals primarily from members of the chemokine family but also from other factors such as platelet-activating factor (PAF) or the complement split product C5a. Such factors may either act in a paracrine manner or be immobilized within the endothelial glycocalyx allowing them to bind to their complementary G-protein-linked receptors on the leukocyte cell surface (9). Approximately 50 chemokines have been identified in humans to date and their selective expression and recognition are believed to contribute to the specificity of leukocyte recruitment to particular tissues. Activation of chemokine receptors leads to conformational changes of integrin receptors



Figure 1 Schematic representation of the principal steps during leukocyte extravasation. Leukocytes are tethered by EC selectins and roll along the endothelium. Note that in the central and peripheral neurovasculature the initial capture may not be mediated by selectins. Subsequently, endothelial-triggered leukocyte migration results in firm adhesion mediated by the interaction between leukocyte integrins and endothelial Ig superfamily adhesion molecules. A dynamic leukocyte–endothelial crosstalk precedes and accompanies spreading/crawling on and migration across the endothelial vessel wall. Further details of this process are described in the text.

on the leukocyte cell surface resulting in a switch from a low-affinity to a high-affinity state. Furthermore, following activation integrins are organized into high avidity clusters (10), which may be important in eliciting similar clustering of endothelial counter receptors and this event may be critical to trigger EC signaling cascades (see below).

Thus a circulating leukocyte is slowed down by transient interactions, which subsequently allow firm adhesion to be established between opposing cells. As a consequence the leukocyte spreads on the EC surface. Firm leukocyte–endothelial interaction is mediated by leukocyte integrins binding to endothelial counter receptors, primarily members of the Ig superfamily. Integrins are heterodimeric cell adhesion molecules composed of noncovalently associated α and β subunits. Of particular importance to leukocyte migration across the vascular barriers are the β 2 integrins $\alpha L\beta$ 2 (lymphocyte function antigen-1, LFA-1) and $\alpha M\beta$ 2 (Mac-1) that bind to the Ig superfamily cell adhesion molecules intercellular adhesion molecule (ICAM)-1 (CD54) and ICAM-2 (CD102) and the β 1 integrin $\alpha 4\beta$ 1 (very late antigen-4, VLA-4) that binds to vascular cell adhesion molecule (VCAM)-1 (CD106) (1,2). These interactions are responsible for the firm adhesion of leukocytes to ECs and their arrest within the bloodstream.

ICAM-1, a 76–115 kDa transmembrane glycoprotein, belongs to a subset of the Ig superfamily of proteins specialized in binding integrins (11). ICAM-1 is expressed by epithelial and ECs, fibroblasts, monocytes, macrophages as well as T, and B-lymphocytes. Basal ICAM-1 expression is low in most cell types but can be up-regulated by a wide variety of stimuli including pro-inflammatory cytokines [such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and interferon (IFN)- γ], lipopolysaccharide (LPS), phorbol esters, and insulin growth factor (IGF)-I, but also during viral and intracellular bacterial infection or cell stress (12). ICAM-1 is composed of five extracellular Ig-like domains, a transmembrane domain, and a short cytoplasmic tail (29 amino acids in humans). It is able to bind many ligands including β2 integrins such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/ CD18) on leukocytes as well as fibrinogen, hyaluronan, CD43, p150/95, plasmodium falciparum-infected erythrocytes, and coxsackie A13 rhinovirus. The binding of LFA-1 to the first and second, and of Mac-1 to the third of ICAM-1s Ig-like domains is largely responsible for the firm adhesion of circulating leukocytes to the endothelial surface of post-capillary venules.

The expression of ICAM-1 on ECs, therefore, is seen as a critical element for successful recruitment of leukocytes from the circulation. However VCAM-1, another member of the Ig superfamily, is also intimately involved in the differential recruitment of leukocytes during both normal traffic and disease. VCAM-1 is a transmembrane glycoprotein with either six or seven immunoglobulin-like extracellular domains that bind to the integrin $\alpha 4\beta 1$ on leukocytes. Under inflammatory conditions, the expression of VCAM-1 can be induced by pro-inflammatory cytokines and may be responsible for the increased traffic of leukocytes into the inflammatory lesion. Apart from their role as docking molecules for integrins on circulating leukocytes, both ICAM-1 (3,4) and VCAM-1 (13,14) have also been implicated in initiating EC signaling cascades that are required for the successful transvascular migration of leukocytes (see below).

Further Ig superfamily adhesion molecules identified to participate in leukocyte transendothelial migration, are platelet endothelial adhesion molecule (PECAM)-1 and Junctional Adhesion Molecules (JAMs). Both are localized to the endothelial cell–cell junctions and may therefore be involved during later stages of leukocyte emigration. PECAM-1 is a 120 kDa glycoprotein expressed by ECs, platelets, monocytes, neutrophils, natural killer (NK), and T cells. Within confluent monolayers of ECs, PECAM-1 localizes to the region of cell–cell contact and to the apical lumen-facing aspect of blood vessels where it is engaged in homophilic interaction across the paracellular cleft (15). The importance of PECAM-1 to leukocyte adhesion and transendothelial migration has been demonstrated both in vitro and in vivo where anti-PECAM-1 blocking antibodies directed to either the ECs or neutrophils/monocytes inhibited leukocyte transmigration (16–21). In support of these studies, in PECAM-1 gene knockout mice, leukocytes show a transient arrest between the vascular endothelium and the basal lamina during transmigration at inflammatory foci (20,22).

Three members of the JAM family of Ig superfamily adhesion molecules have been described (23) and are now designated as JAM-A, JAM-B, and JAM-C. These ~40–50 kDa transmembrane proteins associate in trans across the paracellular cleft (23). They contain two Ig-like domains in their extracellular moiety, a transmembrane domain, and a short cytoplasmic tail containing a PDZ domain, through which JAMs interact with adaptor proteins such as zona occludens (ZO)-1, cingulin, AF-6, and Cas kinase. JAMs are integral components of tight junction strands in epithelial and ECs and their main function may be in the regulation of leukocyte diapedesis rather than adhesive integrity.

Indeed, treatment of endothelial monolayers with blocking anti-JAM antibodies inhibited transendothelial migration of both monocytes and neutrophils (24). Significantly, JAM-A has been identified as a ligand for the integrin LFA-1 expressed on many circulating leukocytes including lymphocytes and JAM-B has been demonstrated to be an endothelial receptor for JAM-C present on the cell surface of circulating dendritic, NK, and CD8⁺ T cells (23). The JAMs may therefore, in addition to their ability to interact with and organize tight junctions, function as junctional cell adhesion molecules that "guide" firmly adhered leukocytes through the paracellular cleft.

Once the leukocyte has firmly adhered it crawls along the endothelial surface and ultimately penetrates the vessel wall in a process called diapedesis. This final stage in leukocyte extravasation is the least well-understood step. It is thought to occur primarily through paracellular routes but there is evidence, at least in the case of neutrophil migration, for a transcellular pathway (25–28). Whichever route is taken by the migrating leukocyte, active co-operation of the EC is required; for instance modulation of EC junctions is thought to be necessary to support paracellular diapedesis.

Finally, chemokines also play a pivotal role in determining leukocyte migration through the provision of chemotactic gradients (9). These appear to play a major role once the leukocyte has passed through the EC lining. Infiltrating leukocytes must ultimately cross the basement membrane that

surrounds the blood vessels, a process thought to be mediated by matrix metalloproteinases.

3. ENDOTHELIAL ADHESION MOLECULES AND LEUKOCYTE MIGRATION ACROSS THE BLOOD-BRAIN BARRIER

The generic principles governing leukocyte migration through the blood vessel wall described above hold true for the CNS. Nevertheless, as with other vascular beds, there are subtle differences that contribute towards the differential migration of leukocytes into the CNS and the neuroretina. A notable difference within the brain is the extremely low number of leukocytes found in healthy tissue, which has contributed to the view of the CNS being immunologically privileged. A reason for this may reside in the low basal expression of cell adhesion molecules on the surface of brain ECs. The profile of leukocyte recruitment to the CNS is also inherently different from many other tissues in that neutrophil infiltration is rare. The ability to recruit neutrophils to the CNS appears to be lost in adults and is thought to be determined by changes in the brain's capacity to synthesize particular cytokines and/or chemokines (29,30). In addition to these differences there has also been speculation over the role of selectins in the capture of leukocytes within CNS vasculature. Thus, it has been reported that the initial phase of T-cell migration at the BBB does not involve selectin-mediated rolling (31) but involves firm adhesion via integrins (32,33).

As with vascular endothelium from other tissues, expression of the Ig superfamily molecules ICAM-1 and VCAM-1 on brain EC plays a pivotal role in supporting leukocyte adhesion and migration. The importance of brain endothelial ICAM-1 in facilitating lymphocyte migration into the CNS is illustrated by the reduced migration observed across endothelium of ICAM-1-deficient mice both in vitro (34) and in vivo (35). The only other adhesion molecule found so far to be of significance in facilitating lymphocyte migration is ICAM-2 (36). In addition, antibodies neutralizing the LFA-1/Mac-1 interaction with ICAM-1 also significantly reduce leukocyte adhesion and migration at the BBB in vitro and in vivo (33,35,37–40). Together, these data demonstrate irrevocably the importance of ICAM-1 in lymphocyte migration across the BBB.

Unlike ICAM-1, which is constitutively expressed at low levels, VCAM-1 is only induced on brain ECs following activation with cytokines such as TNF- α and IFN- γ . Unlike ICAM-1, VCAM-1 does not appear to play a major role in facilitating lymphocyte migration even though it may assist in the earlier stage of adhesion (41). However, the differential role of these Ig superfamily adhesion molecules in supporting different specific subsets of leukocytes can be exemplified by the finding that VCAM-1, and not ICAM-1, is the principle receptor initiating monocyte migration to the brain (42).

JAM may also be important in supporting leukocyte migration into the brain, albeit primarily at the level of the meninges and pia, as antibody blocking studies attenuate leukocyte recruitment in a cytokine-induced animal model of meningitis (43). However, in bacterial or viral induced models, anti-JAM antibodies do not prevent disease (44).

4. SIGNAL TRANSDUCTION BY ENDOTHELIAL CELL ICAM-1

Since EC ICAM-1 is a key player in leukocyte adhesion and migration, its potential role as a signaling molecule has been the subject of much investigation. Superficially ICAM-1 does not appear to be an obvious candidate for a signal transducing protein, since its short cytoplasmic tail is devoid of intrinsic catalytic activity and does not bear canonical consensus sequences to other known protein domains. Nevertheless, heterologous ICAM-1 expression in chinese hamster ovary cells has unambiguously established that ICAM-1, through its cytoplasmic tail alone, is capable of eliciting intracellular signals (45). In brain EC, the expression of C-terminal mutants of ICAM-1 attenuates EC signaling and transendothelial lymphocyte migration. Similarly, introducing peptides mimicking the C-terminal domain also leads to abolition of EC signaling and greatly reduced lymphocyte migration. These series of experiments delivered compelling evidence that the endodomain of ICAM-1 triggers intracellular signal transduction cascades, which are required for successful transendothelial lymphocyte migration (46,47). Indeed, ligation of cell surface ICAM-1 elicits a plethora of intracellular signals that are intimately involved in mediating leukocyte migration (3,4). The capacity to trigger intracellular signals is not restricted to ICAM-1 but also found for other Ig superfamily cell adhesion molecules such as ICAM-2, VCAM-1, PECAM-1, MHC class II, and carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1). Mimicking leukocyte adhesion and activation of ICAM-1 has mainly been investigated in vitro by receptor crosslinking: ECs are treated with anti-ICAM-1 antibodies followed by inducing surface aggregation of the antibody-ICAM-1 complexes by secondary anti-isotype antibodies (48). Alternatively, fibrinogen has also been used to activate ICAM-1 (49). Interestingly, intracellular signaling elicited by these two stimuli differs (see below) which suggests that leukocytes can induce a differentiated endothelial response by binding to the different extracellular domains of ICAM-1 through distinct integrins (such as LFA-1 or Mac-1). Moreover, the signaling pathways activated by ICAM-1 appear to vary between ECs derived from different vascular beds, allowing great diversity and plasticity at the level of ICAM-1 alone.

4.1. The Leukocyte Docking Complex and Primary Signaling

Following the capture and firm adhesion of the leukocyte, the EC can be seen to envelop the leukocyte partially in a cup-like membrane structure that is highly concentrated in cell adhesion molecules, submembranous actin, and ezrin/radixin/moesin (ERM) proteins (50,51). The clustering of ICAM-1 within this docking module is thought to be essential for the propagation of a signal within the EC (Fig. 2A). How the initial signal is generated remains ill-defined but the cytoskeleton appears to be a central mediator. Following crosslinking, ICAM-1 has been demonstrated to partition into a detergent-insoluble subcellular fraction. This observation has been interpreted as an increased association of ICAM-1 with either the cytoskeleton (52) or lipid-raft membrane domains (53). In light of the complex and dynamic nature of the docking module both interpretations may well be true and indeed describe the same cellular structure. Biochemically the cytoplasmic tail of ICAM-1 is able to interact with the cytoskeleton through several cytoskeletal-associated proteins including the actinbundling protein α -actin (54), ezrin, along with other ERM family members, an organizer of cortical actin (55), β -tubulin as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved in bundling of microtubules (56). ICAM-1 co-localizes with the ERM proteins and F-actin (50,55,57), although unlike the case with ICAM-2, ERM proteins from brain ECs cannot be co-precipitated with ICAM-1 antibodies (58).

Bona fide signal transduction molecules interact with ICAM-1 as well. Thus, the tyrosine kinase p60src and its phosphorylated substrate cortactin have been detected in ICAM-1 immunoprecipitates (IPs) from crosslinked EC extracts (59). In HUVEC, fibrinogen binding to ICAM-1 results in tyrosine phosphorylation of its transmembrane domain and an increase in its association with the Src-homology-2 protein tyrosine phosphatase-2 (SHP-2) (49). In contrast, neither antibody crosslinking of brain EC ICAM-1 nor lymphocyte adhesion lead to ICAM-1 phosphorylation on tyrosine or interaction with SHP-2 (46). This may suggest that these events are specific to the fibrinogen-ICAM-1 interaction or yet another manifestation of differential signaling in ECs derived from different vascular beds.

Many proteins interact with ICAM-1 in vitro and their identification will surely lead to a more complete understanding of how ICAM-1 associates with the cytoskeleton, and how the cytoskeleton is remodeled to provide and sustain the endothelial "docking structure" that allows efficient leukocyte diapedesis.

4.2. Secondary Signaling Events

A decade ago one of the first effects observed following crosslinking of brain EC ICAM-1 was the activation of the tyrosine kinase p60src with a subsequent tyrosine phosphorylation of the actin binding protein cortactin (48);



Figure 2 ICAM-1-mediated signaling in endothelial cells. (A) Generation of an intracellular signal. Lymphocyte- or antibody-mediated clustering of ICAM-1 leads to the formation of a docking complex rich in cytoskeletal components. Ezrin or other ERM proteins are thought to generate and transduce a signal to Rho or PKC. Association with the docking complex of the protein kinase src has also been shown and may critically contribute to the initial signal. Subsequently, a number of master signal transduction molecules, including ROS, PKC, src, and rho, propagate the ICAM-1 signal. (B) Downstream signaling and end point modulation. Primary signaling triggered by ICAM-1 ligation leads to modulation of the cytoskeleton and possibly the intercellular junctions, to activation of endothelial integrins but also of transcription. For further details see the accompanying text.

both now thought to associate with activated ICAM-1. These initial studies were at the beginning of a plethora of observations of intracellular signaling events that occur following either ICAM-1 crosslinking and/or lymphocyte adhesion, further demonstrating the capacity of ICAM-1 to initiate a cascade of intracellular events (Fig. 2B). These include enhanced tyrosine phosphorylation of the proteins focal adhesion kinase (FAK), paxillin (PAX), and p130Crk-associated substrate (CAS) (60). These tyrosinephosphorylated molecules become organized into a multimolecular complex with both PAX and CAS associating with the adapter protein CRK that in turn combines with the GTP exchange factor C3G. Part of the downstream effect of these changes is the stimulation of the mitogen-activated protein (MAP) c-jun N-terminal kinase (JNK). In addition to phosphorylation on tyrosine, ICAM-1 crosslinking also induces the formation of actin stress fibers via a protein kinase C (PKC)- and rho-dependent pathway (61). The full physiological relevance of this observation is not clear but believed to be an important component of the signaling pathway responsible for facilitating lymphocyte migration, possibly through mediating the disassembly of lateral cell-cell junctions. The formation of actin stress fibers following ICAM-1 crosslinking in brain EC is similar to that observed in fibroblasts following activation of the small GTPase rho. Significantly, treatment of brain endothelial monolayers with the bacterial exotoxin C3 transferase, that specifically inhibits rho proteins by ADP-ribosylation, not only prevents ICAM-1-mediated activation of rho, subsequent stress fiber formation but also other downstream events such as tyrosine phosphorylation of FAK, CAS, paxillin, and JNK (60,61). Therefore, as has been reported for other cellular responses, rho appears to be a master regulator of ICAM-1-mediated signaling. Significantly, functional rho is also critical for lymphocyte migration as pre-treatment of brain endothelial monolayers with C3 transferase greatly reduces (by approximately 80%) T-cell migration in vitro (61).

Although ICAM-1 ligation leads to activation of rho in most if not all cells, signaling through rho does not appear to be required for leukocyte migration across all EC types. For instance, T-cell migration across macro-vascular aortic ECs is unaffected by C3 transferase pre-treatment (62).

It is currently not known how rho proteins are activated following ICAM-1 crosslinking. A pathway involving PKC-mediated cytoskeletal rearrangements, leading to rho activation via p60src has been described in non-ECs (63). Alternatively ERM proteins, whose activities at the cell membrane initially require activated rho, may support sustained rho activation by sequestration of an inhibitor of rho activation, namely rhoGDI (64). Finally, the generation of reactive oxygen species following ICAM-1 ligation has also been described (65) and may well be a central trigger for rho and MAP kinase activation (see below).

From the work described above it clearly transpires that the efficient transduction of ICAM-1 mediated signaling responses in CNS EC, and consequently transendothelial migration of T-lymphocytes, is critically dependent on functional EC rho GTPases. This has further led to the hypothesis that pharmacological inhibition of rho GTPases may in turn inhibit the ICAM-1 signaling pathway and subsequent lymphocyte migration to the brain. A number of studies have investigated the possibility that inhibition of rho function will inhibit leukocyte migration across the BBB and attenuate neuroinflammatory disease in animal models of multiple sclerosis. The bacterial exotoxin C3 transferase, which ribosylates and inactivates rho, cannot be used in vivo because of its high toxicity precluding its use as a therapeutic agent. An alternative approach to inhibiting rho proteins is to prevent their post-translational prenvlation, which is required for membrane localization and functional activation (66,67). The isoprenoid moieties required for prenvlation are derived from isoprenoid pyrophosphate substrates synthesized as part of the cholesterol synthesis pathway. Thus, inhibition of the cholesterol synthesis pathway with HMG-CoA reductase inhibitors (statins) should deplete production of isoprenoid pyrophosphates, prevent prenvlation of rho, and result in the inhibition of the ICAM-1 mediated signaling pathway. Treatment of brain EC with statins in vitro has indeed been shown to result in a loss of rho protein prenylation and significant inhibition of lymphocyte transmonolayer migration (68). Even more compelling was the observation that ectopic expression of a mutant rho protein engineered to be a substrate for myristoylation (acylation), and which is capable of mimicking the effects of isoprenylation, was able to render cells insensitive to the inhibitory effects of statin treatment in restricting transendothelial migration of T-lymphocytes. Such an observation firmly places endothelial rho protein prenylation as a candidate mechanism for the action of statins in CNS inflammation. When applied in vivo to animals induced for experimental autoimmune encephalopathy (EAE), an animal model of multiple sclerosis, statins were found to attenuate disease and reduce the migration of leukocytes into the brain and spinal cord (68–73). Although statins act upon the immune system in other ways that may also impact on the pathogenesis of neuroinflammatory diseases (74), inhibition of the ICAM-1-mediated signaling necessary for effective leukocyte migration across the BBB appears to be major target of statin action. Direct inhibition of prenyl transferases, responsible for the prenylation of rho, has also been shown to inhibit lymphocyte migration in vitro and the development of EAE (62) providing further evidence for the importance of Rho function in neuroinflammation. Interestingly, extravasation of lymphocytes across other vascular beds is less affected by inhibition of Rho prenylation induced by either protein prenyltransferases or statins, which may offer some selective benefit in attenuating neuroinflammation rather than inflammation occurring in peripheral sites (62,68).

The induction of calcium transients in ECs following their interaction with polymorphonuclear (PMN) leukocytes (75) or lymphocytes (76) is at the heart of another key event in the EC response to leukocyte adhesion. Elevation of intracellular calcium has also been described following ICAM-1 crosslinking in a variety of cell types including fibroblasts (77) and brain ECs (78). In brain ECs, crosslinking ICAM-1 results in the tyrosine phosphorylation of phospholipase $C\gamma$ (PLC γ), inositol phosphate production, and increased intracellular calcium that were detectable after 2 min (78). It has been proposed that increased intracellular calcium, via the actin cytoskeleton and PKC, leads to the activation of the tyrosine kinase p60src and phosphorylation of cortactin. How this relates temporally and spatially to the activated p60src and phosphorylated cortactin found in the primary ICAM-1 signaling module described above has vet to be established. PKC activation is also required for the described induction of actin stress fibers. tyrosine phosphorylation of FAK and paxillin, and the activation of JNK. but not for the tyrosine phosphorylation of p130-CAS or its association with the adaptor protein Crk.

Much evidence of ICAM-1-mediated signaling to the nucleus via MAP kinase cascades has been gathered as well. Within brain microvascular ECs, there is activation of JNK (78) whereas activation of p38 is reported in pulmonary microvascular ECs (79) and activation of extracellular signal-related protein kinase (ERK) in human umbilical vein ECs (HUVEC) and non-ECs (80). In HUVEC, crosslinking of ICAM-1 also induces expression of VCAM-1 and the chemokines IL-8 and RANTES (80) whilst in astrocytes it leads to the elaboration of the pro-inflammatory cytokines TNF- α , IL-1 α , IL-1 β , and IL-6 (81,82).

From this it is tempting to speculate that the outcome of ICAM-1mediated signaling is cell type dependent and raises the possibility to pharmacologically target inflammation in a given tissue through inactivation of its unique EC signaling cascades. The identification of endothelial-specific, and in the case of neuro-inflammation brain endothelial-specific, signaling pathways such as MAP kinase cascades and their inhibitors will hopefully allow rational development of more specifically targeted pharmacotherapies for inflammatory disease.

5. OTHER EC SIGNALING EVENTS INITIATED BY ADHERENT LEUKOCYTES

Signaling through ICAM-1 is clearly a pivotal component in the EC response to adherent leukocytes. Nonetheless, signaling from leukocytes to the EC does not appear to be restricted to the engagement and micro-aggregation of ICAM-1. This would seem reasonable, as ICAM-1 is ubiquitously expressed on endothelium and is unlikely to accommodate fully the differential migratory behavior of leukocytes through different

vascular beds. Moreover, cross talk between differently induced EC signaling pathways may enable different vascular beds to recruit a specific profile of leukocytes to the tissue. Evidence from non-CNS vascular endothelium and other cell types has implicated other members of the Ig superfamily of adhesion molecules.

VCAM-1 is clearly important in recruiting leukocytes to the brain (41,42). Crosslinking VCAM-1 on ECs has been reported to lead to elevated intracellular calcium levels (13,83). As treatment of brain ECs with intracellular calcium chelators significantly reduces neutrophil (75) and lymphocyte transendothelial migration (78), it is likely that such calcium signaling is critical to the migration event. Another potentially important signaling cascade propagated through VCAM-1 crosslinking is the activation of NADPH oxidase and the production of reactive oxygen species (ROS) (14,84) the latter now recognized as playing a major role in cell signaling pathways. Indeed, inhibition of lymph node EC NADPH oxidase or scavenging ROS results in the inhibition of transendothelial lymphocyte migration placing this pathway at the center of EC control of leukocyte migration. How this is achieved is unclear but appears to involve activation of the small GTPase Rho and remodeling of the actin cytoskeleton (14,83,84). In addition to Rho activation, VCAM-1 crosslinking also leads to the activation of the small GTPase Rac1 and p38 MAPK, the production of ROS and the loss of cell-cell adhesion (84). These studies demonstrate that VCAM-1 signaling in EC is a key component in supporting leukocyte recruitment although our understanding of such signaling pathways remains limited.

Engagement of cell-surface PECAM-1 results in the induction of various signaling pathways (85) that may regulate leukocyte adhesion and migration across ECs. Following both biochemical and mechanical stimulation of ECs, PECAM-1 has been demonstrated to undergo phosphorylation on a set of tyrosine residues located within an immunoreceptor tyrosinebased activation motif (ITAM) of the cytoplasmic tail (86). This has been shown to mediate selective recruitment of several signaling molecules including the tyrosine phosphatases SHP-1 (87) and -2 (88), the inositol-5phosphatase SHIP, phospholipase (PL) C- γ (89), and phosphoinositide 3-kinase (PI3K) (90). Both β - and γ -catenin are also able to associate with PECAM and it has been suggested that via association with γ -catenin, PECAM-1 may be a constituent and regulator of the complexus adhaerentes adhesion system within ECs (91,92).

Separate studies have also identified pathways independent of the Ig superfamily molecules that are essential for lymphocyte migration. In particular it has been shown that treatment of brain EC monolayers with pertussis toxin, which inhibits the G α i heterotrimeric G-protein, prevents lymphocyte migration (93). This raises the intriguing possibility that differential control of leukocyte migration may be under the influence of pertussis toxin-sensitive G-protein coupled receptors such as the chemokine receptors. This is especially interesting as leukocyte chemokine profiles differ considerably between subsets and raises the prospect that responsiveness to chemokines may also operate in the counter direction to that generally reported.

6. POTENTIAL EFFECTOR MECHANISMS DOWNSTREAM OF ICAM-1 SIGNALING

The most tantalizing but largely unresolved aspect of this new area of research involves the endothelial effectors that lie downstream of leukocyte migration. Most obvious candidates for end-point modulation are the junctional molecules, but integrin and transcriptional activation may also play a role (Fig. 2B). Transendothelial migration of leukocytes via a paracellular pathway must involve modulation of the EC lateral junctions so as to allow the migrating cell physical passage through the monolayer.

Assembly, maintenance and modulation of tight and adherens junctions have been shown to be under the control of a number of distinct signaling cascades (94,95) many of which overlap with those found within EC following leukocyte adhesion and/or ICAM-1 ligation. Thus control through both calcium and phosphorylation/dephosphorylation events have been shown. Recent data have indicated that JNK activation and tyrosine phosphorylation of junctional proteins can lead to alterations in junctional integrity. Moreover, junctional complexes are closely linked to the cytoskeleton and regulation by small GTPases such as rho has also been demonstrated. Taken together it is tempting to speculate that ICAM-1mediated signals may also lead to alterations of intercellular junctions.

Nevertheless, junctional proteins downstream of leukocyte adhesionmediated signaling cascades remain to be identified although in the last few years the first indications of such modulation have been reported. Immunohistochemical studies of brain tissue following induction of an IL-1ß inflammatory response show increased phosphotyrosine labeling of leukocytes and ECs in areas of extensive recruitment as well as focal disorganization of occludin, ZO-1, and vinculin (96). The permeability of HUVEC monolayers and isolated coronary microvessels has been shown to increase following co-culture with activated PMNs and this was negated by treatment with either tyrosine or serine kinase inhibitors (97). This study also found increased actin stress fiber formation and disorganization of VE-cadherin and β-catenin, both of which also exhibited increased tyrosine phosphorylation. A subsequent investigation identified the tyrosine kinase p60src as responsible for the modification of β -catenin (98). We have recently found that VE-cadherin is also phosphorylated on tyrosine following ICAM-1 ligation or leukocyte adhesion (our unpublished observations). Tyrosine phosphorylation of VE-cadherin has been shown to be enhanced following treatment of EC with permeability factors such as vascular

endothelial growth factor or histamine (99,100). Under these experimental conditions tyrosine phosphorylation coincides with hyperpermeability and a functional link has been proposed. It remains to be established whether ICAM-1- or leukocyte-induced modulation of VE-cadherin impinges on EC permeability or even leukocyte migration. Current expansion in our knowledge regarding junctional structure and function should lead, in the foreseeable future, to a clearer understanding how junctions are modulated following signaling initiated through molecules such as ICAM-1.

As described earlier, EC ICAM-1 activation also leads to phosphorylation of focal adhesion-associated proteins such as FAK and paxillin. These proteins are intimately involved in integrin modulation and it is therefore tempting to speculate that leukocytes, through endothelial ICAM-1, induce weakening of the interaction between basolateral EC integrins and the basement membrane, thus possibly preparing and facilitating late stages of leukocyte emigration. Yet another requirement of EC integrins may be found on the apical face of the endothelium within the leukocyte-docking module or even for the elusive machinery that mediates migration via a transcellular route.

Lastly, the activation of MAP kinase modules following ICAM-1 ligation suggests that transcription is modulated following leukocyte adhesion. Indeed, activation of nuclear factor (NF) kappaB following ICAM-1 ligation has been reported in HUVEC (80) and brain microvascular EC (our unpublished observations). Activation appears to be significantly slower than the time lymphocytes need to transmigrate in vitro, and in accordance we have so far found no evidence that transcription of any gene is required for successful diapedesis in vitro. The transcriptional programs induced may rather set the scene for sustained inflammation and subsequent infiltration of additional leukocytes. However, it is possible that the time taken to extravasate in vivo may be considerably longer (B. Engelhardt, personal communication, 2004), and in that case, products of immediate-early endothelial gene activation could play a pivotal role in lymphocyte extravasation as well. It will be an exciting challenge to identify the transcriptional targets of ERK, JNK, and p38 in EC, since, as stated earlier, activation of these MAP kinases may differ in EC from different vascular beds and therefore also the transcriptional program induced.

7. SUMMARY

In this chapter we have reviewed current knowledge relating to the signaling role EC ICAM-1 plays in controlling leukocyte migration to the CNS. It is not intended to be an exhaustive review of the general mechanism of leukocyte migration, as these are adequately provided for elsewhere, but aims to highlight the burgeoning importance of ICAM-1 signaling in the overall inflammatory process. Although we have reported that the possible downstream effect of ICAM-1 signaling is to modify the cell–cell junction and thus to allow for leukocyte extravasation, many of the signaling cascades so far described would be equally consistent with the EC facilitating a transcellular route for leukocyte diapedesis. Finally, by gaining a greater understanding of the endothelial mechanisms that are essential for successful leukocyte migration, it may be possible to identify new therapeutic targets for the treatment of inflammatory disease. The next decade promises to provide exciting new insights into the dynamic role ECs play in recruiting leukocytes to the CNS and the neuroretina.

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Monocyte Migration Across the Blood–Brain Barrier: Implications for New Lesion Formation in Multiple Sclerosis

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Monocytes are bone marrow derived cells that belong to the so-called innate immune system, along with central nervous system (CNS) microglia, dendritic cells, and polymorphonuclear leukocytes. Monocytes are phagocytic cells and secrete an array of inflammatory and cytotoxic mediators. Monocytes/ macrophages are not antigen specific, but when activated, macrophages express major histocompatibility (MHC) class II molecules that serve as antigen presenters to lymphocytes. Although the antigen binding to monocyteexpressed MHC structures is less discriminatory than the antigen-specific receptors of T and B lymphocytes, they represent a good example of the overlap that exists between the innate and adaptive immune system. Along with B and T lymphocytes, cells of the monocytes/macrophage lineage are hematogenous cells referred to as mononuclear cells (MNCs). Lymphocytes and monocytes exhibit a similar nuclear morphology and nuclear-to-cytoplasmic ratio which is certainly relevant to the history of multiple sclerosis (MS), but also to the neuropathology of the disease. For years, lesions of MS have been recognized and described as areas of demyelination and perivascular accumulation of MNCs. It is only in the last two decades that the inflammatory infiltrate of MS lesions has been further defined in terms of the relative distribution of T and B lymphocytes, of monocytes and, of different subsets of these cells, based on recent advances in the molecular definition of immune cells subsets and advances in microscopy techniques.

In most forms of MS, the early stage of lesion formation is characterized by infiltration of MNCs across the blood-brain barrier (BBB) and accumulation of such cells in the perivascular region of the affected CNS area. Monocyte-derived macrophages contribute largely to the initiation of disease, as well as to its propagation and severity. Molecular mechanisms governing monocyte transmigration through brain microvascular endothelium are not as well known as those regulating lymphocyte adhesion and migration. In this chapter we discuss the role of monocytes in the development of MS and address potential therapies specifically aimed at interfering with monocyte migration across the brain endothelium.

1. MULTIPLE SCLEROSIS

1.1. Clinical Symptoms and Etiology

MS is a chronic idiopathic inflammatory disease of the CNS clinically characterized by the sudden onset of neurological dysfunction, which is followed by periods of remission. The visual, sensory, and motor systems are affected most often, resulting in transient and incomplete loss of vision, episodes of blurred vision, anesthesia or paralysis of a limb, lack of coordination, and difficulty with gait (1-3). After a period of a few weeks, most symptoms will remit spontaneously, at least in the initial stage of the disease. It is a disease of young adults that affects women more than men, in a ratio of about 2:1. The precise etiology of MS remains largely unknown (3,4). Although MS is often viewed as an autoimmune disease, we prefer to use the term neuro-inflammatory disease of the CNS, because it is still not certain that the cause of the disease is a primary immune system dysregulation (5-7). Current hypothesis about the etiology of the disease include viral, bacterial, and genetic/metabolic causes of myelin damage that leads to subsequent immune cell entry into the CNS (3,7). Epidemiological studies support the notion that both environmental and genetic factors, which likely interact, influence the individual disease susceptibility, as well as disease course (8,9) and most likely response to treatment.

Clinically, MS is a heterogeneous disease in terms of presenting symptoms, course of the illness, response to therapy, and even in neuropathological descriptions of lesions (10-14). While 85% of the patients exhibit a relapsingremitting (RR) phenotype, as suggested by the complete resolution of neurological deficits in-between relapses, approximately half of them will evolve into a secondary progressive course in the 10 years following the first symptoms. A minority of patients (10-15%) will develop a form of MS characterized by progressive neurological deterioration and decline from the start of the disease. This disease is called primary progressive (PP) MS. It is not currently clear if the RR and PP patterns share the same patho-physiological mechanisms in terms of etiology, disease initiation, and neuropathology. Based on pathological, experimental and clinical observations it is obvious that they do not share the same response to the apeutic interventions, leaving the patients affected with PP-MS with no effective therapy to modulate the course of the disease. Recently, a careful and extensive re-examination of the neuropathological findings in MS has highlighted the important differences that could be found at the level of the plaques in between individuals, describing at least four different patterns of demyelination (10–16). Although details of this pathological definition of the disease are still debated, this neuropathological re-examination of the disease's substrate confirms the initial clinical impression that MS is a heterogeneous disease.

Because there is such variability in the clinical manifestation of MS and because of the lack of specific biological markers of disease, the diagnosis of MS still relies on clinical observation and requires the presence of two independent relapses. Magnetic resonance imaging (MRI) techniques have contributed to significantly improve the early diagnosis of MS and have also been used to monitor disease activity in clinical trials (14–17). MRI also contributed to define new pathological mechanisms of neurological dysfunction, as it is the case for axonal damage, and to confirm the involvement of BBB disruption as an early event in lesion development (18–26).

Nowadays, it is assumed that MS is a CNS-targeted inflammatory disorder probably caused by a combination of risk factors (environmental factors, viruses, and dietary conditions), in conjunction with a genetic susceptibility (4).

1.2. Neuropathology of MS

MS is a disease of the myelin sheet and its cell of origin, the oligodendrocyte. Oligodendrocytes are CNS glial cells that differentiate from a glial cell progenitor common to both astrocytes and oligodendrocytes. Mature oligodendrocytes casts several long processes that will attach to, and ensheath axons, creating a biological segmental area of insulation that facilitates and potentiates electrical transmission along the axon. The oligodendrocyte processes form the myelin sheet, a multi-lamellar structure composed of lipid-rich membranes that are interrupted at regular intervals, called nodes of Ranvier. These correspond to inter-cellular spaces between oligodendrocyte processes. Myelin sheet formation around axons not only promotes electrical conductance, but also impacts on neuronal (axonal) membrane stability by means of paracrine growth factor production and local ion channel distribution. Most of the axons of the CNS are myelinated, at least at one point in their course. The process of myelin formation is called myelination and is a developmental event that begins late in the prenatal period and extends during childhood. Primary myelination disorders that affect the CNS are called leukodystrophies. Most leukodystrophies are genetically determined and are caused by an enzyme defect involved in myelin metabolism. They include globoid cell leukodystrophy (Krabbe's disease), metachromatic leukodystrophy, and adrenoleukodystrophy, diseases that often present in pediatric populations. In these inherited leukodystrophies, the immune reaction that occurs within the CNS is considered to be secondary to the presence of aberrant myelin products and related to phagocytosis of cellular debris.

In MS, most of the current scientific evidence points to oligodendrocyte myelin proteins as being the target of the immune driven attack (10). In strong support for this notion is the animal model of MS, experimental allergic encephalomyelitis (EAE), in which the disease is induced by immunization of the animals with either myelin product, purified myelin proteins, synthetic myelin immuno-dominant peptides, or even T lymphocytes that bear a T-cell receptor which recognizes myelin epitopes. Although the animal and the human disease are certainly not clinically identical, the neuropathological description of animal and human CNS lesions are so closely related, that EAE is considered to be the best animal model of MS. The human counterpart of this experimental animal model has been described by Pasteur in 1885 and Bareggi in 1889 following immunization of humans with rabbit-derived spinal cord homogenates, to treat patients for rabies. Hurst later demonstrated that the post-vaccinal fatal neuroparalytic incidents were the consequence of the injection of CNS products leading to a CNS-directed immune reaction (27). However, recent evidence suggests that the myelin sheath destruction in MS might be the consequence of an aberrant apoptosis of the oligodendrocytes (28) and that secondary inflammation might arise from this primary oligodendrocyte death. Although this notion is supported by neuropathological observations and represents the type-IV lesions as defined by Lucchinetti et al. (13) it does not explain the multi focal and circumscribed nature of MS lesions. If primary oligodendrogliopathy was the cause of MS, one would expect a more global, diffuse form of leukodystrophy.

In the last decade, the notion that the oligodendrocyte is the main target of immune mediated damage has also been challenged by pathological and radiological observation showing that axonal (neuronal) damage also occurs in MS. The initial description of axonal damage in MS lesions was made by Hoeber as early as 1922, and has recently been investigated by independent groups from the United States (29), Europe (21,30,31), and Canada (32–34). While early signs of axonal damage have been reported in MS lesions as well as in normal appearing white matter, it is not yet clear if this is a consequence of the myelin sheath disruption, with subsequent loss of trophic factors, or a direct consequence of immune-mediated damage to neuronal structures. The resulting effect is a major influx of immune cells from the peripheral vascular compartment to the CNS parenchyma, across the vascular endothelial cells (ECs) of the BBB. Current knowledge about the mechanisms of tissue damage that occurs in MS is still incomplete and is insufficient to organize the array of experimental observations into a step-by-step mechanistic explanation that fits the actual disease process.

In general, MS lesions are scattered throughout the brain with a preferential location in the white matter of the corpus callosum, the optic nerve, the periventricular white matter region, the brainstem, and the spinal cord. Whereas the primary cause of MS is unknown, the neuropathological description of MS lesions is more defined. The major pathological characteristic of MS is the presence of inflammatory lesions or plaques, scattered throughout the brain. In these lesions, extensive inflammation can be found, with a clear predominance of MNC infiltrates. In MS, lymphocytes and macrophages are typically located around small blood vessels in perivascular cuffs and these infiltrated cells may further traffic into the CNS parenchyma, causing demyelination and oligodendrocyte loss. With ageing of the lesions, astrogliosis and axonal pathology become more pronounced. Recently, several classification methods were introduced to estimate lesion type, age and activity, using various (immuno) histochemical markers (35,36). An active demyelinating MS lesion is often characterized by perivascular infiltrates consisting of CD14⁺ monocytes loaded with myelin debris and activated CD4⁺ and CD8⁺ T cells that accumulate around small cerebral blood vessels, together with few CD19⁺ B cells. The presence of large numbers of macrophages filled with myelin debris is often regarded as a unique indicator of inflammatory activity (37,38). After migration through brain ECs, these monocyte-derived macrophages are able to phagocytose myelin products released from disrupted myelin sheats and probably attack intact myelin membranes that are in the vicinity. In the later stage, CNS inflammation resolves and leads to the formation of a local area of scar tissue composed of astrogliosis and hypertrophied astrocytes that fill the demyelinated area. Although it is still a matter of controversy, it has been reported that surviving oligodendrocytes or oligodendrocyte progenitors (39–42) may contribute to re-myelination of axons by generating thin myelin sheaths, which might play a role in the clinical recovery, at least for some patients.

From neuropathological findings in human MS and from findings obtained from the animal model EAE, ideas about the patho-physiology of MS have emerged. Once in the CNS, T cells may recognize a myelin antigen with their T-cell receptor. If the T cell is of the CD4 phenotype, such an antigen must be presented within a Class II MHC complex molecule expressed by an antigen-presenting cell (APC). If the T cell is of the CD8 (cytotoxic) phenotype, the activation process requires the presentation of the antigen in a MHC class-I molecule. In the CNS, perivascular macrophages, microglia, dendritic cells, and, to some extend, astrocytes have been described as putative APCs (Chapters 12 and 8). When MHC class-II antigen presentation occurs in conjunction with an appropriate co-stimulation, CD4 and CD8 T-cell differentiation, specialization, and proliferation ensue (8), leading to the local release of cytokines, chemokines, inflammatory mediators, growth factors and neurotrophins that will create an inflammatory milieu. This phenomenon is the basis for the inflammatory cascade leading to the recruitment of nonantigen-specific bystander MNCs for which attraction to the CNS is facilitated by the focal activation of the BBB endothelium. Subsequently, infiltrated MNCs secrete additional immune factors; cytokines and chemokines that may further enhance CNS inflammation or promote a gradual resolution of the local inflammatory state by secreting antiinflammatory cytokines or chemokines that may attract anti-inflammatory Th₂ lymphocytes towards the lesion site.

1.3. Animal Models of MS

As previously mentioned, most of our current thinking about the pathogenesis of MS is extrapolated from an animal model of the human disease called EAE. EAE is an experimentally induced demyelinating disease of the CNS and can be induced by active immunization of animals with myelin products or purified components of myelin. For immunization one can use the whole spinal cord or brain homogenate, purified myelin, single myelin proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) or even smaller peptide fragments derived from these proteins (43). The MBP fragment at amino acid position MBP 89-94 was shown to be the immuno-dominant peptide in both MS and EAE, i.e., the peptide that induces the strongest immune response against the myelin sheath in vivo. Immunization with myelin or myelin components together with a strong adjuvant, like Freunds' adjuvant, leads to the generation of T cells that recognize autologous myelin proteins. Transferring these autoreactive T cells into naïve animals, the so-called adoptive transfer-EAE model, induces similar clinical signs as EAE induced by active immunization (44). Most of the transfer studies performed using this model demonstrated that the phenotype of the transferred lymphocytes had a significant impact on disease initiation and course. In mouse, MBP specific lymphocytes that are of the CD4 Th1 phenotype (i.e., secrete IFN- γ) are potent inducers of the disease. Conversely, cells of the CD4 Th2 phenotype (i.e., secrete IL-4 and IL-5) are not encephalitogenic and can even protect against Th1 induced disease. It has also been recently demonstrated that

CD8 T cells that bear a T-cell receptor specific for MBP can induce EAE when injected into recipient animals (45,46) and that clonally expanded CD8 T cells can be recovered from MS lesions in human (47). In Chapter 10, Fabry describes in great detail the role of T lymphocytes in the pathology of MS.

The model of transfer EAE is most frequently induced in disease susceptible strains of rodents and in non-human primates like marmosets and rhesus monkeys (48,49). Although EAE is still the best animal model for the human disease, different models can be found. On clinical grounds, depending on the immunization, disease patterns may vary from a monophasic type of disease, with only minor myelin damage (acute EAE and transfer EAE) to the demyelinating and relapsing remitting form (chronic EAE). Generally, clinical signs in EAE are predominantly motor deficits and manifest themselves in an ascending manner, beginning with loss of tail tone followed by paralysis of the hind limbs, and the disease may progress to the front limbs and occasionally even to death of the animal. Neuropathologically, EAE is characterized by the presence of inflammatory lesions consisting of perivascular infiltrates of mononuclear leukocytes (48,49) that are mostly located in the spinal cord of the affected animals, whereas in humans the disease is scattered throughout the brain and spinal cord. Finally, although the existence of myelin-reactive T cells in the peripheral circulation of MS patients is now well established, the frequency of such cells is not different from the one found in the blood of healthy donors. It has not yet been demonstrated if human MBP-reactive T lymphocytes are encephalitogenic, i.e., they promote CNS demyelination leading to signs and symptoms of MS. Perhaps the strongest evidence that myelin reactive T cells can induce lesion formation and provoke clinical attack in humans was derived from the so-called altered ligand peptide clinical trial. In this trial, MS patients were "immunized" with a synthetically altered MBP derived peptide in the hope that immune tolerance to MBP protein will develop. However, three out of eight patients developed cross-reactive T-cell responses to MBP and had an increased disease activity both clinically and on MRI (50). Nevertheless, a clear demonstration of the encephalitogenic potential of human MBP-specific T cells remains to be made. For these reasons, and although we think that EAE is still the best available model for MS. correlation, and extrapolations between the animal EAE model and the human disease remain difficult.

1.4. The Blood-Brain Barrier in MS and EAE

Although infiltrated T cells and monocyte-derived macrophages play an essential role in lesion development, several reports also suggest that a dysfunction of the BBB precedes the MNC infiltration and may even initiate lesion formation. It is generally accepted that BBB disruption is an early phenomenon consistently observed in new active lesions, and that BBB breakdown correlates with active inflammation and myelin damage (51,52). The use of MRI technology along with the intravenous injection of the contrast agent Gadolinium diethylenetriaminepentaacid (Gd-DTPA) is a fairly reliable indicator of BBB leakage and an indirect indicator of the presence of inflammation (for an excellent review of the MRI techniques to measure BBB integrity, see Chapter 19).

In MS patients, the impermeable character of the BBB is compromised during the formation of new lesions, as demonstrated by several MRI studies. Extravascular distribution of Gd-DTPA, indicating BBB disturbance, is often the first indicator of new lesions. Lesions may remain Gd-DTPA enhancing for months, but enhancement usually disappears within a few weeks (53). MRI studies applying different protocols or using a triple dosage of Gd-DTPA markedly improved the detection of areas of focal BBB leakage and revealed that subtle BBB changes may occur in normal appearing white matter (NAWM) of MS patients (54–56). These studies support the notion that BBB dysfunction may precede myelin damage and leukocyte infiltration in MS patients.

In addition to MRI based reports, a number of pathological studies have shown that BBB disruption is the hallmark of active and chronic active MS lesions by demonstrating profound leakage of the serum protein fibrinogen (57–59). A dysfunction of the BBB in inactive MS plaques was previously documented in pathological studies that report ultrastructural abnormalities in the brain endothelium and in the vascular basal lamina (60). Moreover, abnormalities of the expression and structural composition of the brain endothelial tight junction proteins zona-occludens-1 and occludin is common in active demyelinating lesions, and was associated with disturbance of the BBB as marked by the presence of fibrinogen in the brain parenchyma (58,59).

In a number of animal models, MRI has been used to monitor BBB integrity in relation to disease development. In acute EAE, Gd-DTPA enhancement preceded the clinical signs and monocyte infiltration (61). In transfer EAE, Gd-DTPA enhancement was associated with the presence of infiltrating encephalitogenic T cells (62). Not only T cells, but also infiltrated macrophages can induce changes in BBB integrity (63). The observed disruption of the BBB may therefore also be due to locally produced macrophage products like interleukin-1 beta (IL-1 β), TNF- α , chemokines (64), and reactive oxygen species (ROS) (65). Indeed, BBB leakage can be induced in vitro (66) as well as in vivo by intrathecal injection of IL-1 β and TNF- α (67,68). Furthermore, these cytokines influence the expression of adhesion molecule by ECs of the BBB, which may further enhance the infiltration of leukocytes.

Thus, both in MS and EAE, loss of BBB integrity seems to be related to clinical disease activity. However, whether BBB disruption is directly related to cellular infiltration remains unclear. Reports suggest that dysfunction of the BBB precedes the cellular infiltration and may even initiate lesion formation, although clear evidence is lacking.

1.5. Current Therapies for MS

Most of the current therapies in MS have a rather broad mechanism of action, and are based on anti-inflammatory, immunosuppressive, or immunomodulating strategies.

Acute attacks in MS are sometimes treated with corticosteroids (prednisone or methylprednisolone) (69) used as general immunosuppressors. Corticosteroid treatments are reported to shorten the duration of the relapse and accelerate recovery, but whether the overall degree of recovery is improved or the long-term course is altered is still a matter of debate. To date the exact mechanism by which corticosteroids exert their beneficial effects in MS remains unclear. Corticosteroids may diminish BBB disruption, as determined by Gd-DTPA enhanced MRI, via a reduction of capillary permeability and inhibition of inflammatory edema (70). Furthermore, corticosteroids can also decrease transmigration of peripheral blood MNCs across the BBB (71), by diminishing endothelial adhesion molecule expression (72) or via a reduction of metalloproteinase (MMP) 9 production (73).

Currently, two forms of interferon-beta (IFN- β) have been approved by United States and European regulatory authorities for the treatment of RR-MS. Interferon- β -1a (Avonex, Rebif) and interferon- β -1b (Betaseron/ Betaferon) have been studied in large clinical trials and induced a clear reduction in both frequency and severity of clinical exacerbations (74–76). These observations were supported by convincing MRI data showing both a reduction of the number of active lesions and a positive effect on total lesion load in the brain. The rational for the use of IFNs for the treatment of MS was mainly based on the hypothesis that the disease was caused by a persistent or a latent viral infection of the CNS (77,78). Despite the clinical advances, the mechanisms by which IFN- β exerts its beneficial effects in MS remain unclear and there is a wide range of possible sites of action (79). One of these includes a down-regulatory effect on the migration of lymphocytes and monocytes across the BBB (80) and a stabilizing effect on BBB permeability to soluble molecules (81).

Glatimer acetate (GA), previously known as copolymer-1 (trade name Copaxone), is a mixture of random synthetic polypeptides composed of Lalanine, L-glutamic acid, L-lysine, and L-tyrosine. Despite the well-documented clinical effects of GA in patients with RRMS (82) the mechanism of action has not been fully clarified. The proposed actions of GA include the generation of antigen-specific suppressor T cells and/or competition with encephalitogenic antigens, i.e., MBP, MOG, and PLP for binding with MHC class-II antigen on the surface of APCs (83). Neuroradiological studies suggest that GA treatment leads to the reduction of active lesions as assessed by Gd-DTPA-enhanced MRI (84). Currently, there is only limited data on the role of GA as a modulator of BBB permeability. In vitro, endothelial adhesion molecule expression and bulk T-lymphocyte migration (85) remained unaffected after GA treatment. We have recently shown that the migration of specific subsets of Th1 and Th2 lymphocytes across human brain endothelium is differentially modulated by GA and by IFN- β (86). As GA seems to enhance the migration of Th2 cells across BBB ECs, IFN- β elicited a powerful antimigratory effect on Th1 lymphocytes.

There are a number of emerging therapies for MS. One of these strategies is aimed at the interference of the migration of inflammatory cells into the CNS at the level of the BBB. The interaction of the leukocyte integrin VLA-4 (Very late antigen 4, α 4 β 1 integrin) and VCAM is of importance for cellular entry into the brain. Recently a humanized monoclonal antibody against the α 4 chain of α 4 β 1 integrin was developed, and named Natalizumab/Antegren/Tysabri. Treatment with Natalizumab had a significant effect on the primary outcome measure of MS lesion formation—reduction in the number of new brain lesions on gadolinium enhancement MRI over the 6-month treatment period—and significantly reduced the number of clinical relapses (87). Tysabri was approved in the United States for the treatment of relapsing remitting MS in 2004, and removed in 2005 because of the reactivation of CNS JC virus infection (progressive multifocal leukoencephalopathy) in 3 MS patients.

Recently, the lipid-lowering statins were recognized to have immunomodulatory effects. Statins can exert this action by the modulation of the expression of adhesion molecules, by inhibiting the proliferative activity of T cells and B cells and also by affecting signaling pathways involved in cellular migration RhoA isoprenylation. Statins have been shown to ameliorate signs of disease in EAE (61,88–90), by the inhibition of cellular influx across the BBB as monitored by MRI (61). Using gene array analysis, it was revealed that lovastatin treatment of EAE animals suppressed the expression of immune related genes associated with EAE (91).

Other new developments in the treatment of MS patients include the use of cannabinoids and oestriols (92), although it is not yet known whether they act at the level of the BBB.

2. MONOCYTE MIGRATION ACROSS BRAIN ENDOTHELIUM

Before entering the CNS parenchyma, leukocytes have to adhere to, and transmigrate across the BBB (as described in Chapters 9 and 10). Molecular mechanisms governing monocyte transmigration through brain microvascular endothelium are not as well known as those regulating lymphocyte adhesion and migration. Adhesion molecules and inflammatory mediators that

are involved in the diapedesis of monocytes and lymphocytes seem to be different. Monocytes recruited from the blood circulation to the brain rapidly differentiate into macrophages and contribute to inflammatory responses in the CNS. As recently reviewed by Engelhardt and Wolburg (93), it is still not clear if the process of immune cell migration across the BBB is taking place at intercellular junctions (paracellular route) or through the ECs (by a transcellular mechanism called emperipolesis). Once passed across the BBB-EC, the newly formed macrophage has to degrade the thin but extremely compact extracellular matrix or basal lamina, in order to reach the parenchyma. Similar to the findings in MS lesions in humans, the perivascular infiltrate in EAE rats and mice contains numerous macrophages (49). In EAE, infiltrated macrophages were shown to play a crucial role in the initiation of the immune response. Depletion of systemic macrophages using silica particles or clodronate-liposome resulted in a marked reduction or even complete absence of neurological deficits (94-96) supporting a key role for macrophages in the pathogenesis of demyelinating diseases.

2.1. Adhesion Molecules

The role of adhesion molecules in leukocyte transmigration has been a subject of intense research in the last decade. So far, in vitro studies on cellular migration across brain endothelium have focused on lymphocyte migration. Numerous authors insist on the fact that lymphocyte transmigration is a multi-step process that involves an initial step of rolling followed by adhesion to ECs, transmigration through EC and extra-cellular matrix (ECM) breakdown. These studies reported that activated lymphocytes adhere to activated brain endothelium through lymphocyte function-associated antigen (LFA-1) and VLA-4, and that their subsequent transendothelial migration seems to be mainly regulated by ICAM-1, and partly through PECAM-1 (97–99).

The β 2 integrin complement receptor-3 (CR-3 or Mac-1), which is highly expressed on monocytes, has been shown to mediate monocyte migration across peripheral endothelial monolayers (100,101). In EAE, anti-CR3 antibodies have been shown to significantly delay the onset and diminish the severity of clinical signs of EAE, even when injections are given at the first appearance of clinical signs (102). However, in these examples disease palliation was not accompanied by reduced cellular infiltration, implying that other CR3-mediated cellular functions are involved in EAE, such as myelin phagocytosis and ROS production (102,103). Owens et al. reported that following monocyte depletion in EAE, although intense polymorphonuclear infiltrates could be found in the CNS, immunized animals were protected against disease. Taken together, these results suggest that monocyte migration to the CNS is a prerequisite for myelin damage to occur and for the initiation of clinical disease. In the absence of monocytes, the perivascular infiltrate changes from an MNC dominant infiltrate to a PMN based infiltrate. Studies on peripheral endothelium have suggested that the interaction of VLA-4 with VCAM-1 is required for firm adhesion to, and subsequent migration of monocytes through the peripheral EC barrier (100,101). Similarly, an important role for the VLA-4/VCAM-1 pathway has been demonstrated for monocyte migration across brain microvascular endothelial cells (80,104). Using human peripheral blood monocytes and human brain ECs, we have shown that monocyte migration across the BBB was dependent on VLA-4 but not on VCAM-1 (104,105).

An alternative pathway has also been implicated in the infiltration of monocytes into the CNS. Monocytes could access the CNS through integrin $\alpha_D\beta_2$ binding to VCAM-1. Treatment with blocking antibodies directed against α_D was shown to reduce macrophage infiltration at the lesion site spinal cord-injured rats (106). Whether this integrin is involved in monocyte recruitment into the CNS during EAE remains to be established. Together, these studies suggest that VCAM-1 plays an important role in the infiltration of monocytes to the CNS.

Other members of the immunoglobulin super family have been shown to be involved in monocyte infiltration into the CNS. For example, de Vries et al. (107) reported that the widely expressed CD47, also known as integrinassociated protein, mediates the final post-adhesion step of monocyte migration into the CNS by interacting with its monocytic ligand signal regulatory protein- α .

Junctional adhesion molecules (JAM) and other junctional proteins are reported to mediate monocyte migration across brain endothelium. Monocyte migration into murine brain during experimental meningitis is inhibited by anti-JAM monoclonal antibodies (mAbs), and these antibodies have similarly been shown to antagonize monocyte migration across cultured peripheral vascular ECs (108). It is postulated that JAM guides monocytes through EC junctions, since it is expressed at the level of TJ complexes.

The role of JAM-A in the process of transmigration has until recently been contradictory. For example, Liu and co-workers (2000) (109) demonstrated that mAb against the human form of JAM-A did not affect leukocyte migration. However, human JAM-A was described to have a part in transmigration of T cells and polymorphonuclear leukocytes (PMNs) across brain ECs in vitro (110). It was postulated that JAM-A was engaging the leukocyte-integrin LFA-1 in a heterophilic manner, thereby influencing lymphocyte migration.

JAM-B has not yet been directly implicated in leukocyte–EC interactions, although this is likely since JAM-B interacts with JAM-C (111) and with VLA-4; 112, which is expressed on lymphocytes, monocytes, and eosinophils. This binding, however, is only efficient if JAM-B has already engaged JAM-C (112). Antibodies against JAM-C or soluble JAM-C blocked migration of human PMNs across human umbilical vein EC monolayers in response of a chemotactic gradient of stromal derived factor-1 (SDF-1; 113). Because JAM-C is also expressed by subpopulations of human B cells, T cells, and most monocytes (113), one might predict that homophilic interactions between leukocyte JAM-C and EC JAM-C were involved in this process, similar to what has been shown for PECAM-1 and CD99.

PECAM-1 is expressed diffusely on the surface of most leukocytes and is concentrated at the borders of ECs (114,115). Homophilic interaction of the amino terminal part of leukocyte PECAM-1 with the similar domains of EC PECAM-1 is required for diapedesis. Blocking this interaction with a domain-specific mAb or with a soluble form of PECAM-1 as a competitive inhibitor, blocks diapedesis by approximately 90% in vitro (80,115) and in vivo (116). CD99 was recently described to be involved in monocyte migration across perivascular endothelium. CD99 appears to act at the level of diapedesis through the TJ (115). Similar to PECAM-1, CD99 functions in a homophilic manner in transmigration. It was demonstrated that Fab fragments of anti-CD99 mAb blocked diapedesis by approximately 90% in an in vitro model of transendothelial migration. Furthermore, blocking both CD99 and PECAM-1 abolished diapedesis almost completely (117). We have explored the expression of CD99 in cultured human cerebral microvascular EC and found no expression of CD99 either by RT-PCR or by immunocytochemistry (Alexandre Prat, unpublished observation). We believe that the role of CD99 in leukocyte transmigration across brain endothelium in human still needs to be confirmed.

The current concept of monocyte migration through brain endothelium involves signaling events that occur following adhesion molecule interactions, both in monocytes and in the ECs. However, the list of adhesion molecules is growing and many players involved in monocyte migration across the BBB remain to be identified. We believe that adhesion molecules that are concentrated at the lateral borders of ECs and that may be involved in TJ physiology, may also have a role in the process of leukocyte transmigration.

2.2. Monocytic Inflammatory Mediators

Monocytes recruited from the blood into the CNS differentiate into macrophages, and contribute to neuro-inflammatory processes by producing a wide range of mediators that stimulate the inflammatory cascade. Monocyte-derived inflammatory products, like pro-inflammatory cytokines, reactive oxygen species (ROS), or nitric oxide (NO) can further recruit leukocytes into the CNS. Different studies have shown that chemokines (MCP-1; 118) and cytokines (TNF- α and IL-1 β ; 119) can increase the permeability of the BBB and alter the junctional organization of brain vascular endothelium. Macrophage produced TNF- α is also known to impact on the level of expression of ICAM-1 and VCAM-1 on brain ECs, two molecules that have been implicated in the recruitment of immune cells to the CNS.

Accumulating data indicate that oxidative stress plays a major role in the pathogenesis of MS. ROS, generated primarily by macrophages, lead to oxidative stress and have been implicated as mediators of demyelination and axonal damage in both MS and EAE (120). The reduction of oxygen to superoxide and the formation of other ROS occur during the respiratory burst of activated cells of the immune system. Consequently, ROS cause damage to cardinal cellular components such as lipids, proteins and nucleic acids, resulting in cell death either by necrosis or apoptosis. Based on these findings, anti-oxidant agents have been utilized in EAE with controversial results, being protective or detrimental under different circumstances. Anti-oxidant therapies include the use of curcumin, bilirubin, lipoic acid and flavonoids that are described to ameliorate the clinical signs of EAE (121–124).

ROS can also play a role in the migration process of monocytes across brain endothelium. Under normal conditions, oxygen-derived free radicals are constantly produced and scavenged by endogenous anti-oxidants such as superoxide dismutase and glutathione peroxidase. However, superoxide was shown to be produced upon firm adhesion of monocytes to ECs and to act as a signaling molecule inducing endothelial cytoskeletal rearrangements. This event was shown to lead to disruption of tight junction integrity as well as to increased transendothelial migration (43).

In addition to superoxide, NO is a free radical synthesized by the inducible enzyme nitric oxide synthase (iNOS). Inflammatory mediators released in the CNS are able to induce expression of iNOS in ECs, astrocytes and brain macrophages (125,126). NO was shown to modulate permeability of the human endothelium and to partake in the disease process at the level of the BBB (42). The BBB can also be affected by exposure of the EC to exogenous NO (127).

During the extravasation processes, migrating cells not only have to rapidly traverse the tight EC monolayer, but also the basement membrane of the blood vessel endothelium, and migrate into the underlying interstitial ECM. Infiltrating leukocytes therefore, produce MMPs which degrade ECM proteins like fibronectin, collagen, and laminin. MMPs (Chapter 14) belong to the family of zinc-dependent MMPs, and serve as effectors for cell migration, cytotoxicity, and tissue remodeling via degradation of ECM components. The MMPs comprise a family of over 20 endopeptidases, which are synthesized as inactive zymogens. They share a common N-terminal, an inhibitory pro-peptide sequence with a Cys that chelates the Zn^{2+} binding ("cysteine switch") and leads to intermediate activation, followed by autocatalytic cleavage of the pro-peptide from the core protein, and results in a fully active enzyme. Tissue inhibitors of metalloproteinases (TIMPs), the specific endogenous inhibitors of MMPs, form complexes with pro- and activated forms of MMPs and inhibit enzymatic activity of the latter.

In chronic inflammatory diseases of the CNS, such as MS, focal damage of the BBB, perivascular cell infiltration, and neuronal damage occur. There is accumulating evidence that MMPs are involved in all these processes (see chapter 15 by Rosenberg). Resident brain cells (astrocytes, microglia, and EC) and immune cells (T cells and macrophages) secrete various MMPs that contribute to BBB opening (Chapter 15). Immunohistochemistry of brain tissue of MS patients showed that the production of MMPs (MMP-1, -2, -3, -7, and -9) is increased in and around plaques (128). Of all MMPs, MMP-9 has drawn attention, as elevated levels were found both in cerebrospinal fluid (CSF) (129) and in serum (130) of MS patients. A recent study has given evidence for a role for MMPs in endothelial tight junction regulation at the BBB in particular, and probably at TJs in general (131). This study demonstrated that the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) induced increased MMP activity, which was paralleled by severe disruption of cell-cell contacts, as well as proteolysis of occludin, and a marked reduction in transendothelial electrical resistance. Interestingly, in our in vitro migration model we observed a significant reduction of monocyte transendothelial migration in the presence of a broad range MMP inhibitor (De Vries et al., unpublished data).

2.3. Anti-inflammatory Agents and Monocyte Migration

In contrast to cytokines, ROS and adhesion molecules, neurotrophic factors such as nerve growth factor may act as anti-inflammatory agents, since it limits the transendothelial migration of monocytes across the BBB (132). Various findings strongly suggest that neurotrophins and the p75 low affinity neurotrophin receptor (p75^{NTR}) are both involved in pathogenic processes of MS. MS patients show a dramatic increase in the level of NGF in the CSF during acute attacks, whereas NGF levels decrease during remission. Increased levels of NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) have also been observed in the CNS of rats with EAE. A number of studies in EAE rat models have shown the expression of p75^{NTR} in various cell types in perivascular tissue, including perivascular macrophages, pericytes, ECs, and infiltrating monocytes (132). The deficient expression of $p75^{NTR}$ correlated with a greater extent of infiltration and higher clinical scores in EAE animals (133). These observations of p75^{NTR} expression in ECs in the CNS during EAE, and the notion that exacerbation of clinical symptoms correlates with cell infiltration in p75^{NTR} knockout mice, suggest a role for this receptor in maintaining proper permeability of the BBB, at least in EAE. We have also recently shown that IFN- β increases the secretion of NGF, but not BDNF, by human brain microvascular ECs, via an autocrine feedback loop (86), suggesting that brain ECs contribute not only to inflammatory cell infiltration but also in the global protection mechanism via the production of neurotrophins.

Current treatments of neuro-inflammatory diseases aim at dampening inflammatory cascade in the CNS. For instance, IFN- β treatment leads to the reduction of new MS lesions as assessed by MRI (134). Reduced cellular infiltration may be the result of attenuated expression of adhesion molecules on the brain endothelium, as has been reported in EAE animals (61). IFN- β was convincingly shown to reduce monocyte transmigration across brain EC monolayers in animal models and in humans. Experiments conducted separately in two different laboratories in Canada and in The Netherlands have shown a similar effect of IFN- β on the in vitro migration of monocytes across human brain microvascular ECs and rat brain microvascular ECs. These experiments showed that the effects of IFN- β are mediated through the inhibition of adhesion molecules on brain ECs and on monocytes, as well as through a reduction in the secretion of TNF- α and MMPs by the monocytes (104).

Cannabis, now under consideration as a potential therapeutic for MS patients, reduced spasticity and clinical signs in EAE (135), and may influence the migration of monocytes across the BBB. The psychoactive form of cannabis, Δ or δ -9-tetrahydrocannabinol, has been shown to influence macrophage functions, such as phagocytosis, antigen presentation and migratory capacity across endothelium (136).

Lovastatin, a potent inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the cholesterol biosynthesis pathway, may also be a promising new therapeutic agent. Lovastatin was shown to suppress the clinical course of EAE by inhibiting monocyte infiltration into the CNS (80,88).

2.4. Fate and Function of Migrated Monocytes in MS and EAE

Once inside the perivascular space, macrophages probably partake in the phagocytosis of myelin debris, the presentation of antigen to lymphocytes and the perpetuation of inflammation. They are known for their ability to release TNF- α that will in turn stimulate the secretion of RANTES and MCP-1 by brain ECs, two chemokines that are potent chemoattractants for immune cells.

Apart from their production of pro-inflammatory mediators, macrophages also contribute to the recruitment of T lymphocytes towards the CNS parenchyma. Although T-cell activation and cytokine production remains unaffected by macrophage depletion, their infiltration into the CNS parenchyma is almost completely blocked in macrophage depleted EAE rats and mice (96). The effect of CNS tissue macrophages on T-lymphocyte-mediated responses is also well characterized. Through autocrine and paracrine feedback loops between macrophages and lymphocytes, the macrophages can influence the faith of migrated lymphocytes. In the context of MHC class II restricted antigen presentation, T lymphocytes will trigger macrophage activation through the CD40L–CD40 system. This activation leads to the local secretion of IL-12 and IL-23 by macrophages, two important cyto-kines that favors the skewing of the lymphocyte population towards a Th1 (pro-inflammatory) phenotype.

Macrophages are also important mediators of tissue damage. Within the CNS, macrophages secrete CD95L, a molecule of the TNF family, or can induce CD4T lymphocytes to secrete CD95L through TNF- α -mediated activation. CD95L was reported to kill human and mouse oligodendrocytes in vitro (137,138). CD95, the receptor for CD95L, is present on oligodendrocytes in MS lesions in situ (137). Macrophage-produced TNF- α was also reported to impact on oligodendrocyte death in one study, while it was shown to mediate a protective effect in another.

Altogether, these studies identify macrophages as interesting targets for therapy in MS. Therapeutic targets for MS may evolve from in vitro studies aimed at interfering with macrophage infiltration at the level of the BBB.

2.5. Monocyte Migration In Vivo

Neuropathological changes in patients with CNS diseases are determined by MRI. One of the major advantages of MRI is that it is a non-invasive method and serial studies can be performed, enabling a unique dynamic evaluation of cellular events within the same patient or animal. Gd-DTPA enhanced MRI is a powerful diagnostic tool to detect BBB dysfunction. Gd-DTPA enhancement, however, provides no direct information about the trafficking of MNCs across the junctional complexes between ECs. Application of a new magnetic resonance contrast agent based on ultra small particles of iron oxide (USPIO), can be used as a sensitive method for biological imaging (140). Upon the use of USPIOs in EAE animals, lesions became apparent in the brain stem and cerebellum and the upper part of the spinal cord, correlating with the immunohistochemical detection of lesions. Electron microscopy showed that these iron particles were solely present in infiltrated monocyte-derived macrophages in the newly formed lesions, whereas no particles were found in the interstitial space (61,141). Further optimization of MRI techniques to image macrophage migration and activity in MS is therefore of great importance to understand the process of new lesion formation.

2.6. Transcellular Migration Regulation of Endothelial Paracellular Permeability by Monocytes

The paracellular route is not an exclusive pathway for transmigrating leukocytes. Ultrastructural studies have suggested that leukocytes could pass through the body of an EC (142,143). Feng and co-workers (144) showed that transmigrating neutrophils used a transcytotic pathway that did not involve interendothelial junctions. They proposed that active remodeling of the EC membrane caused invagination and fusion of the luminal and abluminal membrane to create a reversible pore at the leading edge of the migrating leukocyte. Electron microscopy pictures taken by Wong et al. (145) have shown that T lymphocytes can move either through the EC cytoplasm or between adjacent ECs, across intercellular contacts. They also showed that there was no evidence of disruption and that the intercellular junctions appeared intact over the migrated T lymphocyte. The exact pathway of migration through BBB-ECs still remains unclear. It appears that monocytes and lymphocytes potentially use both paracellular and transcellular pathways to cross the BBB in association with cup-like structures that are enriched in ICAM-1, VCAM-1, and caveolin (146). However, in vitro evidence favors the paracellular route for PMN migration, whereas in vivo studies implicate both the paracellular and transcellular route for monocytes and lymphocytes (143, 144, 147).

3. CONCLUDING REMARKS

Monocyte extravasation into the brain is presumably controlled by a number of adhesion molecule, as well as by signaling events that act in concert and can be considered as "go/no-go" decision points, which eventually determine whether a monocyte can infiltrate the brain. Inflammatory mediators and signaling events in the infiltrating monocytes may also influence their migratory capacity in response to, for instance chemokines, or ROS. Identification of the players that affect monocytic migratory capacities is of great importance for the development of therapeutic strategies to diminish MS lesion formation at the level of the BBB.

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Cerebral Perivascular Macrophages and the Blood–Brain Barrier

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1. PERIVASCULAR MACROPHAGES IN THE CENTRAL NERVOUS SYSTEM

The blood-brain barrier (BBB) is a composite structure formed by the fusion of several different cell types and the extracellular matrix (ECM). The major constituents of the BBB are endothelial cells, which undergo barrier specialization as a result of their interaction with the foot processes of astrocytes (glia limitans). The perivascular space is a tightly regulated microenvironment and harbors three cell types: perivascular macrophages (PVM), pericytes, and smooth muscle cells. This chapter deals with PVM, which are distinct from their myeloid counterparts found within the brain parenchyma (microglia) and outside the brain parenchyma (monocytes). Due to their strategic location at the BBB, PVM have important cellular functions including recognition, phagocytosis, degradation, and transport of pathogens, antigen presentation to T lymphocytes, and production of immune regulatory mediators. Furthermore, some aspects of the phenotype of PVM suggest that they contribute to the maintenance of the BBB.

1.1. Morphological Characteristics of Perivascular Macrophages

The PVM are the second most prevalent type of resident tissue macrophages in the central nervous system (CNS), surpassed only by microglia. The PVM are readily identified by their location between the endothelial basement membrane and the glia limitans in the perivascular space of arteries, medium sized vessels and capillaries (1,2). However, PVM are often missed during routine stainings because of their relative scarcity. They have an elongated bipolar morphology and contain lysosomes in their cytoplasm (Fig. 1). The nomenclature of these cells is confusing because they have been branded with many different names such as fluorescent granular perithelial cells (3), perivascular microglia (4), pericytes (5,6), ED2 positive perivascular cells (7) or perivascular monocytes (8,9).

The PVM are different from pericytes, which are fibromyocytic cells within the blood vessel wall, however some authors do not make the distinction between PVM and pericytes (10). Pericytes, like PVM are cellular constituents of the BBB, but they lie completely enclosed within the vascular basement membrane on the abluminal side of the endothelium of small blood vessels (Figs. 2 and 3) (1). Although pericytes have an oval to elongated cell body, similar to PVM, their bodies are much thinner. Pericytes possess branching processes that encircle the blood vessel (11,12). They are thought to provide vasodynamic capacity and structural support to the microvasculature of the brain (13).

The PVM are distinct from microglia, which are the other resident mononuclear phagocyte population in the CNS. Microglial cells are ramified cells found in the CNS parenchyma and their processes are arranged either longitudinally along nerve fiber tracts in the white matter or in stellate pattern in the gray matter. Unlike PVM, microglia dramatically change their morphology upon activation: their processes become shorter and stouter their cell bodies become more rounded. In many circumstances microglia cannot be distinguished from blood-derived macrophages (14). In fact, it has been shown by use of bone marrow chimeras that both PVM and microglia are derived from blood monocytes (12). Microglial cells arrive before birth and persist in the brain parenchyma for long periods with little repopulation from the bone marrow (15). The PVM, on the other hand, are replaced every 2 or 3 months by monocytes migrating from the bone marrow (4,12). The fate of PVMs that are replaced is so far unknown, they could either die through apoptosis or migrate to other sites.

1.2. Cellular Markers Specific for Perivascular Macrophages

Apart from morphology, PVM express cellular markers that distinguish them from microglial cells and pericytes (summarized in Table 1) (16). In the rat and human CNS, PVM can typically be recognized by antibodies



Figure 1 Perivascular macrophage network. (A) The blood vessel wall consists of endothelial (End) and pericyte/smooth muscle (Per/SMC) layers. In addition, this blood vessel shows a continuous macrophage (Mac) layer. The macrophages contact one another by their processes (arrowheads) to form a network. A point of contact (large arrowhead) was identified under high magnification. A basal lamina (BL) projection encloses parenchymal cell processes (arrow). Top inset: 2× magnification of the area indicated by a long arrow. The BL covering the large Mac is over 1 µm in thickness. Note the thin projections of the BL (arrowhead) arising from the thick BL (arrow). Bottom inset: $3 \times$ magnification of a zone showing the spreading out of the BL in the BV periphery. One can distinguish the first perivascular BL (covering the Macs, arrowhead) from the BL projections (double arrowhead) enclosing processes of parenchymal cells. (B) A $5.5 \times 7 \mu m$ capillary showing the nucleus of a Mac and a portion of another Mac containing a large lysosome (lys). Both cells are covered by the perivascular BL (arrows). (C) A 4 \times 6 µm capillary showing no perivascular cell. (D) Location of the images A, B, and C. Abbreviations: CC, corpus callosum; CPu, caudate putamen; Ep, Ependyma; LV, lateral ventricle. Scale bars = 5 µm in A, 1 µm in B and C, and 50 µm in D. Source: From Ref. 2 and Fredric Mercier, who kindly gave his permission to incorporate this figure in the current review.



Perivascular macrophage (PVM)

Figure 2 A schematic drawing of the spatial interrelationships between the various structures in and around the blood vessel wall.

against CD163 (rat: mAb ED2 (7), M. Polfliet, manuscript in preparation. human: mAb EDhu1) (17), manuscript in preparation), which is not expressed by any other cell in the CNS. In the mouse and human CNS, the mannose receptor is specific for PVM (17,18).

Furthermore, PVM have higher levels of major histocompatibility complexes (MHC) I and II than microglial cells (6) and are CD45^{high} [leukocyte common antigen (LCA)] whereas microglial cells are CD45^{low}. This condition appears to be the case in both macaques (19) and rodents (20). In macaques, CD14 is only expressed by PVM and not microglia and CD14 has been used to distinguish between these two cell populations in simian immunodeficiency virus (SIV) encephalitis (19). However, in humans CD14 and CD45 are not discriminatory markers in pathology such as human immunodeficiency virus (HIV)-1 encephalitis (21). Moreover, in the latter study, CD45 was not specific for PVM in normal brains (21).

Pericytes can be distinguished from PVM by the presence of smooth muscle actin (SMA) (22) and their lack of macrophage markers. Unlike PVM, pericytes express receptors for vasoactive mediators like catecholamines, endothelin-1, vasoactive intestinal peptide, vasopressin, and angiotensin II, which suggests that pericytes are involved in cerebrovascular tone and blood flow regulation (13).

Apart from their morphology and expression of cellular markers, the PVMs phagocytic function can be used as a marker since PVMs are the only constitutively phagocytic cells in the absence of inflammation. This



Figure 3 Normal human brain cerebral white matter, (A, B). Immunohistochemistry using the immunoperoxidase technique with MAb EDhul against CD163. (A) Frozen tissue section. (B) Paraffin embedded section. The perivascular macrophages are EDhul positive and have an elongated shape around the small vessels and around medium sized blood vessels in the meningeal protrusions in the white matter (objective $20 \times$). (C–E) Double immunofluorescent histochemistry for CD163 and laminin (C), CD68 (KP1; D), smooth muscle actin (SMA; E) and CD45.

characteristic may explain the fact that not all PVMs express conventional markers such as CD163, which would lead to an underestimation of their actual number. Various substrates have been used to prove this phenomena including intrastriatally injected Indian ink (11) and including

	manner					
	PVM	Microglia resting/Active	Macrophages	Pericytes	Astrocytes	References
Macrophage						
CD163	+++++	Human –/+ rat–	Ι	Ι	I	7 ^a
CR3	+	++/+	++	Ι	Ι	43,114
FcR	+	+/-	+	Ι	Ι	44
CD68	+++++	+/-	+++	I	I	7,43,114
MR	+++++	1	Ι	I	Ι	Galea et al., in press
APC						
MHC I	++	+/-	+	I	I	115
MHC II	++	+/-	++	Ι	Ι	12, 43, 57, 60, 115
DC SIGN	q++	. 1	+	I	I	116^{a}
CD80	م +	1	+	I	+/-	$61,117^{a}$
CD86	م +	1	+	I	- (+)c	$61,117^{a}$
CD40	q++	1	+	I	- (+)c	118^{a}
LCA	++	+	++	Ι	1	43
Microglia						
Griffonia		+++/+++	++	Ι	I	119,120
Symplicifolia						
RCA		+++/+++	++	+/-	+/-	114
Pericytes						
SMA	I	I	I	++	I	20
Astrocytes						
GFAP	I	1	I	I	++	121

Table 1 Markers to Distinguish Between PVM and Other Brain Cells

^aPersonal observation. ^bA subpopulation of the perivascular macrophages is positive for this marker. ^cReactive astrocytes.

intracerebroventricularly injected horseradish peroxidase (HRP) (23), fluorescent dextrans (24), Fluorogold (25), the green fluorescent cell tracker CFDA (24), DiI-labeled liposomes (26) and fluorescent microspheres (own unpublished observation). A drawback of the latter method is that it cannot be used on human specimens to identify PVM.

2. PHENOTYPE AND FUNCTION OF PVM IN THE HEALTHY CNS

There are few studies that identify the role of PVM in the CNS. Their function has been largely inferred from their surface molecule expression in descriptive studies. Although it is fair to assume that an existing marker equates to a particular known function, this assumption has cause for concerns. This transfer of function does not take into account possible unknown functions of the molecule in question or its interdependence, cooperation or inhibition with other molecules. Moreover, this approach is unable to accurately define the extent of the PVMs contribution to the function in question or whether the function is redundant.

On the other hand, functional studies address these issues directly by manipulating the PVM population selectively. Several tools have been used to this end, including bone marrow chimerism and clodronate liposomes. Bone marrow chimerism capitalizes on the rapid turnover of PVM compared to microglia resulting in a selective replacement of PVM with donor bone marrow derived cells (4,15,27,28). Clodronate liposomes are injected intracerebroventricularly and are only phagocytosed by PVM resulting in their selective depletion via apoptosis (26). Scavenger, chemotactic, and antigen presenting functions of PVM have been probed in this way, as shall be discussed below.

2.1. Pathogen Recognition

The PVM express a multitude of receptors for pathogen-associated molecular patterns (PAMP) as well as for complement and IgG. Therefore, they form an integral part of the innate immune defenses at the BBB. Such receptors enable the recognition and uptake of blood-borne or invading foreign particles as a result of ligation of PAMP or opsonizing complement and IgG.

Both human and rodent PVM express the scavenger receptor (SR) A types I and II (29,30). These receptors are known to bind Lipid A, lipoteichoic acid (LTA) and CpG DNA, which can result in the internalization of both Gram-positive and Gram-negative bacteria (31). In SR A I/II knockout mice, PVM have been shown to have fewer lysosomal inclusions, indicating that these SRs are constitutionally active (32).
Our group recently showed that PVM in the human CNS express DC-SIGN [dendritic cell-specific ICAM (intracellular adhesion molecule)-3 grabbing nonintegrin] (17). DC-SIGN is a C-type lectin present on dendritic cells and involved in antigen capture as well as in T-cell synapse formation (31). The DC-SIGN recognizes high mannose ligands on various pathogens, and may be responsible for scavenging pathogens that have penetrated the perivascular space in the CNS. More importantly, DC-SIGN acts as a receptor for HIV (34,35) and studies have shown that perivascular cells in the brain are the primary infection points of HIV (19).

Another important receptor in the recognition of pathogens and has recently been described in the murine CNS is the mannose receptor (MR) (18). The MR recognizes branched mannose containing carbohydrate structures on microbial antigens such as *Mycobacterium tuberculosis, Candida albicans, Pneumocystis carinii, Klebsiella pneumoniae,* and *Streptococcus pneumoniae*. This recognition leads to receptor-mediated phagocytosis and enhanced microbicidal activity (36). Interestingly most of these pathogens are known to infect the CNS. Indeed PVM depletion using clodronate liposomes in a *S. pneumoniae* model of meningitis in the rat resulted in worsening of symptoms (37).

The CD14 is expressed by PVM in humans (21) and possibly constitutively expressed by PVM in the rat (38), although the latter study used morphology and OX-42 immunoreactivity [i.e., complement receptor (CR) 3 positivity] to identify PVM. CD14 is known to bind lipopolysaccharide (LPS) complexed with LPS-binding protein. It is a glycosylphosphatidylinositol-anchored cell surface protein with no intracellular signaling domain but serves to concentrate and present LPS to toll-like receptor 4 (TLR4). There are few studies studying the detailed in situ cellular expression of toll-like receptors in the brain. In the rat, TLR4 gene expression was shown to occur constitutively in circumventricular organs and some parenchymal structures but no colocalization with CD14 was observed, probably due to low levels of transcript (39). Although TLR4 expression should theoretically parallel CD14, this does not seem to be the case, since CD14 positive areas were TLR4 negative especially after endotoxin challenge (37). The TLR4 was not immunocytochemically detectable in healthy human brain but was evident in all active multiple sclerosis (MS) lesions, especially in perivascular areas (40). Colocalization studies were not performed in this case. In this study, expression of TLR3, which recognizes double stranded RNA paralled the expression of TLR4. The TLR2, which is the PAMP receptor for lipoteichoic acid and peptidoglycan from Gram-positive bacteria, is expressed in the brain but has not been properly localized on a cellular basis (41). Furthermore, rodent, and human PVM express CD18/CD11b (Mac-1, CR3) and CD18/CD11c of the B2 integrin family, which act as receptors for complement fragments (iC3b), CD54 (ICAM-1), fibrinogen, bacterial LPS and

altered proteins (42). Human PVM also display FcR enabling them to recognize IgG coated targets (43).

2.3. Antigen Presentation

The presentation of antigens by PVM is of great importance since the normal healthy brain parenchyma lacks other antigen-presenting cells. These cells can initiate, amplify or regulate immune responses within the CNS. Microglial cells are drastically down-regulated in the CNS parenchyma and hardly express MHC molecules under normal conditions (4,44). Small numbers of dendritic cells in the healthy CNS are found in the meninges, in the choroid plexus and occasionally in the perivascular space at the level of the BBB (45,46) (see Chapter 10).

The location of the PVM at the BBB, together with its expression of MHC class II in several species (1,12,40,42,45) and of several costimulatory molecules like CD40 and B7-2 (at least in humans) (17,46), supports an antigen presenting role of these cells. Moreover, PVM have been shown to contact T cells in both rat (23) and human brain (17). Overall, this indicates that PVM may play an important role in the recognition of pathogens and degradation products transported through the bloodstream and therefore, form the first line of defense of the brain once the endothelial integrity of the barrier is damaged or circumvented (25).

There is functional evidence that PVM present brain-derived antigen to T cells that have been activated peripherally. Experimental autoimmune encephalomyelitis (EAE) developed normally in rat radiation bone marrow chimeras where the only potential antigen-presenting cells in the brain expressing the appropriate MHC molecules were PVM (4). The disease in chimeras was clinically and neuropathologically identical to that of non-chimeric controls (49) suggesting a functional role in antigen presentation by the PVM. Moreover, when rat brain macrophages were obtained in vitro and sorted into CD45^{high} (bone-marrow derived macrophages including PVM) and CD45^{low} (microglia) populations using flow cytometry, the CD45^{high} population was the only one cell subset capable of stimulating myelin basic protein (MBP) -specific T cells to proliferate and secrete interleukin (IL)-2 (20).

2.4. Antigen Transport

As previously noted, PVM are professional scavengers of the perivascular space in the CNS. It is still unknown whether PVM can transport antigens to draining lymphoid organs. In peripheral tissues the transport of antigens to draining lymphoid organs is performed by dendritic cells, which acquire their antigen in the target tissue. They then leave the tissue, especially if inflammation is present, and travel through the afferent lymph, where they

are present as veiled cells to finally reach the draining lymphoid organs. The activation of naïve T lymphocytes in the lymph nodes may result in an antigen specific immune response or in an antigen specific tolerance induction (50,51). It has been proposed that this mechanism also applies to brainderived antigens, and that these antigens drain into the cervical lymph nodes (CLN) and are presented to recirculating lymphocytes causing an induction of T cells to target the brain (52).

When CLN were removed from rats with EAE, this resulted in a reduced load of cerebral disease by 40%, possibly due to a lack of T-cell priming in the CLN (52). Furthermore, by injecting the soluble antigen albumin in the gray matter of the rat brain resulted in antibody formation in the CLN (53). These studies all suggest that the CLN play a key role in lymphocyte-mediated immune reactions in the brain. A study performed in the CLN of monkeys with EAE and in post-mortem CLN of MS patients showed the presence of myelin antigens in cells expressing dendritic cell and macrophage markers as well as costimulatory molecules in the CLN (54). These findings are in agreement with a study performed by our group in MS patients in which in vivo ultrasound guided fine needle aspiration of the CLN was performed. Myelin antigens were found within macrophages in these lymph nodes (55). Brain-derived antigens may potentially drain into the cervical lymph vessels either in cell-bound/intracellular form or extracellularly by bulk flow to be taken up by dendritic cells or macrophages inside the CLN.

Possible candidates for the cellular transport of antigens out of the brain are infiltrating monocytes or dendritic cells, microglia, and dendritic cells from the meninges or choroids plexus (56,57) as well as PVM. The latter might travel via the perivascular space towards draining lymph vessels and then to the draining lymph node. However, this assertion is merely speculative.

Additional data on the MR support the notion that PVM are involved in the movement of antigen and may provide important information on the role of PVM in antigen transport. Ligand binding sites for the cystein-rich domain of the MR have been detected in developing germinal centers in the spleen, and have been localized on dendritic-like cells migrating from the subcapsular area of lymph nodes into follicular areas (58). Therefore, it has been suggested that the MR directs antigen (bound by the carbohydrate recognition domains) towards sites of developing clonal immune responses (by the cystein-rich domain). A fully functional soluble form of MR is generated by shedding of cellular MR by metalloprotease activity (59) and a similar antigen transport potential has been suggested for soluble MR. Such a scenario is possible during CNS inflammation since interstitial fluid from the perivascular space is known to drain directly into nasal lymphatics and thus into CLN through channels in the cribriform plate (60). Functional studies investigating PVM mediated antigen drainage are lacking. Kida et al. (11) have observed that PVM in healthy rats labeled with intrastriatally injected Indian ink can persist for years but this does not necessarily eliminate the possibility that PVM may exit from the brain. On the other hand, pathology seems to indicate that few PVMs can exit from the brain. Donor MHC I positive cells were observed in the lymph nodes and spleen of rats that received an allogeneic CNS graft in the forebrain and the rats underwent rejection (61). In the periphery, it is known that pathogen products such as LPS or cytokines such as IL-1 or TNF- α induce dendritic cell (DC) migration into the T-cell area of lymphoid organs (62,63).

2.5. Maintenance of the BBB

Macrophages in peripheral tissues are a heterogeneous cell population and can be activated via various routes, including classical activation routes induced by LPS or interferon (IFN) γ and alternative activation routes induced by IL-4 or IL-13 (64). The MR and CD163 expression in macrophages denote a state of "alternative activation" (64). This state is characterized by marked up-regulation of arginase-1 that results in proline production and gives the alternatively activated type 2 macrophages a pro-fibrotic predisposition (62). In normal physiology the production of ECM might contribute to maintenance of the BBB since the PVM is sandwiched between glial and endothelial basement membranes. Indeed, SR A knockout mice which have an abnormal number and phenotype of PVM are characterized by a thin, absent or discontinuous glial basement membrane (32).

2.6. Immune-to-Brain Signaling

Peripheral infection or inflammation results in a collection of symptoms known as sickness behavior, which includes fever, lethargy, anhedonia, hyperalgesia, reduced physical activity and social withdrawal. These symptoms are normal physiological responses and do not represent a diseased state of the CNS. Due to the presence of the BBB, mechanisms must be in place to allow the brain to sense circulating PAMP and cytokines. Numerous studies have shown that these mediators activate cells lying at key areas of the brain–immune interface (circumventricular organs, meninges, and BBB) initiating a wave of inflammation, which then penetrates the brain parenchyma, thus constituting a biphasic response. The first phase usually occurs at around 1 to 2 hours following the peripheral immune stimulus and then dwindles only to be followed by a second phase a few hours later. This has been shown through the use of different markers including c-fos mRNA (66), $I-\kappa B$ mRNA (67,68), IL-1 β mRNA (69), IL-1 β protein (70), tumour necrosis factor (TNF)- α mRNA (71,72), and CD14 mRNA (38). Normally, PVM are in a state of readiness to respond to inflammatory stimuli. They express the entire plethora of PAMP receptors discussed above as well as the type 1 IL-1 receptor (73). Once stimulated, they can secrete IL-1 β (16,74,75) and TNF- α (76) and express immunocytochemically detectable amounts of cyclo-oxygenase-2 (COX-2) (73,76) and inducible nitric oxide synthase (76). Cerebral endothelial cells, while capable of responding to inflammatory stimuli (75,76), are not as immunologically alert as PVM, which are the most constitutively immunophenotypically activated cells in the CNS (80). Therefore, PVM have the potential to amplify weak peripheral cytokine signals at the BBB. Using double immunocytochemistry, it has been shown that PVM up-regulate COX-2 as quickly as 30 minutes following intravenous IL-1 β injection in the rat, reaching a peak at 2 hours, and persisting as long as 4 to 6 hours post-injection (73,77). This response suggests that PVM activation bridges both phases of the innate immune response at the BBB and suggests an amplification role connecting the two phases.

3. PVM IN DISEASE

We feel it is important to distinguish between PVM in health and in disease. We have already noted situations in which phenotype and function of PVM differ depending on the presence or absence of inflammation, as it is the case for TLR3 and TLR4 expression. Moreover, the vast majority of surface molecules, which have been discussed, are up-regulated in disease (81).

3.1. Bacterial Meningitis

Perivascular and meningeal macrophages when selectively depleted in a rat model of pneumococcal meningitis resulted in a deterioration of clinical symptoms due to a diminished recruitment of polymorphonuclear cells to the meninges and a reduced elimination of pneumococci (37). This study indicates that PVM are involved in the recruitment of granulocytes during meningitis, though this was not due to decreased production of the predominant neutrophil chemokine MIP-2 (macrophage inflammatory protein-2). Disease worsening could also have occurred as a result of ineffective MR-dependent bacterial clearance (see above). Nevertheless, PVM are known to secrete chemokines such as MIP-1 α and RANTES (regulated upon activation, normal T cell expressed and secreted) under pathological conditions (23,82). Functional studies of PVMs chemotactic potential are however lacking.

3.2. Autoimmune Disease

EAE is an autoimmune inflammatory disorder of the CNS commonly used as a model of MS. When acute EAE is induced in the Lewis rat by

immunization with MBP in complete Freund's adjuvant, this results in showed perivascular inflammation in the CNS, characterized by mononuclear cell infiltrates consisting of T lymphocytes and macrophages. The animals also develop transient neurological deficits around day 10 after immunization (83). However, a robust increase in ED2 expression following immunization occurs before any cellular infiltration or clinical signs of EAE (83). Interestingly, it has been shown that EAE in mice is similarly preceded by a behavioral syndrome (84), which is in support of an immune-to-brain signaling role for PVM.

Experiments performed in our lab showed that after the selective depletion of PVM and meningeal macrophages in EAE, the initial phase of the disease was suppressed (83). This might suggest (1) that there is an important early chemotactic role of PVM and (2) that once activated T cells have migrated to the perivascular space, there is self-perpetuating chemo-kine secretion (85), which over-rides any contribution from PVM. However, it is clear from this functional study that the PVMs chemotactic role is largely redundant. Another mechanism by which PVM contribute to cellular infiltration is through their early expression of IL-1 during EAE, which is known to up-regulate ICAM-1 on the endothelial cells of the BBB (83).

We also observed an increased number of CD163 positive macrophages in MS brains similar to our results from EAE experiments (17). Moreover, PVM up-regulated the expression of costimulatory molecules CD40, CD80, CD86, and the antigen recognition molecule DC-SIGN (17). Both in vivo animal and post-mortem human studies therefore point towards a significant role of PVM in CNS autoimmunity.

3.3. Retroviral Encephalitis

The HIV encephalitis is clinically identified as HIV-associated dementia. Given the presence of the BBB, the entry of HIV into the CNS must involve an alternative route. In SIV encephalitis, it has been shown that PVM are the primary cell type that is successfully infected (19). Indeed, PVM express a variety of surface molecules, which are used as receptors by HIV/SIV to gain entry into the cell. These include CD4, several chemokine receptors (CCR3, CCR5, and CXCR4) (86) and DC-SIGN (34,35). Moreover, there is recent circumstantial evidence that the physiological replenishment of PVM by circulating monocytes, which is augmented in inflammation, might be a mechanism behind primary CNS infection by the virus (87). Thus HIV-infected bone marrow derived monocytes probably act as "Trojan horses" that traffic HIV particles from the peripheral blood to the CNS.

3.4. Lysosomal Storage Disorders

The G_{M2} gangliosidoses, such as Tay-Sachs disease, are lysosomal storage disorders characterized by the intracellular accumulation of the ganglioside

 G_{M2} and related glycosphingolipids in the CNS (88). Clinical symptoms include epilepsy, motor/cerebellar abnormalities, and cognitive dysfunction. These diseases are caused by inherited deficiencies in lysosomal β -hexosaminidase, which results in the accumulation of neuronal inclusions finally leading to neurodegeneration. In a mouse model of Tay-Sachs disease, PVM have been shown to contain microcorpuscular material morphologically identical to that seen in neurons (89). This suggests that PVM might be directly involved in processing secreted material or dying neurons.

4. CONCLUSION

There is reasonable circumstantial evidence suggesting that PVM represent an important immunological player at the BBB. However, solid experimental evidence proving the role of PVMs in CNS inflammation is often limited. The recent identification of markers that are specific for this cell population and the constant development of methods to study their functional role in vivo in animals open new perspectives for research on this very interesting cell population.

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Chemokines at the Blood–Brain Barrier

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1. INTRODUCTION

Circulating leukocytes cross an endothelium in a multi-step, reversible process that involves sequential interaction of selectins, integrins, and chemokines. In the first step, interactions between selectins on the surface of the endothelium with carbohydrate counter receptors on leukocytes initiate a "tethering" of cells along the endothelium within the shear forces of the bloodstream, resulting in dramatic slowing and rolling along the endothelium. Integrins, located on the surface of leukocytes, are next very rapidly activated by chemokines, a process that requires signaling through Gai-linked receptors. The activation of integrins allows for the firm arrest of leukocytes through interactions with cell adhesion molecules (CAMs) on the endothelium. Once arrested, leukocytes can penetrate the endothelial lining and travel into tissue via diapadesis. During this process leukocytes are directed by chemoattractant gradients to migrate across the endothelium and through the basal lamina and extracellular matrix into tissue (1).

Chemokine-driven migration was long believed to occur through a soluble gradient, however this idea seems unlikely considering that leukocytes must encounter chemokines while in the bloodstream, where a soluble gradient could not be easily established or maintained. In addition, soluble chemokine gradients would activate leukocytes in circulation before initial selectin-mediated interactions with endothelial cells, resulting in reduced ability of leukocytes to initiate endothelial cell adhesion and migration (2). For this reason, researchers began to look for alternative mechanisms that would support the interaction of chemokines with leukocytes in circulation. These possible mechanisms have come from endothelial cells themselves, which in the last decade have proven to be highly instrumental in the transendothelial migration of leukocytes across their barrier, rather than being a passive barrier as previously believed. Direct evidence comes from in vitro where transendothelial migration is eliminated when endothelial cells are fixed with paraformaldehyde, indicating that both transmigrating leukocytes and the endothelial cells of the endothelium are needed for migration to occur. Two further observations, namely the in situ binding of CXCL8 to human skin endothelium (3) and the ability of immobilized CXCL8 to attract leukocytes in vitro (4), led to the suggestion that chemokines are immobilized on the surface of endothelial cells, where they are presented to leukocytes.

But the question remains: how do chemokines get from the abluminal side of the endothelium, where they are produced by cells within tissue, to the luminal side of the endothelial cells, where they can be immobilized and presented to leukocytes. Recently, the non-signaling chemokine-binding "interceptors" have been implicated as having an active role in chemokine transport from the abluminal side of the endothelium to the luminal side. Together with charged glycosaminoglycans (GAGs), these molecules may present chemokines on the surface of endothelial cells and therefore mediate chemokine-driven transendothelial migration. To date there is strong evident to support this idea, gathered mainly in studies performed with cutaneous tissue models and associated vascular endothelium. Here we will review what is currently known about mechanisms of chemokine transport and presentation in vascular endothelium as a stimulus to addressing these issues in the context of the endothelium of the blood-brain barrier (BBB). The goal of this chapter is to highlight the unique nature of this specialized barrier and to direct future efforts to complete its story.

2. CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines are small, soluble proteins (6-10 kDa) that have been implicated in several physiologic processes including immune cell differentiation, leukocyte trafficking, and regulation of inflammation. Their best characterized function is that of trafficking immune cells, and they have been implicated in numerous homeostatic and inflammation models of leukocyte recruitment. These phenomena have been recently and adequately reviewed elsewhere, and will not be a primary focus of this chapter (5–7).

There are over 40 chemokines identified in mammalian species such as humans and mice, and although they exhibit relatively low sequence homology, they retain a highly conserved tertiary structure (8,9). There are four chemokine subfamilies categorized based on their number of amino acids located between the first two, of four, cysteine residues. Two disulfide bonds are formed between these four cysteine residues, one between the first and third residue and another between the second and fourth, which account for the structural homology. The first subfamily, termed CC or β chemokines, contain two adjacent cysteine residues, while the second subfamily, named CXC or α chemokines, are separated by a single amino acid between their first two cysteine residues. The structural differences between these two main chemokine subfamilies impart functional disparity. The CC chemokines are chemotactic to mononuclear leukocytes, including monocytes and lymphocytes, as well as eosinophilic and basophilic granulocytes. The CXC chemokines are divided into two groups. The first contains an ELR domain (glutamic acid-leucine-arginine) and are chemotactic for neutrophils, while the second group lacks this ELR domain and are chemotactic for lymphocytes. The final two chemokine subfamilies are C and CX₃C, which contain one cysteine residue or two separated by three amino acids, respectively.

Chemokines work through specific heptahelical G-protein-coupled receptors that exhibit distinct chemokine affinity and cell expression profiles, thus enabling an enormous diversity of interactions (Table 1). There are four corresponding receptor subfamilies for the chemokine subfamilies, termed CCR, CXCR, CR, and CX₃CR. To date there are 10 CC receptors and six CXC receptors identified, and only one each of CR and CX₃CR. Many chemokines can interact with more than one chemokine, however, this is not true of all of them. Chemokine promiscuity is almost always limited to the same subfamily of receptors (Table 1).

3. CHEMOKINE PRESENTATION ON ENDOTHELIAL CELLS

Increasing evidence suggests that endothelial cells actively transcytose chemokines from the abluminal side of the endothelium to the luminal side, where they are presented to leukocytes (Fig. 1). Using immuno-electron microscopy, Middleton et al. (10) showed that labeled CXCL8 and CCL5 bound the abluminal side of endothelium and were transcytosed to the luminal side within 30 minutes of being injected into human or rabbit skin. This transcytosis was not specific since either chemokine was able to displace the other. They further showed that the transcytosed CXCL8 was presented to neutrophils on endothelial cells microvillous processes, contributing to increased neutrophil migration. Chemokine transcytosis is not limited to inflammatory chemokines, as it was also seen with CCL19 in the HEV of lymphoid tissue (11). Chemokines immobilized and presented on the endothelial cell surface in this manner contribute to haptotactic gradients of extracellular matrix components, adhesion molecules and chemoattracts,

Receptor	Cell expression	Chemokine
CXCR1	M, N	CXCL 6,8
CXCR2	M, Eos, N	CXCL 1,2,3,5,6,7,8
CXCR3	BL, TL	CXCL 9,10,11
CXCR4	Thym, BL, TL, imDC, M, N, pl	CXCL 12
CXCR5	BL, TL	CXCL 13
CXCR6	TL	CXCL 16 ^a
CCR1	NK, TL, iDC, M, N, B, Eos	CCL 3,5,7,8,13,14,15,16,23
CCR2	NK, BL, TL, M, N	CCL 2,7,8,13
CCR3	TL, M, Eos	CCL 5,7,8,11,13,15,24,26,28
CCR4	Thym, NK, TL, iDC	CCL 17,22
CCR5	Thym, BL, TL, imDC, M	CCL 3,4,5,8
CCR6	BL, TL, iDC	CCL 20
CCR7	BL, TL, mDC	CCL 19,21
CCR8	Thym, BL, TL, M	CCL 1
CCR9	Thym, TL	CCL 25
CCR10	TL	CCL 27,28
XCR1	NK, TL	XCL 1,2
CX ₃ CR1	NK, TL, M	CX ₃ CL 1 ^a
Duffy	rbc, EC	CXCL 1,8,9; CCL 2,5
D6	TL	CCL 3,5,7,8,11,13,14

 Table 1
 Chemokine Receptor Expression and Affinity for Chemokines

^aDenotes chemokines that can be produced as membrane bound proteins (8,16). *Abbreviations*: M- macrophage; N- neutrophil; BL- B lymphocyte; TL- T lymphocyte; Thymthymocyte; Eos- eosinophil; Pl- platelet; iDC- immature dendritic cell; mDC- mature dendritic

cell; B- basophil; NK- natural killer cell; EC- endothelial cell; rbc- erythrocytes

facilitating leukocyte interaction and migration (12). There appears to be more than one mechanism to immobilize chemokines on the surface of endothelial cells, of which some mechanisms are specific and others being less so.

3.1. Membrane-Bound Chemokines

Although most chemokines exist primarily in a soluble, secreted form, two (CX₃CL1 and CXCL16), at least, also exist as membrane-bound proteins. Fractalkine/CX₃CL1 is unique in that it can function as either a chemoat-tractant or an adhesion molecule. It is expressed predominantly by epithelial cells, and within the CNS it is expressed on neurons (13). The CX₃CL1 may also be expressed by endothelial cells upon activation by pro-inflammatory cytokines such as IFN- γ and TNF- α . The CX₃CL1 can be released by ADAM-mediated proteolysis. Soluble CX₃CL1 is chemotactic to lymphocytes, NK cells, and macrophages, while membrane-bound CX₃CL1 is credited with capturing and enhancing migration of these cells upon



Figure 1 Three non-mutually exclusive mechanisms for chemokines to be presented on the surface of endothelial cells. *Left*: Type-I membrane glycoproteins with N-terminal chemokine domains, such as CX_3CL1 and CXCL16. *Middle*: Chemokines are taken up abluminally by unknown mechanisms and transported to the luminal side where they bind to GAGs on the surface of the endothelial cells. *Right*: Interceptors bind chemokines abluminally, transport them via calveolae, and present them on the luminal surface of endothelial cells.

secondary stimulation with additional chemotactic factors. The corresponding receptor, CX_3CR1 , is expressed on cytotoxic effector lymphocytes and NK cells. An advantage afforded to this chemokine is the self-reliance in establishing a haptotactic gradient, since the chemokine, itself, may be displayed on the endothelial cell surface (14,15).

The CXCL16 is also unique in that it has attributes of two chemokine subfamilies. It is a CXC chemokine, however it also contains a transmembrane domain and a mucin-like stalk, both of which enable CXCL16 to be specifically localized to the cell surface. The CXCL16 is expressed on the surface of antigen presenting cells (B lymphocytes, macrophages and dendritic cells) and cells in the splenic red pulp. Functional CXCL16 is expressed on memory and activated effector T lymphocytes. Although the biological role of CXCL16 and CXCR6 is not known, it is speculated that they are functional in thymocyte development as well as T-cell recruitment during inflammation (16).

3.2. Chemokine Immobilization on Glycosaminoglycans

One well-documented mechanism that allows chemokines to be immobilized on the endothelial cell surface is via binding to GAGs. The ability of chemokines to elicit transmigration when immobilized on the endothelial cell surface via GAGs is a decade-old paradigm, and has been supported experimentally (10.17). The GAGs are polysaccharides with a high negative charge that are located on the surface of cells and in the extracellular matrix, usually associated with proteins to form proteoglycans. The most abundant GAGs on the cell surface are heparan sulfate; the highly sulfated heparan sulfate heparin; chondroitan sulfate; dermatan sulfate; keratin sulfate; and hvaluronic acids (18). Because chemokines are basic molecules, they interact electrostatically with GAGs, which exhibit an overall negative charge due to the presence of carboxylate and sulfate groups. The interaction between heparan sulfate and CCL2 was recently demonstrated in a transwell chemotaxis assay when CCL2, applied apically or basally in the transwell system, was co-localized with heparan sulfate into distinct regions on the apical surface of endothelial cells under conditions that induced strong migratory response of monocytes (19).

The primary proteoglycan on the surface of endothelial cells is heparan sulfate, which comprises 50–90% of total endothelial cell proteoglycans (20). However, there is much variation in GAG composition and GAG binding to chemokines on endothelial cells of different origins (21), which may lend selectivity in chemokine presentation and therefore leukocyte recruitment. A sub fraction of heparan sulfate, for example, was shown by affinity co-electrophoresis to preferentially bind CXCL8 and CXCL1 but not CXCL4 or CXCL7 (22,23). The CCL5, CCL2, CCL8, and CCL3, respectively, exhibit decreasing affinity for heparin (24,25). Individual chemokines also exhibit diverse affinities for different GAGs. The CCL2 and CXCL8 bind heparin > heparan sulfate > chondroitan sulfate = dermatan sulfate, contrasting to CCL5, which binds GAGs in the order of heparin > dermatan sulfate >heparan sulfate > chondroitan sulfate (23,24). Thus, the story cannot end here, as there is too much variety to attribute chemokine-driven migration simply to GAG-chemokine interaction. To further complicate matters, some chemokines, such as CCL4, appear not to require GAG binding to mediate transmigration (26). Rather, interaction with GAGs serves to enhance the activity of chemokines at low concentrations (27).

Some of the differential activity seen with different chemokine–GAG interactions may stem from the varied binding conformations which are only recently being examined using specifically modified chemokines (Table 2). There is some evidence that chemokines must oligomerize to bind GAGs and mediate chemotactic activity in vivo (17). When GAG-binding domains of CCL2, CCL5 or CCL4 were mutated so that heparin binding was diminished, the mutant chemokines were almost as effective as wild-type

Mutant	Chemokine	Mutant characterization	Effect on migration
⁴⁴ AANA ⁴⁷ RANTES	RANTES	Less binding to heparin than WT ^a	in vitro: active in vivo: inert
[Nme ⁷ Thr] RANTES	RANTES	Obligate monomer	in vivo: inert
[Ala ⁶⁶] RANTES	RANTES	Dimeric variant	in vivo: inert
[Ala ²⁶] RANTES	RANTES	Tetrameric variant	in vivo: active
¹⁸ AA ¹⁹ MCP-1	MCP-1	Less binding to heparin than WT	in vitro: active in vivo: inert
[Ala ⁸] MCP-1/P8A	MCP-1	Obligate monomer	in vivo: inert
K58A/H66A MCP-1	MCP-1	Less binding to heparin than WT	in vitro: active in vivo: inert
⁴⁵ AASA ⁴⁸ MIP-1β	MIP-1β	No heparin binding	in vitro: active in vivo: inert
[Ala ⁸] MIP-1β	MIP-1β	Obligate monomer	in vivo: inert

 Table 2.
 Migration Effects Mediated by GAG Binding Mutants of Chemokines

Various single and double mutants have been used to show a high correlation between chemokine ability to bind GAGs and form higher order oligomers with their ability to effect leukocyte recruitment in vivo. In vitro assays were performed using standard transwell assays; in vivo assays were performed as intra-peritoneal migration assays in mice (17,18,28). Dimeric and tetrameric variants are able to form dimers or tetramers, respectively, but not higher order oligomers in solution (17).

^aChemokine binding to heparin in vitro correlates with binding to extracellular matrix and other structures in vivo (17,74).

chemokines during in vitro chemotaxis. When, however, these same mutants were utilized in intra-peritoneal recruitment assays, they were unable to stimulate migration in vivo (17). Supporting the link between oligomerization and GAG-binding, an obligate monomeric CCL2 mutant (P8A) exhibited identical behavior as a GAG-binding deficient mutant (28). Specifically, P8A is an agonist in vitro but a potent antagonist in vivo.

The GAG binding epitopes of CCL2 were further identified using a panel of surface alanine mutants in heparin-binding assays (Table 2; Ref. 18). In the presence of octasaccharide, wild-type CCL2 was shown to form tetramers that exhibited a continuous ring of GAG binding residues encircling the tetramer. Such an arrangement would be highly advantageous for GAG binding (18). The authors suggested that the tetramer forms the fundamental oligomeric form in the heparin-bound state, despite CCL2 existence as a dimer in solution (18). Together, these results suggest that both GAG binding and higher order oligomerization are necessary for chemokine function in vivo and further indicate the need to expand beyond the traditional in vitro chemotaxis assays to fully understand the complex effects of GAG binding on chemokine function and structure.

3.3. Chemokine Binding to Decoys and Interceptors

Although the role of GAGs in immobilizing and presenting chemokines to circulating leukocytes is strongly supported by these data, it is not understood how chemokines (which are usually produced by parenchymal cells) travel from the abluminal side of the endothelium to the luminal side, where they encounter leukocytes. Recently, two candidates have emerged as mediators of chemokine transcytosis and, possibly, presentation on endothelial cells. The D6 and Duffy/DARC (Duffy antigen receptor for chemokines) are able to bind chemokines but are not associated with GPCRs and so do not mediate cell signaling. These non-signaling receptors, named "interceptors," are proposed to regulate the distribution of chemokines across the endothelial cell surface and influence biological activity (29). This new hypothetical function rests on their ability to serve as chemokine sinks or scavengers, which accomplish the removal of extraneous chemokines from the blood stream.

The Duffy antigen, or Duffy antigen receptor for chemokines (DARC), was first identified on erythrocytes and is the site of entry for the malaria parasite Plasmodium vivax. In addition, DARC is expressed on endothelial cells of post capillary venules in kidney, lung, thyroid, and spleen (30,31). Duffy expression on post-capillary venules is retained in individuals whose erythrocyte-lineage cells are Duffy negative, the so called African allele, which may impart immunity to malaria (32,33). The DARC is able to bind CXCL8 and other CC and CXC chemokines, although not CCL3 (34), yet it does not initiate signaling. Structurally, DARC retains the seven transmembrane structure of G-protein linked chemokine receptors, however it lacks the DRY motif needed for GPCR association. Because DARC is able to internalize chemokines without signaling, it has been suggested that it may regulate the subsequent fate of chemokines, in particular whether they are transcytosed or degraded. Recently evidence that DARC may transport chemokines from the abluminal side of endothelial cells to the luminal side, where they can be presented to leukocytes, was shown by immuno-electron microscopy using CXCL8 in both human and rabbit skin by Middleton et al. (10). Radio-labeled CXCL8, when injected abluminally, was later detected in Duffy containing caveolae and collected on the luminal side of the endothelial cells within 30 minutes of injection (10,30). In a separate report, Duffy antigen transfected into human endothelial cells facilitated transcytosis of CXCL1 across the endothelial cell monolayer and stimulated enhanced neutrophil migration in vitro (35). The DARC was also preferentially localized to endothelial cell junctions, and may serve to limit diffusion and therefore loss of chemokines by pericellular routes (29). Instead, DARC may mediate internalization of chemokines and subsequent guidance to sites of chemokine presentation (29).

Establishing a role for DARC in chemokine transcytosis and presentation is not necessarily mutually exclusive of GAG-mediated presentation. Treatment of endothelial cells with heparanase abridged the ability to transcytose chemokines and affected the migration of neutrophils in the above mentioned human skin and CXL8 system (10). It seems likely, therefore, that the two mechanisms act together.

Specific evidence in support of DARC involvement in chemokine presentation comes from studies of genetically altered mice. Several groups have generated Duffy-antigen deficient mice, and collectively their results hint at a complex role for Duffy in chemokine regulation. In one study, Duffy deficient mice exhibited decreased recruitment of neutrophils to the lungs, gut, and peritoneal cavity after lipopolysaccharide (LPS) injection (36), suggesting that Duffy has a pro-inflammatory role. Conversely, an anti-inflammatory role for Duffy was proposed by a second group when LPS injection led to increased accumulation of neutrophils in the lungs and hepatic sinusoids (37). Both groups used comparable doses of LPS, however their time of analysis differed greatly and may shed some light on their divergent results. The first group evaluated the mice 24 hr post-LPS injection, while the second assessed at 2 hr. The diversity of results seen between these two systems indicate that Duffy may play an important role in regulating chemokine kinetics in leukocyte recruitment. These seemingly conflicting results may be due to the absence of Duffy on erythrocytes and may reflect the ability of Duffy to act as a chemokine reservoir in addition to its postulated role as a chemokine sink (29). Evidence for this comes from the observation that injected chemokines are depleted from plasma in Duffy-antigen deficient mice, but not in wild-type controls (38). This observation is consistent with those seen in humans where Duffy-antigen deficiency is associated with decreased levels of plasma CCL2 (39). Nibbs et al. (29) have proposed that Duffy may therefore act as a "buffer" which can minimize chemokine plasma concentrations in acute inflammation while maintaining them in chronic inflammation. Although none of the above studies examined Duffy-antigen deficiency on endothelial cells, one other report suggests that mice that lack Duffy antigen on endothelial cells but retain it on erythrocytes exhibit impaired neutrophil recruitment into the peritoneal cavity upon chemokine injection when compared to wild-type controls (29). These findings suggest that Duffy is able to act as an accessory molecule in the in vivo recruitment of neutrophils.

The interceptor D6 is very similar in structure to GPCRs, although it lacks the DYR motif, and instead has a DKY motif that prevents it from being able to signal through G proteins. The D6 functions as a CC chemokine scavenger (40). The D6 binds to at least nine inflammatory CC chemokines (29), and mediates internalization and degradation of CCL2 (41). The D6 is expressed by endothelial cells lining the afferent lymphatic venules (ALV) and other lymphatic channels (42), however notably not by vascular ECs. Because of its ability to bind chemokines and its convenient location around ALVs, it has been suggested that D6 may influence the inflammatory CC chemokine content of draining lymph nodes (29,43). Very recently, it was shown that D6 functions to clear β -chemokines from inflammed skin, thereby contributing to the resolution of inflammation and minimizing the recruitment of inflammatory cells that can lead to aberrant pathology (74).

Another non-signaling chemokine receptor identified to date is CCX-CKR. Initially, this receptor was identified by two separate groups that called it CCR10 (44) and CCR11 (45). However, neither of these preliminary designations was subsequently confirmed since the receptor did not demonstrate signaling capability based on chemotaxis assays and Ca^{2+} flux. The CCX-CKR binds CCL19, CCL21, and CCL25, and its closest relatives are CCR6, CCR7, and CCR9. Along with D6 and DARC this receptor is proposed to act as a non-signaling decoy in mammalian systems, and it will be interesting to monitor this receptor as its role in the organism is revealed (46).

4. SPECIAL CHALLENGES OF ENTERING THE CNS

The multi-step paradigm of leukocyte extravasation likely is applicable to migration across the BBB, although far less is known about the process at this specialized barrier. The BBB, located along the central nervous system (CNS) capillary endothelium, refers to specialized endothelial cells, which provide both anatomical and physiologic protection to the CNS by limiting the passage of substances and cells into and out of the brain parenchyma. Two characteristics of the BBB impart the effectiveness of this barrier: tight junctions between cells that prohibit paracellular transport, and a relative lack of endocytic vesicles in brain microvascular endothelial cells (BMECs), which preclude transcellular transport (47,48). The physiologic significance of this barrier is evident considering the relative intolerance of the CNS to inflammation. The CNS lacks both antigen presenting cells and structural lymphatics in the brain parenchyma. Furthermore, since both the brain and spinal cord are enclosed in bone, the anatomical nature of the CNS is not permissive for inflammation. Swelling caused by inflammation within the CNS would produce dangerous pressure on these non-regenerating tissues (49).

Despite these limitations, neuropathologies exist that clearly demonstrate robust response to inflammatory stimuli. Diseases as diverse as HIV-associated dementia and multiple sclerosis, as well as localized inflammation as seen with cortical injury and stroke, all demonstrate the accumulation of inflammatory leukocytes within the CNS. Therefore, pathways must exist that allow the entrance of leukocytes into the CNS. One such mechanism is transendothelial migration across the BBB. Studies over the past 20 years have shown that activated T lymphocytes cross the BBB independent of antigen specificity, although it remains uncertain whether the endothelium needs first to be activated as mimicked in experimental systems by LPS or TNF- α treatment (50,51).

5. CHEMOKINES OF THE CNS

The CNS, like many tissues in the body, has its own patterns of chemokine expression and receptor homing. Several chemokines have been implicated in mediating specific inflammatory homing to either the CSF or brain parenchyma. The CCL2, produced by astrocytes in adult murine models of cortical trauma, is necessary for the recruitment of monocytes to sites of injury (52). The CCL2 has also proven crucial for macrophage recruitment in murine experimental autoimmune encephalomyelitis (EAE), a model for autoimmune demyelination of the CNS. The CCL2 deficient mice develop effector T cells that transfer disease to naïve wild-type recipients despite being resistant to EAE themselves (53).

One feature of chemokines unique to the CNS is their requirement for non-immune functions (54). The CXCR4 and its chemokine CXCL12, both highly expressed in the early stages of the developing nervous system, are necessary for proper CNS development, particularly for neuronal precursor cell distribution within the cerebellum (54). The CXCR4 or CXCL12 knock out mice exhibit embryonic lethality due to numerous defects in organogenesis including significant alterations to the CNS (55,56).

The chemokine CXCL1, along with its receptor CXCR2, also contribute to CNS development. Oligodendrocytes, the cells responsible for myelin production in the CNS, originate as precursor cells that migrate to developing regions of the CNS through a complex process that is followed by subsequent proliferation. The CXCL1, expressed focally within the developing CNS, inhibits oligodendrocyte precursor cell migration via CXCR2, thereby promoting increased cell-substrate interactions and cell localization in the optimal environment for proliferation (57). Yet another chemokine, CX_3CL1 , is constitutively expressed by neurons and acts towards microglia through its receptor CX_3CR1 . The biological significance of this interaction, however, is not known (58,59).

In addition to the normal physiologic role of chemokines in inflammation and CNS development, chemokines have been implicated in a wide variety of CNS disease and injuries including multiple sclerosis (MS), Alzheimers, HIV-associate dementia, head trauma, and stroke. Current knowledge on these topics has been widely and adequately reviewed elsewhere, and so will not be discussed further here (5,6,52,60,61). Both chemokines and chemokine receptors are excellent potential drug targets, and so the enormous attention given to these proteins is well placed (60,62–64). A full understanding of "chemokine neurobiology" is necessary to understand how to minimize adverse effects and apply drugs appropriately.

6. CHEMOKINE PRESENTATION AT THE BBB

To date there is very little known about the ability of BMECs to transcytose chemokines or their mechanism, if any, of immobilizing chemokines on their surface. It is tempting to speculate that the mechanisms employed in the peripheral vascular endothelium are also utilized in the BBB. However, considering that a large portion of studies to date have looked at neutrophil migration, which are virtually non-existant in both brain and CSF infiltrates, these mechanisms may differ greatly in crossing the BBB.

One apparently unique mechanism of chemokine transcytosis in BMEC has been proposed to be mediated by specific chemokine receptors. Both CCL2 and CCL3 bound to high-affinity saturable binding sites along human brain microvessels and could be specifically inhibited by unlabeled cognate chemokine, so that CCL2 and CCL7, but not CCL3, inhibited CCL2 binding to vessels, and CCL3 but not CCL2 inhibited binding of radiolabeled CCL3 (65,66). The two chemokines displayed very different binding patterns: CCL2 demonstrated almost continuous binding along the abluminal surface (65), while CCL3 exhibited a very punctate distribution along the abluminal side (66). Binding was resistant to heparinase treatment, suggesting that it was not a GAG-mediated event. Therefore, although presentation of chemokines may be GAG-dependent at the luminal surface of endothelial cells, it appears in the BBB that at least some abluminal chemokine binding is mediated via the other mechanisms. Furthermore, when CCL2 binding to brain microvessels was compared between wild-type and CCR2-/- mice, CCL2 was shown to be internalized via non-clathrin-coated mechanisms in wild-type mice but not in CCR2-/- mice (67). The authors speculated that chemokines cross the BBB through high affinity specialized interaction of CCL2 with its receptor, CCR2 (68).

Although DARC is expressed by Purkinjie cells of the cerebellum (69), it is not reportedly expressed by BMECs. Nor is D6, although characterization of this receptor in the CNS is lacking. One group has reported an increase in Duffy antigen expression in MS lesion tissue as compared to normal white matter by cDNA microarray, although this analysis is thus far limited to one patient and the cellular location of Duffy alteration is not known (70). Thus the most striking observation characterizing potential mechanisms of chemokine transcytosis and presentation at the BBB is that, to date, there is relatively little. Therefore, extensive review of the literature serves to highlight the need for focused effort at clarifying this aspect of chemokine biology.

To this end, we have developed a model of the BBB using SV40 transformed human BMEC (THBMEC) generously provided by Monique Stins of Johns Hopkins University to examine transendothelial migration in vitro (71). When cultured for 4 to 7 days in 24 well transwell plates coated with collagen, THBMEC form a monolayer that exhibits high TEER, low permeability to fluoresceinated dextran, and the expression of occludin and ZO-1 at cell-cell interfaces, all of which are consistent with tight junction formation that verifies the successful recapitulation of the BBB microenvironment (72,73). Our THBMEC model also support the transmigration of peripheral blood mononuclear cells (PBMC) to yields sufficient for flow cytometric analysis of migrated populations. We have demonstrated elevated migration of PBMCs towards CCL2 and CCL3 when placed in the basal chamber of transwell plates in a pertussis-toxin sensitive manner. In this system, soluble CCL2 is necessary in the basal chamber, as incubation with THBMEC and CCL2 for 1 hour followed by removal of CCL2 during the course of a transmigration assay did not result in migration rates above basal levels. Therefore, in this system, transport of CCL2 across the THBMEC was not sufficient to drive migration. Although this model will undoubtedly prove useful in further examination of chemokine-presentation and binding, especially with respect to GAG binding and polarized chemokine binding sites, an eventual progression to in vivo models of chemokine interaction and leukocyte infiltration in the CNS is foreseen.

7. CONCLUSIONS

Although there has been considerable progress in elucidating the mechanisms of chemokine-presentation in directing leukocyte extravasation, much less is known of this process at the BBB. In non-BBB models, chemokines bind to GAGs on the luminal surface of endothelial cells in a manner dependent upon their oligomerization state and mutual, specific affinity between chemokines and GAGs. Several non-signaling chemokine receptors, termed interceptors, have been implicated in transporting chemokines from the abluminal to the luminal surface of endothelial cells, where they can be utilized by circulating leukocytes. However, none of these molecules or receptors have been characterized in BMECs. We have developed an in vitro BBB model that has great potential for characterizing the interaction of chemokines with BMEC of the BBB. This model will undoubtedly continue to prove valuable in extending what have hitherto been limited to descriptive observations of chemokines and chemokine receptors at the BBB. Future studies in the BBB will require the extrapolation of mechanisms characterized in other systems, with the consideration that unique processes may occur at this very specialized barrier.

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Matrix Metalloproteinases and Proteolytic Opening of the Blood–Brain Barrier in Neuroinflammation

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"Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are in more danger from them than from the intruders. We live in the midst of explosive devices; we are minded."

Lewis Thomas, "Germs", in Lives of a Cell, 1974.

1. INTRODUCTION

Cerebral blood vessels are damaged in a wide variety of neurological illnesses. When permeability of the blood vessels is altered, substances normally excluded from the brain are allowed to enter, causing brain edema. Severe disruption of the vasculature leads to hemorrhage. Both edema and hemorrhage are life threatening. Proteases and free radicals participate in the final common pathway that leads to blood vessel breakdown. Neutral proteases, such as the matrix metalloproteinases (MMPs) and the plasminogen/plasmin system interact with free radicals to cause damage to endothelial cells and the extracellular matrix and other cells surrounding the vessels. For the past several years our laboratory has been studying the MMPs, which are a gene family of neutral proteases that degrade all components of the extracellular matrix. They have been shown by other laboratories and by us to be important in normal development, wound healing, and pathological processes. An important role is to modulate blood-brain barrier (BBB) function during neuro-inflammation. The MMPs can be both produced endogenously by most brain cells and delivered to the brain packaged in leukocytes. They alter BBB permeability by attacking the extracellular matrix around blood vessels.

Collagenase was the first MMP discovered, and it was found to break down the collagen matrix in a tadpole's tail, allowing regrowth of a new one (1). More relevant to human disease, 72 kDa type-IV collagen was shown to be secreted by metastatic cancer cells, disrupting basement membranes, breaking down blood vessel walls, and facilitating metastases (2). The MMPs are tightly regulated; they are secreted as latent enzymes that require activation. Their action is opposed by tissue inhibitors to metalloproteinases (TIMPs) (3), and by synthetic compounds that interact with zinc in the catalytic site (4). There is now a large body of literature that describes the function of the MMPs in brain and has pointed the way to novel forms of therapy in several neuro-inflammatory conditions. Several recent review articles have detailed the broad area of MMP and TIMP function in the brain (5–8). This review will focus on the role of the MMPs in the function of the BBB.

The BBB is series of interfaces between brain tissue and blood, which maintains a stable neuronal environment. Earlier writers saw the intimate relationship of the blood vessels and glia cells and proposed that the astrocyte acted as a transducer to transmit information from the neuron to the vessels (9). These remarkable insights have been refined into more modern concepts that take into account the multiple cell types and the intervening matrix. The "neurovascular unit" refers to the endothelial cells, basal lamina, pericytes, and the astrocyte foot process. When the unit functions properly, there is limited transfer of cells and blood products into the brain, and metabolic products are removed. However, at times of stress, such as traumatic injury, stroke, infection, and autoimmune reactions, there is an increase in permeability, allowing white blood cells and blood products to enter the brain. The cellular response, leading to edema and hemorrhage, is called neuro-inflammation. Prior to antibiotics, an inflammatory response was protective in that it removed the invading organisms and initiated the repair process. During the secondary inflammatory response, there is an increase in proteases and free radicals, which are the final common pathway for cell death.

2. NEUROINFLAMMATION

Inflammation is a response to injury that causes the release of vasoactive substances with an alteration in the vascular permeability and the influx into tissues of leukocytes (10). The process begins with the release of vasoactive

tissue factors that are stored in cells surrounding the site of injury. Amplification of the process by cytokines, free radicals, and other acute phase reactants occurs as the immediate early genes are involved and cells are recruited to the site of the injury. Post-capillary venules are a site of predilection for the initiation of the inflammatory response. Once early vasoactive factors initiate the response, chemotactic factors recruit inflammatory cells, including the neutrophils and lymphocytes. Tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) are pro-inflammatory cytokines that induce downstream inflammatory mediators (11). Chemokines attract leukocytes to the site. Neutrophils are early responders, and release preformed enzymes, such as neutrophil collagenase (MMP-8) and gelatinase B (MMP-9), and free radicals, including hypochlorous acid, that attack membranes both directly and indirectly by activating the proteases (12). In the later stages of the inflammatory response, macrophages and lymphocytes are recruited to remove damaged tissue. During the process of repairing the injury, there is the danger that a chronic inflammatory process is initiated.

Brain inflammation has unique aspects (13). The response to an inflammatory stimulus in the brain is less severe in the early stages than in the peripherial tissues. Proteases are involved at all stages of the inflammatory process in the brain, and are important in the disruption of the BBB. Proteases involved are the MMPs and the plasmin/plasminogen activator system. During the inflammatory response, MMPs are secreted by leukocytes that have adhered to the vessel lumen, facilitating the entry of the inflammatory cells into the brain. Cell membranes and extracellular matrix are attacked by proteases. In addition to the exogenous production of MMPs that are infiltrating the brain, there are endogenously released MMPs, which are stimulated as part of the inflammation, contributing to the proteolytic load. An important, although poorly understood process is the collateral damage to the myelin-covered nerves by the released proteases, which results in secondary or "by-stander" demyelination (14). Besides the role of MMPs in tissue disassembly during the acute response, they are important in movement of cells through tissue toward the site of injury where they participate in the repair process.

The timing and extent of the inflammatory response varies, depending on the type of insult. In multiple sclerosis, for example, an initial change in the cerebral vessels initiates the cellular infiltration of T lymphocytes through the release of MMPs (15,16). On the contrary, an ischemic insult causes a delayed inflammatory response: hypoxia initiates a molecular cascade that includes the influx of neutrophils and the production of endogenous proteases with maximal inflammation from 24 to 72 hours after the insult (17). Cell death by necrosis and apoptosis is prominent in the ischemic core and in the penumbra.


Figure 1 Molecular injury cascade triggered by hypoxia/ischemia. Energy failure with a fall in ATP levels is an early event that leads to the production of glutamate and the influx of calcium. Immediate early genes are induced and the neuroinflammatory reaction is begun. Leukocytes infiltrate the tissues. Free radicals and proteases are produced. Proteases have multiple effects: caspases degrade DNA, leading to apoptosis, lysosomes break down membranes, and matrix metalloproteinases attack the extracellular matrix. The end result is autolysis of the tissues, blood-brain barrier opening with hemorrhage and edema, and cell death.

A molecular cascade is initiated by an insult to the tissues, such as a prolonged episode of ischemia/hypoxia, a significant brain trauma, or an infection that leads to tissue infarction. The loss of oxygen causes energy failure, release of the excitotoxin, glutamate, and the influx of calcium. Immediate early genes are activated by free radicals, hypoxia inducing factors, and elevated calcium. Cytokines induce the late effector genes, which produce the proteases that are involved in the final common pathway of damage. The proteases lead to self-digestion and cell death (Fig. 1) (17).

3. NEUROVASCULAR UNIT

Tight junctions are present between the cells that form the sites of the BBB. Several proteins have been identified in the tight junctions, such as occludins and the gene family of claudins (18). Astrocytes interact with the endothelial cells to induce the tight junctions (19). Tight junctions form the major feature of the endothelial barrier as demonstrated in early ultrastructual experiments with intravenous horseradish peroxidase (20). Other sites where tight junctions are found include the apical border of the choroid plexuses and the arachnoid (21). Astrocytes contribute to the permeability characteristics of the blood vessels. In lower organisms, the glial cells have tight junctions and perform the barrier function. Sharks and other species in the elasmobranch family have a glial barrier rather than an endothelial cell barrier (22).

Evidence supporting a role of the astrocyte in fluid and electrolyte balance has come from studies showing that the astrocyte is polarized with the cell body taking up potassium and the end feet releasing it into the extracellular space (23). The astrocyte end foot, which surrounds the blood vessels is rich in purinergic receptors, P2Y-2 and P2Y-4 and the gap junction protein, Cx43 (24). Pericytes are embedded in the basal lamina (Fig. 2). Astrocyte end feet on endothelial cells are covered with aquaporin-4 molecules, which show denser immunostaining than glial fibrillary acidic protein (GFAP), suggesting a critical role in vascular permeability (25). Several different aquaporins have been found in the brain, and lack of aquaporin-4 in knockout mice protects against brain edema (26). Aquaporin molecules facilitate transfer of water across membranes. It is possible that the absence of aquaporin leads to reduced accumulation of water molecules in the early stages of edema formation, but the same mechanism may be needed for removal of water, raising the possibility that the edema may be aggravated by agents that block the function of the aquaporin molecule.

4. MMPs IN THE NEUROVASCULAR UNIT

The major MMPs in brain are the stromelysins (MMP-3 and MMP-10), matrilysins (MMP-7), gelatinases (MMP-2 and MMP-9), membrane-type MMP (MMP-14), collagenase (MMP-1) (27), and macrophage elastase (MMP-12) (Fig. 3). All brain cells express MMPs with certain ones being more prevalent in one cell type or another. For example, astrocytes predominantly express MMP-2, while MMP-9 is normally found at very low levels in the brain. During inflammation, however, endothelial cells, astrocytes, and neurons express MMP-9. Stromelysin-1 (MMP-3) has been observed by immunohistochemistry in microglial cells and neurons (28). Macrophages express metalloelastase (MMP-12). The finding of differences in expression of MMPs in various cell types and under different conditions, suggests that complex interactions between cells may be occurring.

Both MMP-2 and MMP-9 act on similar substrates and are able to modify the macromolecules in the basal lamina. Latent MMP-2 is constitutively expressed and normally present in brain and cerebrospinal fluid (CSF). Activation of proMMP-2 requires membrane-type metalloproteinases (MT-MMPs, such as MMP-14, and the presence of tissue inhibitor to



Figure 2 The neurovascular unit is composed of an endothelial cell (EC) with tight junctions (TJ), numerous mitochondria, glucose and amino acid transporters (GT and AAT), and ATPase pumps on the abluminal surface that pump out 3 Na⁺ in exchange for 2 K⁺, creating the osmotic gradient used for interstitial fluid formation. Basal lamina (BL) form a layer of macromolecules between the endothelial cell and the astocytic foot process (AFP). Pericytes (PC) are surrounded by BL. Ensheathing the endothelial cells and basal laminas are the AFP with gap junctions (GJ) and aquaporin-4.

metalloproteinase-2 (TIMP-2). Spatially controlled proteolysis by MMP-2 is possible because of the attachment of the activation complex to the membrane. This could be important in restricting normal basal lamina breakdown to small regions as shown schematically in Figure 4. Loss of basal lamina proteins have been shown in ischemic injury, supporting a role of proteases in the basal lamina disruption (29). The endothelial cells have a basal lamina surrounding the epithelial-like cells that separates the foot processes from the endothelial cells. Basal lamina is composed of extracellular matrix



Figure 3 The primary structure of the MMP molecule. A signal peptide and propeptide region contains cysteines that form the cysteine switch, which maintains the latency of the MMPs. A fibronectin binding site and the zinc-containing catalytic domain are followed by the hinge region with a hemopexin and transmembrane domains. The MMPs have different components, and the classification of MMPs is based on the basic protein structures. Matrilysin with only a catalytic domain is the smallest of the MMPs. Stromelysins have a hemopexin added to the catalytic portion. Gelatinases contain the fibronectin binding site. MT-MMPs have a transmembrane domain.

macromolecules, such as type IV collagen, fibronectin, heparan sulfate, and (30). Although the exact function of the basal lamina in the brain is uncertain, in other organs it serves as a structural support and filter for molecules of different sizes and charges. Such a role is likely in the brain where the type IV collagen could provide structure to the thin-walled capillary, and the heparan sulfate molecules may serve as a charge filter.

Regulation of the MMPs occurs at a number of steps. The promoter regions of the genes for the MMPs are similar, but have unique features that separate one MMP from another. Gelatinase A (MMP-2) is constitutively expressed. In the MMP-2 gene's promoter region are found p53, AP-2, SP-1, and PEA3 sites. Gelatinase B (MMP-9), which attacks substrates

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Figure 4 Activation of MMP-2 occurs through the action of the trimolecular complex. In the astrocytic foot processes (AFP), the membrane-type matrix metalloproteinase (MT-MMP) join with tissue inhibitor of metalloproteinases-2 (TIMP-2) to activate proMMP-2 in a spatially constrained manner close to the basal lamina (BL). In the BL are the pericytes (PC). The endothelial cell (EC) have tight junctions (TJ). The activated MMP-2 has direct access to the portion of the BL beneath the AFP and components of the BL are degraded. The manner in which this disruption of the BL leads to increased permeability is unclear since the role of the BL in maintaining the integrity of the blood vessel is uncertain.

similar to those of MMP-2, has a different promoter region composition. The MMP-9 promoter region has two AP-1 sites and a NF- κ B site, defining it as an inflammatory or inducible gene. Other pro-inflammatory MMPs, such as MMP-3 and MMP-7, also have AP-1 sites. The membrane bound MMPs, such as MMP-14, have sites suggestive of both a constitutive and inducible gene (Fig. 5). The promoter regions for the inducible genes, which have AP-1 sites, respond to cytokines and immediate early gene products, such as TNF- α and the c-Fos and c-Jun dimmer. Once the mRNA is formed, it can be degraded or translated into protein in the endoplasmic reticulum.

The MMPs are secreted in a latent form and their activation is accomplished by other proteases and free radicals (Fig. 6). As described above, activation of MMP-2 occurs through the formation of a trimolecular complex (31). The activation process proceeds when TIMP-2 is present at the correct concentration; too low results in no activation, while too much inhibits activation. An intracellular proconvertase, furin, is able to activate the MT-MMP in the endoplasmic reticulum before it translocates to the cell surface. Another activator is plasmin (32). There is evidence, in other tissues, that MT-MMP may have direct proteolytic activity on the cerebral basal lamina in addition to its indirect role as the activator of MMP-2, but its role in brain remains to be defined. Other mechanisms may be involved in



Figure 5 The promoter regions for the human MMP genes identified in the brain are shown. The constitutively expressed, MMP-2, gene has p53, AP-2, and SP1-1 sites, and is continually being formed. Inflammatory genes, such as, MMP-3, MMP-7, MMP-9 have AP-1 sites, and in the case of MMP-3 and MMP-9, a NF- κ B site. MMP-14 (MT-MMP) has a NF- κ B site, but can be induced similarly to the inflammatory genes. *Source:* From Ref. 84.

the activation of the trimolecular complex on the cell surface, for example, thrombin activates MMP-2 through the activation of MT-MMP (33,34).

The third component of the neurovascular unit is the macrophage-like pericyte. This cell has features of both macrophages and smooth muscles as shown by immunostaining, which shows smooth muscle, actin, and macrophage markers (35). Recent studies have shown that pericytes are important in angiogenesis through the release of vascular endothelial growth factor (VEGF) (36). It is interesting that VEGF has been linked with MT-MMPs both as an activator of MT-MMP, and as being activated by MT-MMP. Since VEGF is stimulated by the hypoxia-inducing factor-1 α (HIF-1 α), hypoxia may trigger VEGF release by pericytes, and the activation of the trimolecular complex.

Neurons contribute to the neurovascular unit. One study showed that neurons have vasopressin-containing fibers contiguous with the blood vessels, suggesting that the release of vasoactive substances, such as



Transcription Translation Activation

Figure 6 Regulation of the MMPs occurs at several steps. The gene promoter region has various elements. Shown here are the activator protein-1 (AP-1) and the nuclear factor- κ B (NF- κ B) sites. These promoters are stimulated by immediate early gene products, such as c-Fos and c-Jun, TNF- α , free radicals and lipopolysac-charide (LPS). After transcription of the messenger RNA, the latent protein (proMMP) is made. Activation is the next step, which is performed by other MMPs, plasmin, and free radicals. Other mechanisms of regulation that are not shown are destruction of the mRNA and digestion of the protein before it is secreted. The TIMPs are endogenous inhibitors of the MMPs that contribute to the regulation by interfering with activation and blocking the activated enzymes.

vasopressin, may influence water and electrolyte balance (37). Vasopressin interacts with the aquaporin molecule in the kidney, but evidence of a role of vasopressin in control of aquaporin in the brain is lacking (38). Neuronal activity could indirectly affect the capillary function through an interaction of the neuron and the astrocyte, leading to elevation of intracellular calcium. An interesting link between excessive neuronal function and the disruption of the neurovascular unit was shown recently in rats with cortical spreading depression, which releases a wave of potassium depolarization that spreads at a slow, steady rate across the cortex after an insult to the brain. The MMP-9 mRNA and latent protein along with activated MMP-9 was induced in a pattern that correlated with the opening of the BBB to the tracer dye, Evans blue (39). Seizures lead to excessive neuronal activity and disruption of the BBB when the seizure activity is prolonged. The MMPs have been shown to be elevated in brain tissues after experimentally produced seizures (40,41).

Multiple MMPs are located in the cells that comprise the neurovascular unit. Cerebral vessels have MMP-9, which is normally only seen at very low levels in the brain, but responds dramatically to a wide variety of insults. Endothelial cells in culture released MMP-9 when stimulated with the cytokines, TNF- α and IL-1 β (42). Dexamethasone suppressed the MMP-9 production. There was evidence that zona occludens protein can be degraded by the MMP-9. Pericytes and microglia form MMP-9 and stromelysin-1 (MMP-3) (28).

Astrocytes, which make MMP-2 under normal circumstances, can be induced to produce MMP-9, which is formed in a latent state that requires

activation (43). Stimulation of astrocytes in culture results in the expression of mRNA and protein for MMP-9. Cultured microglia can be stimulated to express MMP-9 as seen by zymograpy (44) and MMP-3, which can be demonstrated by immunohistochemistry (28). We found that LPS stimulated the production of latent MMP-9 in astrocyte cultures, but co-cultures of astrocytes and microglia resulted in the activation of the MMP-9. This activation could be blocked by a broad-spectrum MMP inhibitor, BB-1101 (28). Nitric oxide (NO) was shown to activate MMP-9 through nitroxylation of the cysteine switch (45). Once the MMP-9 is activated, it can have a wider area of tissue destruction than MMP-2 as it diffuses through the extracellular space (Fig. 7).

Most of the studies have focused on the role of the gelatinases, MMP-2 and MMP-9, mainly because of the availability of a sensitive method for their detection in brain tissue (46). However, other MMPs may be involved in BBB damage. Support for this comes from PCR studies to measure the mRNA of MMPs after an insult. Besides MMP-2, MMP-3, and MMP-9, expression of matrilysin (MMP-7), stromelysin-2 (MMP-10), and metalloelastase (MMP-12)



Figure 7 Drawing similar to that shown in Figure 4. Included are the proinflammatory MMPs. An insult, such as, ischemia, trauma, or infection activates the cytokines, TNF- α and IL-1 β . These and other factors induce the production of MMP-9 and MMP-3 from astrocytes and pericytes, respectively. The activation of proMMP-9 proceeds through the action of MMP-3, which is shown and by nitric oxide, which is not shown. Combined with the action of the other gelatinase, MMP-2, it is envisioned that both the basal lamina (BL) and the endothelial cell (EC) tight junctions would be degraded. The MMP-9 has been shown to degrade tight junctional proteins. This more extensive activation mechanism with the formation of the spatially unconstrained, MMP-9 and MMP-3, would cause more extensive an area of injury than the MMP-2. Other proteases and free radicals that contribute to this process are not shown.

have been reported in brain tissues and cells (47–49). A new gene family of metalloproteinases, the ADAMs (a disintegrin and metalloproteinases) have been identified (50). Members of this family, which are similar to the MMPs because of the zinc catalytic site, are mainly active at the cell surface. The TNF- α converting enzyme (TACE or ADAM-17) is the enzyme involved in the processing of the inactive 28-kDa TNF- α molecule into the active 17-kDa form (51). The processing of TNF- α has been shown to be involved in the leukocyte adhesion preceding lesion formation on the MRI in multiple sclerosis patients (52).

5. PROTEOLYTIC DISRUPTION OF THE NVU IN ISCHEMIA AND HEMORRHAGE

Permanent middle cerebral artery occlusion resulted in gradually increasing opening of the BBB to radio-labeled sucrose from 12 hours to 6 days and correlated with the accumulation of water (53). Disruption of the BBB peaked at 8 days and remained open for 14 days in permanent occlusion. Since there was resolution of the brain edema during that time, they concluded that the removal of the excess fluid might be facilitated by the increased permeability. On the other hand, reperfusion produced a different pattern of BBB opening. Reperfusion produced a biphasic opening of the BBB with a refractory period: the initial injury at 3 hours after reperfusion was attributed to a pronounced reactive hyperemia, and the later opening to a maturational process (54). Interestingly, the second more severe opening was not accompanied by loss of tight junctions on electron micrographs. A new method of inducing an ischemic injury by the insertion of a suture thread into the middle cerebral artery through the external carotid enabled investigators to relatively simply control both the time of ischemia and that of reperfusion (55). Using the suture model, a 6 hours permanent MCAO was shown to produced less injury than 3 hours of occlusion and 3 hours of reperfusion, confirming the earlier observation of the increased damage secondary to reperfusion (56).

Cerebral ischemia induces MMP production, which contributes to the reperfusion injury to the cerebral vasculature. We showed an increase in MMP-9 in the first 48 hours after a permanent middle cerebral artery occlusion, and a late increase in MMP-2 (57). In transient ischemia with reperfusion, there is a biphasic opening of the BBB with an initial, reversible opening at 3 hours and a more severe, delayed disruption at 48 hours (Fig. 8A). The initial opening coincides with an increase in MMP-2, and a second increase in MMP-2 occurs at 5 days, persisting for 3 weeks (Fig. 8B). The second, more damaging opening is associated with a marked increase in MMP-9, which begins around 24 hours and continues to 48 hours (Fig. 8C). A similar pattern is seen in the non-human primate with MMP-2 mainly involved in the early BBB opening (58). In the non-human primate, the early opening is associated with MMP-14) (59). The second opening is predominately associated with MMP-9 production. This

sequence of MMP induction suggests that the MMP-2-derived extracellular matrix disruption fails to result in permanent tissue damage. Thus, there is an early, MMP-2-related opening of the BBB, and more severe opening at 24 to 48 hours associated with MMP-9 expression (Fig. 9).

By 5 days after the restoration of blood flow, a marked increase in MMP-2 is seen with a reduction in MMP-9. At this time, immunohistochemistry shows gliosis around the region of necrosis, and the astrocytic processes are stained with the MMP-2 antibody (28). Concomitant GFAP staining shows that only select processes, particularly those associated with blood vessels, contain MMP-2 immunoreactive product. Most likely the MMP-2 is involved in angiogenesis, but very little data is available on this.

Intracerebral hemorrhage creates a different pattern of MMP expression. There is increased production of MMP-9 seen in the first 24 hours. This could come from endogenous production or from recruitment of neutrophils. A recent report showed that MMP-12 was important in the evolution of tissue damage in intracerebral hemorrhage (60). This study was done with the bacterial collagenase-induced hemorrhage model in the rat and there are some elements of inflammation combined with the effects of the bleeding that may have influenced the results.

Other factors contribute to the edema due to inflammation in models of intracerebral hemorrhage. The edema associated with intracranial bleeding is aggravated by the presence of thrombin and other blood products (61). Thrombin converts fibrinogen to fibrin, which stabilizes the clot, but also initiates the activation of the plasminogen/plasmin system to form plasmin for fibrinolysis. Thrombin is present in the site of the bleeding, where it contributes to the production of cerebral edema. Since thrombin and plasmin are potential activators of MMPs, the presence of blood products may worsen edema through MMP-activation.

6. MMPs IN BBB OPENING IN MULTIPLE SCLEROSIS AND INFECTION

Inflammation around the blood vessels causes the opening of the BBB in acute attacks of multiple sclerosis (MS) (62). There is a prominent role for T lymphocytes, suggesting that an autoimmune process is taking place. However, the antigens involved in the initiation of the inflammation are not known and many factors have been implicated, including infection, trauma, and stress. Inflammatory events in blood venules lead to infiltration by leukocytes. The leukocytes release chemokines to recruit additional inflammatory cells and cytokines. Eventually, there is production of the MMPs both by the leukocytes invading the brain, which uses various MMPs to cross the BBB, and endogenously by brain cells.

The first evidence that MMPs were important in MS came from a study of the CSF in patients with MS (63). Patients with acute attacks were

shown to have elevated levels of MMP-9 in the CSF. This was not a finding specific to MS, however, as other inflammatory conditions, such as bacterial meningitis and Guillain-Barre patients also had elevated CSF levels. The



Figure 8 (*Caption on facing page*)

MMP-9 was shown to be elevated in the brains of mice with experimental allergic encephalomyelitis (EAE) and treatment with a non-specific MMP inhibitor, GM6001, was shown to reduce the injury to the BBB (64).

The MMPs were found to augment movement of T lymphocytes across artificial membranes, and interferon-1 β was shown to reduce the flux of activated leukocytes across them (16,65). Acute attacks of MS can be shortened by the use of high-dose methylprednisolone, which is usually given for 3 to 5 days. Gadolinium-DTPA is a paramagnetic substance that decreases the water signal on MRI; it is used to monitor the integrity of the BBB. Normally it remains confined to the lumen of the blood vessels. Treatment with high-dose methylprednisolone, which is used to shorten exacerbations of MS, leads to a rapid resolution of the BBB opening (66). There is marked reduction of the levels of MMP-9 in the CSF after methylprednisone treatment in acute MS; those patients with evidence of Gd-DTPA enhancement on MRI had the greatest reduction in the MMP-9 levels. Steroids have many actions one of which is to block the action of Fos/Jun at the AP-1 site. However, the effects of steroids are transient. Recently, the source of the MMP-9 in the CSF was investigated by simultaneous measurements of MMP-9 in the blood and CSF. A MMP-9 index was calculated similar to that used in assessing the source of IgG in the CSF, which was based on the levels of albumin in both compartments. The source of the MMP-9 appeared to be the CSF (67). Levels of MMP-9 are increased in the blood of patients with acute MS, while those of TIMP-1, its endogenous inhibitor are low (68). Recently, patients with chronic forms of MS were shown to have elevated levels of MMP-2 in the blood (69). In

Figure 8 (Facing page) Opening of the blood-brain barrier (BBB) in a normotensive rat with a 90 minute middle cerebral artery occlusion and reperfusion for the times shown on the x-axis. (A) Sucrose space is measured with 14 C-sucrose injected intravenously 10 minute before sacrifice and radioactivity measured in the brain and blood. Normal values of less that 2% for sucrose space are seen on the non-ischemic hemisphere as shown by the open bars. Reperfusion for 4 hours resulted in a transient opening of the BBB. A second, more prolonged opening was seen at 48 hours, persisting for 5 days, and seen minimally, but significantly, present at 3 weeks. The later changes may be due to angiogenesis. (B) Values for MMP-2 at the same times of ischemia and reperfusion as in A are shown. There was an early increase in MMP-2 in the ischemic side compared to the non-ischemic side. Values decreased at 16 hr, but began to increase at 24 hours, remaining high for 3 weeks. The increase at 5 days and 3 weeks was due to increased expression in astroglial cells forming the scar and participating in the wound healing process. (C) Similar values for MMP-9 as shown in B. The increase in MMP-9 was more prominent at 4, 16, 24, and 48 hours than at the later times. Open bars indicate the non-ischemic side and closed bars the ischemic side. Asterisks show the values that were statistically similar (unpublished data).



Figure 9 A schematic drawing to show the theoretical mechanisms leading to the initial reversible opening of the BBB and the later more slowly reversible opening. It is envisioned that the initial injury is related to the expression of the components of the trimolecular complex, primarily MT-MMP and MMP-2. With the progression of the injury, a threshold is crossed and the induction of the cytokines and other injury mediators, such as neutrophils, leads to the production of MMP-3 and MMP-9. Neutrophils bring in exogenous MMP-9 and activated glial cells and endothelial cells make MMP-9 endogenously. The end result is a more severe injury to the blood vessel, which is more slowly repaired, and leaves signs of permanent damage in the remodeled vessel walls.

addition to the elevated levels of MMP proteins in the blood, there are increases in mRNA for the MMPs (70).

Since MMPs attack the myelin sheath, they have another important role in the pathophysiology of MS. In vitro, MMPs have been shown to break down the myelin molecule (71). The MMP-2 and MMP-3 were the most potent their myelin-degrading potential, but collagenase and MMP-9 also showed some activity against myelin. Metalloelastase (MMP-12), which acts against elastin, was shown to attack myelin; it has proteolytic activity against other extracellular matrix components, including laminin and type IV collagen (72).

Bacterial meningitis results in the expression of the MMPs and contributes to the opening of the BBB and to cells death. In rat pups with experimentally induced bacterial meningitis, there is expression of MMP-9 (73). Agents that inhibit MMP-9 are effective in reducing the injury from meningitis (74). Studies have been done in humans with meningitis that document the increase in MMP-8 and MMP-9 in the CSF (75). These studies suggest that inhibitors to the MMPs may be useful adjunct therapy

for brain infections. Since these are protective in experimental models it does not seem likely that closing the BBB affects the entrance of the antibiotics to the brain, which if it occurred could limit the effectiveness of the primary treatment modality.

Patients with human immunodeficiency virus (HIV) involving the central nervous system have elevated levels of MMP-2, MMP-7, and MMP-9 in the CSF (76). The HIV-1 transactivating protein, Tat, induces MMP-2, MMP-7, and MMP-9, and causes neuronal cell death in culture (77). Treatment with antibodies to MMP-2 and MMP-7, but not MMP-9, blocks neuronal cell death in animals implanted with Tat-producing cells. In addition, an inhibitor to MMPs has a similar beneficial effect, suggesting that MMPs may be important in cellular damage by HIV, and that agents that inhibit MMPs could be used in treating these patients.

Although much is known about the expression of MMPs in a wide variety of neuro-inflammatory conditions, the mechanisms involved in altering vascular permeability remain illusive. The MMPs attack the extracellular macromolecules in the basal lamina and around cells. There is a loss of integrity of the basal lamina as documented by a decline in laminin. There is recent evidence to suggest that the tight junctions are affected, and the BBB is compromised when the site of the barrier is altered (Yang et al., unpublished data). This suggests that hydrolysis of the basal lamina and tight junction proteins may open the transcellular routes.

7. THERAPEUTIC INTERVENTION WITH MMP INHIBITORS

Design of MMP inhibitors has been driven by the potential for the use of these agents for the treatment of cancer and arthritis, which have enormous commercial potential (78). The first agents were developed after the discovery of a role for MMPs in cancer. Hydroxymate-based compounds were found to block the zinc active site in the molecule. A number of compounds were designed with the hydroxymate core. These proved difficult to dissolve, and an early study was done with interperitoneal instillation of the agent for the treatment of ovarian cancer with abdominal metastases. The development of an orally available agent, marimastat, initiated large-scale clinical trials in cancer. Other MMP inhibitors were designed as computer models became available. The goal of these studies was to identify agents that acted against specific MMPs rather than broad-spectrum agents that blocked the zinc in the MMPs.

One of the early broad-spectrum inhibitors, GM6001, was shown to reduce inflammation in EAE in mice (64) by closing the BBB. The opening of the BBB induced by intracerebral injection of TNF- α was blocked by Batimastat (BB-94), another broad-spectrum inhibitor (4). Subsequent studies in a number of animal models have shown the usefulness of these agents as therapies in neurological diseases. An antibody to MMP-9 was found to be effective in cerebral ischemia, reducing infarct size (79). The early opening of the BBB in cerebral ischemia with reperfusion was blocked by BB-1001; an agent similar to BB-94. In addition, the edema seen at 24 hours after reperfusion was blocked by BB-1101, but the second more extensive opening at 48 hours could not be blocked.

Studies in bacterial meningitis, experimental allergic neuritis, experimental models of AIDS, and other neurological diseases have shown the benefit of MMP inhibitors. The role of MMPs in the opening of the BBB in the acute inflammatory phase of MS and their ability to degrade myelin, suggested that inhibitors to MMPs may be useful in the therapy of MS. Several broad-spectrum MMP inhibitors have been shown to be effective in the EAE model, and a tetracycline derivative, minocyclin, that reduced the MMP load, has been shown to reduce the extent of injury in animals with EAE (80).

Recently, several studies have shown that the toxicity of rtPA can be dramatically reduced by BB-94. In a model of multiple emboli in rabbits, the degree of hemorrhage after rtPA reperfusion was reduced with BB-94 (81). Another study has found benefit from BB-94 in rtPA-related mortality (82). We have shown that an important action of the MMP inhibitor, BB-94, is to block the opening of the BBB, which restricted the rtPA to the vascular space. Mortality was drastically reduced in tPA-treated rats that were given BB-94 (83).

8. CONCLUSIONS AND FUTURE DIRECTIONS

The contribution of the MMPs to the inflammatory response provides a mechanistic basis for the use of MMP inhibitors for the control of BBB damage. Since the MMPs act as a final common pathway for the disruption of the BBB in a number of pathological processes, there is great interest in identifying suitable MMP inhibitors that could be used to dampen their impact at the barrier. On the other hand, the MMPs are important in wound healing, contributing to the formation of new blood vessels through angiogenesis. Thus, it will be necessary to block the pathological processes while preserving those that are beneficial.

Challenges remain in identifying appropriate therapeutic agents. Should the drug be a broad-based inhibitor or specific for the MMPs thought to be involved directly in the pathology? If specific, which MMPs should be targeted? Are the agents getting into the brain or are they acting at the level of the blood vessel? What are the diseases that should be targeted initially? As more information emerges in the timing of the MMP expression and as computer-driven processes design more novel agents, the answers to these questions will emerge.

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15

Endothelial Cells, Extracellular Matrix, and Astrocytes: Interplay for Managing the Blood-Brain Barrier

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The blood-brain barrier protects the neural microenvironment from changes in the blood composition. It is located at the endothelium, which is both seamless and interconnected by tight junctions. The restrictive paracellular diffusion barrier is accompanied by an extremely low rate of transcytosis and the expression of a high number of channels and transporters for such molecules that cannot enter or leave the brain paracellularly.

The blood-brain barrier endothelial cells are situated on top of a basal lamina, which contains various molecules of the extracellular matrix. Pericytes and astrocytes directly contact this basal lamina; however, little is known about the signaling pathways between these cell types and the endothelium which possibly are mediated by components of the basal lamina. Heparansulfate proteoglycan agrin is among the numerous components of the extracellular matrix that appears to play an important role, since its expression is up-regulated during blood-brain barrier maturation. The function of agrin includes the polarization of astrocytes, which causes heterogeneity of different membrane domains on the astroglial surface and is strictly connected to an intact blood-brain barrier. To understand the mechanisms of blood-brain barrier formation and maintenance, it is necessary to investigate the molecular interplay between endothelial and perivascular cells, and the basal lamina in between.

1. INTRODUCTION

The original finding of Paul Ehrlich (1) that an infused dye did not stain the brain tissue, together with the observation of his pupil Ernst Goldmann that the very same dye if applied into the cerebrospinal fluid did stain the brain tissue has lead to the concept of a biological barrier between blood and brain. Due to the free access of the dye from brain ventricle to brain tissue, it was concluded that there is no cerebrospinal fluid-brain barrier. However, the staining of circumventricular organs and the choroid plexus in the experiment when applying the dye into the general circulation (Goldmann-I experiment) and the lack of staining of these organs when applying the dye into the cerebrospinal fluid (Goldmann-II experiment) suggested the existence of a barrier between the cerebrospinal fluid and the blood. The cellular basis of these barriers was unclear for decades. Today, we know that in most vertebrates the barrier is located within the endothelium [endothelial bloodbrain barrier (BBB); only in elasmobranchs, the BBB is located in astrocytes] and in the epithelial choroid plexus cells and the tanycytes of the circumventricular organs [glial blood-cerebrospinal fluid barrier (BCSFB)]. In this chapter, we will focus predominantly on structure and function of the endothelial BBB.

2. STRUCTURE OF THE BLOOD-BRAIN BARRIER

The BBB protects the microenvironment of the central nervous system from toxins and buffers fluctuations in blood composition (2–4). The main structures responsible for the barrier properties are the tight junctions (5–9). The cells responsible for the establishment of the barrier and are interconnected by tight junctions is the capillary endothelial cells in case of the BBB, and the epithelial (glial) cells in case of the BCSFB.

Mature BBB capillaries in the mammalian brain are mainly characterized by the small height of endothelial cells (Fig. 1A), the interendothelial tight junctions (2,5); for recent reviews, see References 10 and 11; Figures 1B and 2A, the small number of caveolae at the luminal surface of the cell (12), and the high number of endothelial mitochondria (13). In addition, sub-endothelial pericytes that are completely surrounded by a basal lamina, phagocytic perivascular cells, and astrocytic processes belong to the set of elements directly adjacent to the cerebral vasculature (14,15).

The microvascular endothelial cells are undoubtedly important in the restriction of the BBB-related permeability. From transplantation experiments it became evident that endothelial BBB characteristics are largely determined by intrinsic factors of the brain. It has been shown that neural tissue grafted into a non-neural environment induced BBB characteristics in the host-derived endothelial cells (16,17). Proliferating endothelial cells of the leptomeningeal vascular plexus when invading the neuroectoderm



Figure 1 Electron microscopy of the BBB. (A) Ultrathin section of a mouse brain capillary. The arrow points to a tight junction. A basal lamina separates the pericyte from both the endothelial cell and the astrocyte. The asterisks mark astrocytic endfeet. The molecular structure of these endfeet membranes is illustrated in C. Bar: 2μ m. (B) Freeze-fracture replica of mouse brain capillary. The arrow points to the tight junction (for more details, Fig. 2). Bar: 0.5 μ m. (C) Freeze-fracture replica of a rat brain perivascular astrocytic endfoot. OAP are densely studded in the glial membrane, IF in endfoot cytoplasm. Bar: 0.2 μ m. (D) Freeze-fracture replica of a perivascular astrocytic endfoot membrane from rat brain in higher magnification; however, the OAP density is not as high as in (C). Bar: 50 nm. *Abbreviations:* OAP, orthogonal arrays of particles; IF, intermediate filaments; EC, endothelial cell; P, pericyte.

were suggested to be committed to express the BBB phenotype by an interaction with neuroectodermal cells (18,19).

2.1. Endothelial Tight Junctions

Tight junctions are domains of occluded ("Zonula occludens") intercellular clefts (5,20) between endothelial or epithelial cells. They form intramembrane networks of strands in freeze–fracture replicas (Figs. 1B and 2A,B). If sectioned transversally, the tight junction appears as a system of fusion ("kissing") points each of which represents a sectioned strand. Two



Figure 2 Freeze-fracture replicas of endothelial tight junctions. (A) Endothelial BBB tight junctions from capillary fragments freshly prepared from bovine brain. Most strand particles are associated with the P-face. The arrows point to P-face-associated tight junction particles. Bar: 100 nm. (B) Bovine brain capillary endothelial cells in culture have tight junctions almost completely associated with the E-face. This is also typical for non-BBB endothelial tight junctions. Asterisks label particle-free ridges of tight junctions on the P-face. Arrows point to E-face-associated tight junction particles. Bar: 50 nm. *Abbreviations:* EF, E-face; PF, P-face.

parameters, visualized by freeze–fracture electron microscopy, determine the functional quality of tight junctions: the complexity of strands and the association of the particles with the inner (P-face) or outer (E-face) lipidic leaflet of the membrane. The complexity of the tight junction network is recognized to be related to the transepithelial electrical resistance (21,22). Epithelial tight junctions are commonly associated with the P-face forming a network of strands and leaving grooves at the E-face, which are occupied by only a few particles (23,24). After ATP depletion, MDCK cells suffer from deterioration of paracellular barrier ("gate") function, which is

followed by a reorganization of the actin cytoskeleton (25,26) and a decreased P-face association of the tight junctions. Thus, there seems to be a causal relationship between the degree of particle association to the P-face and the observed transepithelial resistance.

The notion that endothelial cells form an efficient permeability barrier orginated from electron microscopy tracer experiments (5). Then, Nagy et al. (27) investigated the tight junctions of the BBB using the freeze-fracture method and found them to be the most complex junctions in the entire vasculature of the body. In addition to the complexity of the tight junction network, the association of the tight junction particles with the inner (protoplasmatic: P-face) or outer (external: E-face) leaflet of the endothelial membrane has been described as an additional parameter in the quality of the permeability barrier of the brain (28). The BBB tight junctions are unique among all endothelial tight junctions because their P-face association is as high as or even slightly higher than their E-face association (Fig. 2A). Interestingly, the P-face/E-face-ratio of BBB tight junctions continuously increase during development (29). In cell culture, the freeze-fracture morphology of BBB endothelial cells is similar to non-BBB endothelial cells (28,30,31) (Fig. 2B) indicating that the association of the strand particles with the membrane leaflets reflects the quality of the barrier and is under the control of the brain microenvironment. The shift of tight junction particles from the P- to the E-face association seems to be the most sensitive parameter found so far to characterize the compromised barrier: in asymptomatic adult stroke-prone spontaneously hypertensive rats (SHRSP) a strong reduction of the P-face/E-face ratio rather than a reduced expression of tight junction proteins went along with a reduced polarity of BBB endothelial cells as assessed by the distribution of the glucose transporter isoform GLUT-1 (32).

The molecular biology of tight junctions has become extremely complex and will not be considered intensely in this chapter, because we have covered this elsewhere (see Chapter 3 and Refs. 10, 11, and 33). Most data were collected from epithelial cell studies, possibly because the regulation of BBB endothelial tight junctions is considerably more complex than that in epithelial cells.

Briefly, after the detection of tight junction-associated proteins such as ZO-1, ZO-2, ZO-3, and cingulin, intramembrane proteins were found to be directly involved in the restriction of the interendothelial permeability. The first protein found was occludin, a protein with four transmembrane domains (34). However occludin does not seem to be the crucial permeability-restricting tight junction component, since the occludin-deficient mouse mutant has intact biological barriers (35). The discovery of the claudins, a new family of tight junction-related proteins, which also have four transmembrane domains but no homology to occludin (36,37) was of fundamental importance for tight junction research. In addition, members of the

immunoglobulin superfamily, such as the JAMs and ESAM, are components of tight junctions, which fulfill regulatory functions of the barrier. This exciting field was recently reviewed by several groups (e.g., 11,38–44).

2.2. The Extracellular Matrix of Blood-Brain Barrier Microvessels

The BBB is under the strict control of the brain microenvironment that is composed of neuronal and glial cells, pericytes, and the ECM. The basal lamina of cerebral endothelial cells is complex and consists of various collagens, laminins, fibronectin, entactin, thrombospondin, as well as heparan, and chondroitin sulfate proteoglycans (45-48). Many studies on the functional impact of the ECM on the vasculature of the brain came from cell culture studies, or from studies investigating the conditions during angiogenesis, tumor growth, inflammation, or aging (47,49-53). For example, only two isoforms, laminin 8 (composed of laminin $\alpha 4$, $\beta 1$, and $\gamma 1$), and laminin 10 (composed of laminin $\alpha 5$, $\beta 1$, and $\gamma 1$) were found in endothelial basement membranes of most tissues including the CNS (54). The localization of inflammatory cuffs surrounding post-capillary venules during experimental allergic encephalomyelitis allowed scientists to distinguish between endothelial cell and astroglial basement membranes. They found that the endothelial cell basement membrane contains laminins 8 and 10, whereas the astroglial basement membrane contains laminins 1 and 2 (47).

The role of the ECM turnover is particularly remarkable during angiogenic processes by their development and pathology. The invasion of microvascular endothelial cells is characterized by a proteolytic degradation of the ECM. Both u-PA (urokinase-type plasminogen activator) and its inhibitor PAI-1 (plasminogen activator inhibitor type-1) are up-regulated in gliomas (55). The u-PA system has been proven to be necessary for angiogenic processes (56); as reviewed in Reference 57. Furthermore, cathepsin B has been found in proliferating endothelial cells of gliomas (58). The matrix metalloproteinases (MMPs) are a growing family of degrading enzymes, which are associated with tumor cell invasion and blood vessel transmigration (for reviews see Refs. 59 and 60). The MMP-2 (gelatinase A, type IV collagenase, 72 kDa gelatinase), MMP-9 (gelatinase B, type-V collagenase, 92 kDa gelatinase) and MMP-12 (metalloelastase and macrophage elastase) have been found to be up-regulated by glioma cells and MMP-9 and MMP-2 are secreted by proliferating glioma endothelial cells (61-63). Both bFGF and VEGF induce the release of MMP-9 from glioma cells in vitro in a doseand cell density-dependent manner, implicating possible roles of these growth factors to enhance MMP-9 expression levels in gliomas (64). Interestingly, the inhibitors of MMPs, the tissue inhibitors of metalloproteinases types 1 and 4 (TIMP-1 and TIMP-4) are also up-regulated in gliomas, with TIMP-1 mainly expressed by endothelial cells (65). The MMPs may be

involved in BBB-impairment by shedding of growth factors that have been stored in the vessel ECM contributing to angiogenic processes, or by remodeling the vascular ECM via stimulation of integrin receptors. It was demonstrated that $\alpha v\beta \beta$ integrin is up-regulated in glioma endothelial cells (66), and that its binding to these domains is able to induce cell spreading, migration, and angiogenesis. Interestingly, certain BBB-related molecules are involved in the activation of MMPs. Miyamori et al. (67) demonstrated that claudin-5 and claudin-1 promote the activation of pro-MMP-2 through membrane-type matrix metalloproteinases (MT-MMPs), which also are upregulated in gliomas (62). Furthermore, HT7 or EMMPRIN (ECM metalloproteinase inducer), also called neurothelin, CD147, basigin or M6, which is associated with normal BBB function (as reviewed in Ref. 68), is present on the surface of tumor cells and stimulates adjacent cells to produce MMPs (69). The EMMPRIN has been demonstrated in the vascular endothelium of non-neoplastic regions of the brain, whereas it is absent in proliferating blood vessels in malignant gliomas and present in the tumor cells (70). A deficiency of MMP-9 resulted in a protection from transient focal ischemia by attenuation of serum extravasation via the BBB and reduction of the lesion volume demonstrating the beneficial role of ECM components on the integrity of the BBB under these conditions (71).

The heparan sulfate proteoglycan agrin was originally characterized as the essential molecule for clustering acetylcholine receptors at the motor endplate (72,73), but has also been described as being important within the CNS, particularly for the integrity of the BBB (74–76). Two isoforms were described with different amino termini: the short amino terminal isoform with 49 amino acids was called SN-agrin, and the long isoform with 150 amino acids LN-agrin; the LN-agrin assembles in basal laminae, whereas SN-agrin is cell-associated (77). The agrin splicing variant Y0Z0 is reported to be specifically present in the endothelial cell basal lamina of CNS capillaries (78). Agrin binds to α -dystroglycan (79), but also to certain integrins and to heparin. α -Dystroglycan is a member of the dystrophin– dystroglycan complex (DDC) which localizes at glial endfeet membranes (80) (Figs. 3A,C, and 4), but at endothelial cells as well (81).

2.3. The Dystrophin–Dystroglycan Complex

The DDC research has focused mainly on skeletal muscles, where the complex is localized in the cell membrane and links components of the ECM to the sarcolemma, providing stability and structural integrity during contraction and perhaps a way for transducing signals (for a short-cut introduction to the dystrophin-associated complex, see Ref. 82). Mutations in the dystrophin-encoding gene lead to severe alterations in the cell membrane and in consequence to the manifestation of muscular dystrophies, e.g., the Duchenne muscular dystrophy (DMD) (80). Dystrophin and its truncated



Figure 3 Human glioblastoma, immunostained with antibodies against α -dystroglycan (a-Dys; (A), (C)) and aquaporin-4 (AQP4; (B), (D)). (A) and (B), and (C) and (D), are from peripheral and central areas of the tumor, respectively. In peripheral areas (A), (B), where the BBB is still intact, α -dystroglycan and AQP4 are restricted perivascularly. In central areas, where the BBB is damaged, α -dystroglycan remains restricted at the vessel wall, but AQP4 is redistributed across the surface of the glioma cells. For further details, see text.

isoforms such as Dp260, Dp140, Dp116, and Dp71 have also been found in the CNS (83,84) and they are reduced in both DMD patients and the dystrophin-deficient mdx mouse (85). Accordingly, cognitive defects have been recognized in DMD patients (86,87). Although the reasons for these deficits may primarily be explained in the synaptic failure of dystrophin, an effect of the dystrophin deficiency on the BBB compromising general neuronal networks cannot be ruled out. Recently, severe deleterious effects on the integrity of the BBB in mature and developing mdx mice have been described (88,89). In these dystrophin-deficient mice, the authors observed an increase of the vascular permeability, a loss of some tight junctional components, and a reduction in the expression of the water channel protein aquaporin-4 (AQP4). Although there are different components participating in the muscular DDC compared to the DDC in the astrocyte endfeet, the main molecules and functions are identical. In astrocytes, actin filaments



Figure 4 Schematic view of the molecular complex in the astroglial endfoot membrane. AQP4 water channel protein aquaporin-4, OAP orthogonal arrays of particles, α -DG α -dystroglycan, β -DG β -dystroglycan, α -Db α -dystrobrevin, and Syn syntrophin. α -Syntrophin contains a PDZ-binding domain which is both connected to AQP4 and to the inward rectifier potassium channel Kir4.1. The box around AQP4 indicates the general opinion that the OAPs consist of AQP4. In addition, we discuss here the possibility that the OAPs may also contain the dystrophin–dystroglycan complex as well as Kir4.1 (indicated as the *stippled box*).

of the cytoskeleton are either linked to dystrophin or utrophin, which is also called dystrophin-related protein. Dystrophin is connected via its amino-terminal domain to the glycoprotein dystroglycan, a transmembrane spanning protein which consists of a α - and β -subunit. Both subunits are encoded by a single gene and are formed by cleavage of a precursor protein into two mature proteins that form a tight non-covalent complex (90,91). The transmembrane β -dystroglycan anchors α -dystroglycan to the cell membrane and the cytoskeleton via its linkage to the C-terminal domain of dystrophin (Fig. 4). The brain-selective deletion of the dystroglycan gene has been described to cause brain malformations such as disorganization of cortical layering and aberrant migration of granule cells (92). The authors did not exclude abnormalities of the BBB leading to reactive, inflammatory gliosis. In addition to dystroglycan, proteins such as dystrobrevins and syntrophins are also connected to the C-terminus of dystrophin and allow the DDC to interact with channel molecules. Dystrobrevin was described to be immunolocalized at glial and endothelial cells in the rat retina (93) and rat cerebellum (94). A subunit of syntrophin, α 1-syntrophin, contains a PDZ-binding domain in its C-terminal domain that is both connected to AQP4 (95,96) and to the inward rectifier potassium channel Kir4.1 (97) (Fig. 4). The cell surface-associated α -dystroglycan binds ECM-molecules, such as laminins and agrin (79). Laminin has been described to induce the aggregation of Kir4.1 and AQP4 via the DDC in cultured astrocytes (98). The degree of glycosylation of α -dystroglycan seems to be crucial for the binding activity of α -dystroglycan to these extracellular ligands. In the muscle–eye–brain disease (MEB) and the Fukuyama congenital muscular dystrophy, α -dystroglycan was found to be hypo-glycosylated (99). Experimental de-glycosylation of α -dystroglycan disrupted laminin-binding activity (100). Under conditions of BBB disruption, agrin was reported to be lost (75,101). The implications of the loss of this important binding partner of α -dystroglycan for the stability of the whole DDC in the glial endfoot membrane and for the integrity of the BBB will be comprehensively discussed in the last paragraph of this chapter.

2.4. Perivascular Astrocytes

The brain capillaries are surrounded by a basal lamina, in which the pericytes are embedded and which are contacted by the astroglial endfeet (Fig. 1A). The role of pericytes in the establishment of the barrier is widely unclear. However, new data suggest that pericytes, and astrocytes as well (102), secrete angiopoietin-1 (Ang-1), an anti-angiogenic factor, which is known to bind to its receptor tie-2. Pericyte-conditioned medium-induced up-regulation of occludin mRNA could be antagonized in cultured endothelial cells by an angiopoietin-2-neutralizing antibody (103). In contrast to pericytes, astrocytes have been mainly investigated as putative inducers of the BBB and are widely believed to be involved in operating in favor of the BBB formation (9,10,102,104-107). Humoral factors released from astrocytes were suggested to contribute to tight junction formation (107-110) but are not sufficient to induce and maintain BBB characteristics. Nevertheless, the glial cell line-derived neurotrophic factor (GDNF) seems to be necessary for BBB induction (111-113). Inversely, interleukin-1ß secreted by astrocytes and induced by tumor necrosis factor α via an endothelin-1 mediated mechanism has been shown to be responsible for compromising the BBB quality (114). The src-suppressed C-kinase substrate (SSeCKS) in astrocytes has been reported to be responsible for the decreased expression of the angiogenic permeability factor vascular endothelial growth factor (VEGF) and the increased release of the antipermeability factor Ang-1. The SSeCKS over-expression was shown to increase the expression of tight junction molecules and to decrease the paracellular permeability in endothelial cells (102).

However, when the BBB is induced during embryonic development, astrocytes are still undifferentiated. Therefore, if astrocytes have any role in BBB management, it has to be restricted to the maintenance of the barrier properties of the endothelial cells. Recent data propose that the BBB may be repaired also in the absence of direct contact between glial fibrillary acidic protein (GFAP)-positive astrocytes and endothelial cells (115).

The GFAP is a type of intermediate filament protein in glial cells that has been proposed to influence the BBB. In aged GFAP-deficient mice, the BBB was found to be impaired (116). Moreover, astrocytes of these mice failed to induce a functional BBB in aortic endothelial cells in vitro (117). Knockout mice lacking GFAP and vimentin developed dilated blood vessels in the brain and the spinal cord (118). Dystrophic mdx mice showing severe alterations of BBB characteristics such as increased permeability and a reduction in the expression of tight junctional molecules, revealed a clearly diminished content of GFAP in the astrocytes (89). However, the link between altered astroglial intermediate filament equipment and BBB organization or integrity is unknown. Nevertheless, in the fibroblast growth factors 2 and 5 double knockout mouse, Reuss et al. (119) were able to observe a reduction in the expression level of GFAP in the perivascular astroglial endfeet. This defect was accompanied by an increase in albumin extravasation and loss of occludin and ZO-1 immunoreactivities in cerebral blood vessels confirming the role of intermediate filament proteins in glial cells in maintaining the endothelial barrier.

When analyzing the morphology of the glio-vascular complex, it is notable that a mature BBB is characterized by a highly polarized astrocyte. Polarization of an astrocyte means an unequal distribution of membrane compounds which for a long time could only be recognized by means of freeze-fracturing; the orthogonal arrays or assemblies of particles (OAPs; Fig. 1C,D). These arrays are densely packed (about $400/\mu m^2$ membrane area) at the point in which the astrocytic membrane contacts the perivascular or superficial basal lamina, the glial limiting membrane (120). The point at which the membrane bends away from close contact with the basal lamina has been shown to have a reduced density of OAPs $(20-40/\mu m^2 \text{ membrane})$ area). This polarity develops in parallel to the maturation of the BBB (121,122), but is not expressed in cultured astrocytes (for review see Ref. 123). As well, the OAP-related polarity is considerably reduced in glial tumors (120). Today we know that the OAPs contain the water channel protein AQP4. The involvement of AQP4 in OAP formation was demonstrated in three separate experiments. First, by the absence of OAPs in astrocytes of AQP4-deficient mouse (124), second by the formation of OAPs in Chinese hamster ovary cells stably transfected with AQP4 cDNA (125), and third by the immunogold fracture-labeling technique showing that AQP4 is a component of the arrays (126). Moreover, Nielsen et al. (127) were able to demonstrate by immunogold immunocytochemistry that the distribution of the AQP4-related immunoreactivity was identical to that of the OAPs. It should be stressed that AQP4 is the only member of the aquaporin family that is associated with a membrane structure that can be visualized by electron microscopy.

Aquaporins mediate water movements between the intracellular, interstitial, vascular and ventricular compartments that are under the strict control of osmotic and hydrostatic pressure gradients (128–130). This function is conserved in animals, plants, and bacteria. At least 10 isoforms of aquaporins have been identified in mammals, designated AQP0 through AQP9 (130,131). Although most aquaporins, including AQP4, are selectively permeable to water, AQP3, AQP7, and AQP9 (aquaglyceroporins) are also permeable to urea and glycerol (132). In mammals, aquaporins are involved in renal water absorption, generation of pulmonary secretions, lacrimation, secretion, and reabsorption of cerebrospinal fluid and aqueous humor, and development of edema.

3. WATER FLUX THROUGH ASTROCYTIC AQUAPORINS

Brain edemas are classified as vasogenic or cytotoxic depending on their development (133). When the BBB becomes leaky and permits the entry of plasma fluid into the parenchyma, for example in glioblastomas or metastatic brain carcinomas, they are called vasogenic. If intracellular fluid accumulates during water intoxication and anoxia-generating conditions, edema will be classified as cytotoxic. Independent from their development and classification, the amount of edema fluid present in the brain reflects the balance between its production and clearance.

The treatment of vasogenic edema is still one of the most serious clinical problems during the pathogenesis of human glioblastoma. The resulting increase of the intracranial pressure also accounts for much of the morbidity and mortality associated with other primary neuropathological diseases like head trauma, head abscess, and stroke, and with conditions that affect the brain indirectly like sepsis, hyponatremia, kidney, and liver failure (134-137). Treatment options for brain edema are, e.g., osmotic diuretics, corticosteroids, maintenance of normocapnia, decompressive craniectomy, and hypothermia, but none of them corrects the molecular alterations which are responsible for brain swelling. The reason for these limited treatment options is that little is known about the regulatory mechanisms of water transport in the brain and even less about the pathomechanisms leading to edema. During the past decade this dearth of information stimulated further investigations in this field and it is generally accepted now that the astrocyte is the major cell type which reacts stereotypically with swelling to mechanical, physical, and chemical injuries of the brain (138,139).

Recent data indicate that the water channel protein AQP4, which is selectively expressed in the astroglial endfeet around blood vessels (Fig. 3B), plays a decisive role in the volume regulatory mechanisms between blood and brain (4). In rodents, AQP4 expression in astrocytes is

up-regulated in response to cerebral edema caused by brain injury (140,141), focal brain ischemia (142), and hyponatremia (143). The anchorage of AOP4 to syntrophin (95) seems to be important for the correct targeting of the water channel protein at the perivascular site, because syntrophindeficient mice show a marked loss of AOP4-immunoreactivity at perivascular membranes and a considerable swelling of glial endfeet due to a reduced clearance of water (144). However, in human brain tumors the redistribution of AQP4 and α -syntrophin across the whole surface of glioma cells suggests that α -syntrophin alone cannot be responsible for the correct targeting of AQP4 to the perivascular membrane (145). The loss of the water channel-related polarity obviously results in water movement not only between blood and glia but also between glia and brain parenchyma, where the water accumulates and decisively contributes to an increased intracranial pressure. Despite these lines of evidence, there was still no definite proof whether AOP4 contributes to cerebral fluid accumulation or its clearance. The first direct evidence that AQP4 is responsible for edema formation in the brain was provided by several experiments on AOP4-deficient mice, which are phenotypically normal and do not manifest neurological abnormalities, altered BBB properties or impaired osmo-regulation (146). Brains from these mice, however, showed a significant reduction of the osmotic water permeability (131,147) and the survival time of the mice after water intoxication was greatly improved. The deletion of AQP4 protected the brain from fluid accumulation and swelling of the astroglial endfeet, impressively demonstrating the crucial role of the water channel protein in edema formation.

Water fluxes are inevitably coupled to fluxes of osmolytes. In the case of astrocytes in the brain and Müller cells in the retina, it has been repeatedly pointed out that the main water-directing osmolyte is the neuronally released K⁺. Indeed, one of the best-established functions of astrocytes and retinal Müller cells is the spatial buffering and siphoning of extracellular K⁺ that is released into the blood and the vitreous, respectively (148). The weakly rectifying K⁺ channel Kir4.1 is responsible for this release at the glial endfoot membrane. The aggregation of this channel at this membrane domain is of fundamental importance for K⁺ siphoning and therefore under the control of a complex molecular machinery which includes AQP4 and the DDC (98,149–151,161).

Although much of the data come from studies on AQP4, the expression of AQP1, AQP3, AQP5, AQP8, and AQP9 has been described in the rodent brain recently (for review see Ref. 129). In analogy to AQP4 which seems to be co-expressed with other channels or membrane proteins, non-AQP4 water channel proteins may also be associated with ion channels that have not been identified. The AQP1 is found on epithelial cells of the choroid plexus and seems to be involved in cerebrospinal fluid formation (129). But the up-regulation of AQP1-expression in glial tumors indicates that the water channel also contributes to cerebral edema (152). Current results showed an expression of AQP3, AQP5, and AQP8 in neuronal primary cultures and astrocyte cultures. In addition, expression of AOP8 was observed on oligodendrocytes and AOP5-expression on astrocytes in rat brain (153), but their physiological roles have still to be elucidated. Immunolabeling against AOP9 is found on astrocyte processes in the periventricular region of the parenchyma and in the glia limitans (154), suggesting that AQP9 contributes along with AQP4 in facilitating water movements between cerebrospinal fluid and brain parenchyma. The observation that the distribution patterns of AQP4 and AQP9 in the brain are similar indicates that both water channel proteins have similar functions and may act in synergy. This suggestion is supported by experiments on mice after transient middle cerebral artery occlusion (154), in which an increased immunohistochemical signal to AQP9 has been found on astrocytes, indicating that AQP9 is also involved in cerebral edema formation. At present, no data is available in explaining AQP9-expression in malignant brain tumors, but it might be speculated that AOP9 will be found in a similar expression pattern like AQP4 (145,155).

4. AGRIN, THE DYSTROPHIN–DYSTROGLYCAN COMPLEX, AND THE BBB: A WORKING HYPOTHESIS

It is generally accepted that astrocytes play a decisive role in the maintenance of the barrier properties of the brain microcapillary endothelial cells (see above). As pointed out already, an interesting correlation exists between astroglial differentiation and BBB maturation. The polarization of astrocytes occurs concomitantly with the maturation of the BBB (106,156) and is not maintained by reactive (155) or cultured astrocytes (123,157).

An indirect proof of the relationship between the OAP-related polarity of astrocytes and the quality of the BBB is the observation that under brain tumor conditions when the BBB is known to be leaky, the OAP-related polarity of glial cells decreases (120). Remarkably, the density of OAPs in membranes of glioma cells was extremely low (120), and the AQP4 content as detected by immunocytochemistry was increased (145,155) (Fig. 3D). The apparent contradiction in the up-regulation of AOP4 and down-regulation of AOP4-positive OAPs in glioma cells can be explained only by the theory that under glioma conditions AQP4 exists separated from the OAPs in the glial membrane and is no longer restricted to the glial endfeet membranes. The restriction of AOP4-immunoreactivity at the endfoot membrane was maintained only where agrin was present in the perivascular basal lamina (145). If agrin was absent in the basal lamina, AQP4-immunoreactivity was randomly found across the whole surface of the cell. This would suggest that agrin is responsible for the restriction of AQP4 molecules at the glial endfoot membrane. However, agrin has no binding site to AQP4. It does,

bind to α -dystroglycan (79). Thus, α -dystroglycan has to be connected to AQP4. Indeed, they are found to be co-expressed in glial cells (Fig. 3A,B). The only connection between α -dystroglycan and AOP4 known so far is given by the dystrophin/utrophin-system, which includes the family member α 1-syntrophin. α 1-syntrophin has a binding site to dystrophin and a PDZ domain binding to AQP4 (95). As well, in glioblastoma, the binding strength between α 1-syntrophin and AQP4 seems to be strong enough to bind AQP4 and al-syntrophin together during their redistribution across the surface of the glioma cell (145). In contrast, dystrophin remains restricted at the endfoot membrane suggesting a cleavage of dystrophin/- α 1-syntrophin/AQP4-complex. Together with these observations, we found a loss of α -dystroglycan in the perivascular domain of glioma cells (145). Therefore, a loss of agrin corresponds with a loss of α -dystroglycan. a redistribution of AQP4/ α -syntrophin and a loss of OAPs including a severe reduction of OAP-related polarity of glioma cells. All these observations together lean in favor of the theory that the OAPs consist of more than AOP4. They also may contain components of the dystrophin–dystroglycan complex.

It has been shown that the truncated dystrophin isoform Dp71 is essential for the clustered localization of the weakly rectifying potassium channel Kir4.1 in retinal Müller (glial) cells (158). In addition, the PDZdomain of α -syntrophin can also bind to Kir4.1 (97). Kir4.1 is normally restricted to the endfoot membrane in astrocytes and retinal Müller glial cells (159,160). On the basis of co-localization of AQP4 and Kir4.1 in retinal Müller cells, and due the well-known fact that water fluxes are driven by ion fluxes, it was hypothesized that K⁺-clearance is coupled to water flux (127,150,151,161). Accordingly, in the α -syntrophin-deficient mouse in which AQP4 is delocalized across the glial surface, the K⁺-clearance was delayed (144). The authors argue that K^+ uptake would be facilitated if accompanied by water flux. In the hypoxic retina, Kir4.1 is down-regulated in retinal Müller cells compromising the spatial buffering capacity; as a consequence, intracellular K⁺ concentration increases and water flux is causative for the observed cell swelling (150). Alternatively, the cell swelling can also be due to a failure to release water at the endfoot membrane, because the molecular complex consisting of Kir4.1 and AOP4 may dissociate. In preliminary experiments in human glioblastoma tissue we were able to observe a redistribution of both anti-AOP4- and anti-Kir4.1-immunoreactivities across the surface of the glioma cell (Warth et al., in preparation). All of these observations together suggest that Kir4.1 may also be a constituent of the OAPs as well (Fig. 4), which under the conditions of glioma disintegrate from the arrays and redistribute as separate channels. An uncoupling of water transport through K^+ -siphoning has recently been postulated for such pathological conditions as brain contusion, bacterial meningitis and brain tumors (162). These can all arise due to cell swelling.
Interestingly, before the detection of the aquaporins, the OAPs were identified as morphologically similar to potassium channels (3,68,123,163). However, the molecular aggregation of different channels in one morphological structure has also been established at the subpial endfoot membrane and not just at the perivascular endfoot membrane suggesting that the OAP-related molecular arrays have no exclusive significance for the BBB.

In the α -syntrophin-deficient mouse, Amiry-Moghaddam et al. (164) described a prolongation in the survival time of the animals under hyponatremic conditions in comparison to wild type mice. The reason for the longer survival time might be the reduced formation of brain edema that was a consequence of the redistribution of AOP4 after its cleavage from α -syntrophin. However, as we were able to show, the cleavage of α -syntrophin from AQP4 does not seem to be a prerequisite for the redistribution of AOP4 (145). Rather, we believe that the loss of agrin reduces the OAP/AQP4-related polarity of astrocytes. The loss of agrin might be caused by an activation of the metalloproteinase-3 that has recently been reported to occur under conditions of cerebral ischemia (165). Furthermore, this could lead to a redistribution of "free" AQP4 molecules outside the OAP structure, a down-regulation of Kir4.1, a consecutive failure of spatial buffering of K^+ , followed by osmotic water influx and swelling. All of these processes together may represent key events for both the development of an edema and the loss of capability of the glial cell to maintain the BBB properties within the endothelial cell. It has already been suggested that a non-polarized astrocyte is unable to induce or maintain the complete set of BBB properties in endothelial cells, in particular the barrier permeability (166).

The barrier permeability is determined by both the tight junction-controlled paracellular and the caveolae-mediated transcellular permeability. The VEGF plays a central role in triggering angiogenesis and vascular permeability. The VEGF has been shown to induce the phosphorylation of occludin and ZO-1 that could result in both a dissociation of caveolin from the junction (167) followed by targeting to the luminal membrane. The VEGF receptor 2, also known as Flk-1, is closely associated with caveolin-1, the main molecule of caveolae (168). Caveolin-1 has also been shown to coprecipitate with occludin (169). Thus, tight junctions could play a role as a "sink" for caveolin-1, or, put the other way, occludin-bound caveolin-1 may be a stabilisator of tight junctions. Once dissociated from occludin, caveolin-1 could increasingly bind to the DDC, in particular to the NO synthase (170), which, at least in muscle cells, is associated with syntrophin (80). However, any information about a connection between NOS and endothelial dystroglycan is lacking so far. On the other hand, eNOS is present in BBB endothelial cells and its activity increased in permeable blood vessels (171). As well, NO donors were shown to disrupt the BBB (172). This effect of NOS on the permeability seems to lie downstream from the VEGF effect (173, 174).

Up to this point in time, we recognize that we have been unable to identify the regulatory mechanisms of the different signaling cascades that are involved in brain endothelial cell permeability. More research is needed to understand the DDC components in endothelial cells, the signal transduction between these components, the interplay between VEGF and tight junctions, and the signals leading to the loss, cleavage and/or downregulation of agrin.

5. CONCLUSIONS

It has been 120 years since the discovery of the BBB by Paul Ehrlich. Since that discovery. BBB research has focused on the morphological description of the barrier using mainly conventional histological and electron microscopical methods as well as methods to demonstrate the tightness of the barrier against a variety of low and high molecular weight substances (for review see Ref. 175). Tight junctions have been described as a network of protein particles using freeze-fracture electron microscopy. With time, scientists have discovered protein components of the tight junctional complex and identified the protein particles within the freeze-fracture replicas. This discovery was followed by the realization that these tight junctional complexes are dynamic structures. The next step is to characterize the interplay of these molecules and their regulation through an association with cellular signaling cascades and cytoskeletal components. It has been shown that the endothelial barrier in the brain differs from epithelial barriers. The fact that epithelial, but not endothelial cells, are able to form a high resistance and low permeability barrier in vitro sheds light on the significance of the brain microenvironment in the formation and maintenance of the barrier in vivo. This microenvironment consists of endothelial cells, pericytes, microglial cells, astrocytes, neurons, and the ECM which itself forms a microcosmos of its own. All of these components may or may not operate simultaneously or independently during development or during pathological derangements. The greatest challenge in the future will be to characterize the mechanisms involved in BBB differentiation and pathology in order to understand the key principles of barrier formation in the CNS.

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Molecular Modulation of the Blood–Brain Barrier During Stroke

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1. INTRODUCTION

Stroke is the third leading cause of death and a leading cause of long-term disability in Western civilization (1). During stroke, a decrease in oxygen levels (hypoxia) due to a cessation in blood flow and an increase in cerebral vascular permeability and vasogenic cerebral edema occur, which contribute to neurological deficits associated with brain infarct (2). Most of what is known about the effects of stroke on the brain is in neurons. Ischemic stroke is known to cause neuronal cell death and alter the cellular activity of neurons. The ultimate goal of stroke research is the development of therapeutic agents, which will improve patient clinical outcome. Most stroke research currently investigates the cellular mechanisms associated primarily with neuronal damage. Additionally, a majority of these therapies aim at reducing neurological deficit by modulating neuronal intracellular mechanisms. While this research is compelling, recent research investigating ischemia/ reperfusion implicates blood-brain barrier (BBB) integrity as a primary factor in the clinical outcome of stroke patients. It has become increasingly clear that the BBB plays an important role in the pathophysiology of several conditions including stroke, human immunodeficiency virus (HIV) dementia, experimental autoimmune encephalomyelitis (EAE), and inflammation

(3–7). It is now known that the extent of BBB dysfunction has profound effects on pathophysiologies associated with the central nervous system.

We have reviewed the molecular changes that occur at the level of the BBB endothelial cells during ischemia/reperfusion, which result in BBB permeability alterations. Additional discussion of the intracellular signaling pathways involved in these molecular changes is also examined, including commentary on the role of the glial and neuronal factors on the loss of integrity of the BBB. Finally, we discuss the implications of BBB disruption on therapeutic treatments for stroke patients.

1.1. The Blood-Brain Barrier/"Neurovascular Unit"

Brain microvessels, which form the BBB, are lined with specialized endothelial cells, as well as pericytes and astroglia-foot processes. The BBB forms a metabolic and physiological barrier to separate the peripheral circulation from the brain extracellular fluid. Microvessels of the BBB lack vesicular transport and fenestrations, while having a high level of metabolic activity and efflux pumps (8-10). The lack of fenestrations is due to the presence of tight junctions (TJ) and adherens junctions (AJ), which restrict paracellular transport of molecules across the BBB. The presence of efflux pumps, transporters and channels control the flux of ions, water, peptides and drugs across the BBB. The net result is decreased permeability of the cerebral microvasculature and a transendothelial resistance (TEER) of $\sim 2000 \,\Omega^* \,\mathrm{cm}^2$ (8-10). While it was once thought that the BBB was static, it is now understood that the BBB is actually a dynamic structure, which is tightly regulated during physiological and pathophysiological states. This regulation involves modulation of the TJ, AJ, ion channels and transporters at the BBB (11).

While the traditional concept of the BBB remains, our current understanding of the BBB also considers the complex interactions between the endothelial cells, astroglia, pericytes and neurons (i.e., the "neurovascular unit"). Each of these cell types in the "neurovascular unit" contributes to the physiological and pathophysiological activity of the BBB. It is this "neurovascular unit" which responds to specific stimuli in order to induce a change in endothelial cells of the BBB. Current discussions of stroke physiology must now consider the role of the "neurovascular unit," since it is the collective response of all the cells of the central nervous system to ischemia/reperfusion which contributes to the pathophysiology of stroke.

1.1.1. Tight Junctions

The presence of TJs is a hallmark feature of endothelial cells of the BBB. Much of the research on the BBB during stroke focuses on the effects of ischemia/reperfusion on the TJs, since these proteins are extremely important for restricting/controlling paracellular transport. The proteins



Figure 1 Illustration of proteins, which are expressed at the BBB. Each of the structures represented in this illustration have been demonstrated or are potentially modified during ischemic stroke. *Source:* From Ref. 10.

of the TJ are localized to the apical side of the paracellular cleft. Structurally, TJs are complexes of transmembrane proteins and accessory proteins involved in cell–cell adhesion (Fig. 1 and Chapter 3). The major integral membrane proteins of the TJs are occludin, claudin (which there are at least 20 different isoforms) and junctional adhesion molecule (JAM). Occludin and the claudins are connected to the actin cytoskeleton by scaffolding (accessory) proteins, principle of which are the zonula occludens (ZO-1, ZO-2, and ZO-3). Each of these proteins interacts to form structural and functional protein complexes necessary for normal function of the BBB (9–12).

1.1.2. Transporters and Channels

The expression of transporters and channels in brain microvessel endothelial cells is important for maintaining BBB structure and function. These proteins are responsible for regulating transcellular transport across the BBB and many of them have distinct localization to either the luminal or abluminal portion of the endothelial cell. The activity of these transporters and channels are tightly regulated to ensure that physiological and metabolic brain homeostasis is intact. There is a wide variety of transporters and channels which are present at the BBB. They include but are not limited to efflux transporters (MDR, BRC, and ABC), ion channels (TRP channels, K^+ channels and Ca²⁺ channels), ion antiporters (Na⁺/H⁺ exchanger and Na⁺/K⁺/2Cl⁻ co-transporter), energy dependent transporters (Na⁺/K⁺ ATPase) and the aquaporins (Fig. 1 as well as Chapter 16) (8,10,13–24). All of these proteins are necessary to help maintain cellular polarity and regulate the passage of ions, peptides and drugs across the BBB.

1.2. Defining Stroke in Research

Clinically, stroke is categorized as either focal or global ischemia. Focal ischemia is defined as a loss or reduction in blood flow to a specific brain region. Global ischemia is defined as a loss or reduction in blood flow to the entire brain. While this distinction is important in the clinical diagnosis of stroke, it is the duration and degree of reduced blood-flow and the extent of reperfusion, which determines clinical outcome for patients. It is these factors together which alter the BBB during and following stroke. For this reason, both in vitro and in vivo models have been developed to investigate stroke.

1.2.1. Hypoxia/Reoxygenation Models

There are several widely accepted in vitro BBB models. They include endothelial cell lines or primary brain microvessel endothelial cells (BMEC) isolated from human, bovine, porcine and murine species (Fig. 2) (25–28). Modifications of in vitro models involve co-culturing the BMECs with astroglia for a more accurate representation of the BBB. The BMECs are then exposed to normoxia (room-air), hypoxia (low oxygen) or anoxia (no oxygen) followed by reoxygenation (re-exposure to room-air) in order to mimic stroke (i.e., in vitro hypoxia model). Also, an in vivo hypoxia model in which animal subjects are exposed to normoxia, hypoxia and hypoxia/ reoxygenation has been established in our laboratory (29). The exposure time to these hypoxic conditions may vary depending on the experimental paradigm being studied.

1.2.2. Middle Cerebral Artery Occlusion Model

There are several different in vivo models used to investigate stroke. Typically, investigators use variations of the middle cerebral artery occlusion (MCAO) model of stroke (30). Variations can include the length of time the artery is occluded, whether the occlusion is permanent vs. transient, whether a single artery or both arteries are occluded and the reperfusion time following the occlusion. Figure 3 shows the resulting infarct from



Figure 2 Brain microvessel endothelial cells in culture during normoxic conditions. Cells were stained with anti- β -catenin to show the endothelial cell junction.

a sham and permanent MCAO [based on a method developed by Zea-Longa et al. (30)] in Sprague–Dawley rats from our laboratory.

All of these are valid models to investigate the BBB and stroke, with each having strengths and weaknesses. However, it is important to note that physiological and molecular changes may differ between models and experimental paradigms. For example, BBB alterations which occur following ischemia are different from those which occur with post-ischemic reperfusion. For the purposes of this chapter, we will discuss the cellular response of the BBB to ischemic insult and reperfusion injury and how the similarities and differences in this response can present challenges and opportunities for treatment of stroke victims.

2. MODULATION OF THE BBB FOLLOWING HYPOXIA/ISCHEMIA

During hypoxia/ischemia, both in vivo and in vitro stroke models have demonstrated that cerebral vascular permeability increases leading to a subsequent increase in cerebral edema and that these changes are due to a disruption of the BBB (3,4,6). The processes involved in these changes

MCAO



Figure 3 Scanned images of coronal forebrain sections stained with 2% triphenyltetrazolium chloride 4 hr following sham (*left*) or permanent MCAO surgery (*right*) in Sprague–Dawley rats. The planar images correspond to 2 mm section surface of approximately -0.26 to -0.40 mm in reference to Bregma. The unstained regions of the brain indicate ischemic infarct damage due to occlusion of the MCA.

in BBB permeability are not completely understood. However, in the past 10 years studies have begun to elucidate the mechanisms involved in these changes at the level of endothelial cells and at the level of the "neurovascular unit." At the level of the endothelial cell, disruption of the BBB is due to alterations in the TJ and AJ structure and function, as well as changes in ion channel and efflux transporter activity. At the level of the "neurovascular unit," BBB disruption can involve release of neurotransmitters and growth factors, as well as humoral factors that act at the level of the endothelial cell. How these changes at the "neurovascular unit" contribute to the changes at the level of the BBB is a very active field of research.

2.1. Molecular Alterations of the Tight Junctions

As stated earlier, stroke causes an increase in vasogenic edema, which can be attributed to an increase in BBB permeability. Using in vitro models to determine the role of hypoxia/reoxygenation on BMECs, recent investigations have begun to elucidate the molecular changes leading to increases in BBB permeability. In studies by Mark et al. (31), an increase in actin protein levels and actin stress fibers was observed following hypoxic insult, while hypoxia alone had no effect on protein expression of the TJs, occludin,

claudin-1, or ZO-1/2. Following hypoxia/reoxygenation, increases in protein expression of occludin, claudin-1, and ZO-1/2 were observed. These results differ slightly from data of Fischer et al.(32), which demonstrated a decrease in ZO-1 protein expression following hypoxia. Interestingly, experiments using immunofluorescent microscopy demonstrated changes in the cellular localization of the TJ proteins, occludin and ZO-1/2 following hypoxic insult, which returned to control levels upon reoxygenation (31,32). These changes in TJ protein expression and localization correlated well with observed cell permeability changes following hypoxia and hypoxia/reoxygenation (31). Similar results by Fischer et al. showed ZO-1 localization was altered following hypoxia (32). These results are summarized in Table 1. Further confirmation that changes in TJs contribute to hypoxia/reoxygenation-induced changes in cell permeability come from a study by Witt et al. (29). In this newly developed in vivo hypoxia model, a change in occludin expression following hypoxia/reoxygenation correlated extremely well with changes in BBB permeability to ¹⁴C-sucrose (29). Together these results provide evidence that the structure of the TJ is altered causing an increase in BBB permeability (29,31,32). These studies, however, do not explain the cellular-based mechanisms behind the hypoxia-induced and hypoxia/reoxygenation-induced changes in the TJs.

		Hymoryia /
	Hypoxia	Reoxygenation
Actin		
Protein expression	↑	Ť
TJ localization	Stress fibers	Stress fibers
Claudin-1		
Protein expression	\leftrightarrow	\leftrightarrow
TJ localization	\leftrightarrow	\leftrightarrow
Occludin		
Protein expression	\longleftrightarrow	Ť
TJ localization	Away from TJ	Return to TJ
ZO-1		
Protein expression	$\leftrightarrow/\downarrow$	Ť
TJ localization	Away from TJ	Return to TJ
ZO-2		
Protein expression	$\leftrightarrow / \downarrow$	1
TJ localization	Away from TJ	Return to TJ

Table 1 Summary of Tight Junction Protein Changes which Occur in Isolated Brain

 Microvessel Endothelial Cells Following Hypoxia and Hypoxia/Reoxygenation

Data presented in this table is compiled from Refs. 31 and 32. (\uparrow) Increase, (\downarrow) decrease, (\leftrightarrow) no change.

Recently, it has been shown that following hypoxia/ischemia, proteins which compose the TJs are phosphorylated (11). Specific post-translational modifications, such as the phosphorylation of specific residues of the TJ proteins, can regulate the function of a TJ protein in different ways as outlined in Figure 4. All of these changes to the TJ can cause the paracellular cleft to open thus increasing vascular permeability of the BBB. In fact, studies have demonstrated a correlation between increased phosphorylation of TJ proteins and increased permeability in both kidney (Madin–Darby canine kidney, MDCK) and BMEC models (33,34).

Examples of how phosphorylation of the TJ protein occludin can alter location, function and interactions with other proteins are as follows: Occludin is able to be phosphorylated at multiple residues (11). When it is phosphorylated at Ser/Thr residues, localization of the protein is controlled (11,35). Meanwhile, phosphorylation of Tyr residues on occludin appears to target this protein for degradation (36). Furthermore, it is known that phosphorylation of occludin affects its interactions with the accessory scaffolding proteins,



Figure 4 Diagram illustrating potential mechanisms by which post-translational modification can alter the TJ structure and function. Post-translational modifications can (1) alter the interaction of the cytoplasmic domain of transmembrane TJ proteins with intracellular TJ and cytoskeletal proteins, (2) destabilize the extracellular interactions between TJ protein complexes, (3) designate the TJ proteins for breakdown or (4) cause relocalization of the TJ protein to an intracellular compartment or to the basolateral membrane. *Source:* From Dr. Ken Witt, Davis Laboratory, University of Arizona.

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ZO-1, ZO-2, and ZO-3 (37). These structural and functional changes caused by phosphorylation of occludin can lead to increases in cell permeability.

The regulation of the TJ complexes is extremely complicated with the TJ proteins being modified at varying times and to varying degrees. Different post-translational modifications can lead to different TJ structure changes at different times. To add to this complexity, several intracellular signaling molecules, which regulate endothelial cell permeability via alterations in TJ protein expression, phosphorylation, and localization, have been discovered. These signaling molecules include cAMP, cGMP, Ca²⁺, tyrosine kinases, small G-proteins, mitogen activated protein kinases (MAPK), nitric oxide (NO), and protein kinase C (PKC) (11,38–41).

2.1.1. Signaling Mechanisms Leading to Alterations in the TJ

In recent years, the intracellular signaling mechanisms behind changes in BBB permeability within stroke models have been thoroughly investigated. In vitro studies have been extremely useful in helping us determine which signaling mechanisms are being activated during hypoxia/ischemia. Hypoxia-induced changes in TJ structure in BMECs have been shown to involve increases in vascular endothelial growth factor (VEGF), whereas inhibition of VEGF attenuates the hypoxia-induced increase in BBB permeability (42–45). In addition, studies by Mark et al. (46) demonstrated that hypoxia increases NO release in BMECs and that the NO donor DETANONOate mimics the hypoxia-induced cell permeability changes. Furthermore, inhibition of NO synthase by N^{\u03c0}-nitro-L-arginine (L-NAME) and 1400 W reduce the effect of hypoxia on cell permeability (46). The mechanisms involved in the VEGF- and NO-mediated changes in cell permeability are still being investigated. Studies by Fischer et al. (42) suggest that hypoxia increases VEGF, which increases NO production to cause a change in cell permeability by altering the TJ structure and function. It is hypothesized that NO may directly modify the TJ proteins by nitrosylation or nitrosation, or NO may activate an intracellular signaling cascade which can then modify the TJ proteins.

While activation of NO is important in mediating the changes in BBB permeability following hypoxia, it is not the only signaling pathway that is involved. Studies have now demonstrated that the hypoxia-induced changes in BBB permeability also involve activation of phospholipase C γ , phosphatidylinositol 3-kinase (PI3K)/Akt and protein kinase G (PKG) (47). Furthermore, inhibition of these intracellular signaling pathways attenuates the hypoxia-mediated changes in cellular localization of ZO-1/2 (47). Additionally, it is possible that the activation of these signaling pathways occur due to the autocrine action of VEGF on endothelial cells and downstream activation of PLC γ and PI3K/Akt, which in turn upregulates NO release (47). This increase in NO release then activates soluble guanylate cyclase, which stimulates PKG to alter BBB permeability (47). The mechanisms by which PKG alters the localization of ZO-1/2 remain to be established. However, it has recently been shown that hypoxia increases phosphorylation of ZO-1 (11). This may be a potential mechanism by which ZO-1 localization is altered from the membrane to the cytoplasm thus causing an increase in cell permeability.

While this is one potential mechanism by which hypoxia and ischemia alter BBB TJs, several other intracellular signaling pathways are also candidates. Studies by Brown et al. (48) investigated the role of intracellular Ca^{2+} on BMEC cell permeability. These studies showed that increasing intracellular Ca^{2+} alone has no effect on cell permeability. However, during hypoxic insult, increases in intracellular Ca^{2+} are observed and chelation of Ca^{2+} (by BAPTA/AM) significantly attenuates the increase in cell permeability caused by hypoxic insult (48). The actual source of intracellular Ca^{2+} in these studies is under investigation. Additionally, it is not known if these hypoxia-induced increases in intracellular Ca^{2+} are involved in stimulation of down-stream intracellular signaling pathways.

Recently, studies have begun to investigate the activity of PKC in BBB endothelial cells during hypoxia/ischemia. PKC is an enzyme which phosphorylates serine and threonine residues on numerous target proteins including the TJ proteins. There are 11 isozymes of PKC, and they are classified based on their mode of activation, which can include a requirement of intracellular Ca²⁺ for activation (49,50). Studies in epithelial and endothelial cells have shown that the PKC activator, phorbol 12,13 myristate (PMA), increases cell permeability (11).

Preliminary studies from our laboratory have shown that hypoxia stimulates total PKC activity compared to normoxic controls in BMECs. Additionally, overexpression of PKC8 increases cell permeability and causes disruption of the TJs (77). Several studies also suggest that PKC α is important in regulating cell permeability following ischemia and inflammatory stimulation while others have implicated a prominent role for PKC β in modulating endothelial cell permeability (51). Further supporting a role for PKC in TJ regulation are data showing that ischemic preconditioning stimulates a translocation of PKC α to the membrane (52). In addition to a role for the Ca²⁺-dependent PKCs in regulating endothelial cell permeability, the importance of the novel and atypical PKCs is also being investigated. For example, immunofluorescence studies have shown that $PKC\zeta$ co-localizes with ZO-1 in MDCK and Caco-2 cells (53). How these PKCs regulate BBB permeability is not clear. However, it has been demonstrated that PKC is able to phosphorylate ZO-1 and ZO-2 and activation of PKC leads to a dephosphorylation of occludin (11).

Besides direct phosphorylation of the TJs, it is also suggested that PKC may interact with other intracellular signaling molecules to indirectly regulate the tight junctions. For example, studies have demonstrated that PKC is upstream of the NO signaling pathway and thus increases NOS phosphorylation and NO production (51,54). Further support for interaction between PKC and NO is a study in which pharmacological inhibition of NO partially blocks the PKC-induced alterations in permeability of coronary venules (55). Also, PKC δ has been shown to be upregulated following transient focal ischemia in rat (56). This upregulation of PKC δ expression and its sustained kinase activity has been associated with the NF- κ B transcription factor (57). Further studies have also demonstrated an importance for PKC in VEGF-induced increases in cellular permeability (51).

While this discussion has focused on intracellular signaling pathways, which may contribute to the disruption of the BBB, it is also important to consider those intracellular signaling pathways, which strengthen the BBB. Little is known about such pathways, but it is important to remember that the endothelial cell is tightly regulated so that normal function is maintained. For example, Fischer et al. (47) determined that inhibition of the p38 MAPK had no effect on hypoxia- or VEGF-mediated changes in cell permeability in vitro. However, if p38 was inhibited following MCAO there was actually a further increase in vascular leakage compared to that seen with MCAO alone (58). These data suggest that activation of p38 following MCAO has a protective effect on the BBB and that its inhibition actually exacerbates damage following stroke. These data indicate that certain molecular changes which occur following stroke may be beneficial in maintaining BBB integrity.

2.2. Matrix Metalloproteases and the BBB

Recent research has begun to investigate the importance of the extracellular matrix (basal lamina) on BBB permeability during stroke. The basal lamina is composed of collagen, fibronectin, laminin and heparin sulfate. When this lamina is degraded, the blood vessels are weakened. Matrix metalloproteases (MMPs) are zinc dependent and have the ability to degrade basal lamina (59). Following ischemia/reperfusion, MMPs have been shown to be up-regulated in the brain (60,61). More specifically, it has been shown that both MMP9 and to a lesser extent MMP2 levels were increased over time following ischemia/reperfusion (60,61). This increase in MMP2 and MMP9 levels correlates with the increase in sucrose diffusion across the BBB, which is observed following ischemia/reperfusion (61). Additionally, these same studies demonstrated that inhibition of MMP with the pharmacological agent BB-1101 reduced sucrose uptake, which occurred following ischemia/reperfusion (61).

While these studies suggested that the MMPs cause BBB disruption via extracellular matrix degradation, several studies have also investigated whether MMPs degrade TJ proteins. In a study where BMECs were treated with the tyrosine phosphatase inhibitor phenylarsin oxide (PAO) there was an MMP-dependent loss in BBB integrity (62). The authors of this study further demonstrated that the extracellular component of occludin was cleaved following PAO treatment and that inhibition of the MMPs blocked this cleavage. Claudin-5 and ZO-1 remained intact in these studies (62). These data suggest that the up-regulation of the MMPs may also cause breakdown of the TJ structure by cleaving the extracellular domain of occludin, thus disrupting the interaction between occludin on endothelial cells (Fig. 4).

2.3. Modulation of Channels and Transporters

While TJs are important for limiting paracellular transport at the BBB, the channels and transporters are also key components for maintaining cerebral physiological and metabolic homeostasis. While it is understood that these proteins are important for normal physiological function, it is not known what role they play during stroke pathophysiology. However, since a major consequence of stroke is the formation of cerebral vasogenic and cytotoxic edema, it is important to understand the effect of stroke on the function of channels and transporters at the BBB.

2.3.1. Aquaporins

In the early 1990s, the presence of water channels termed aquaporins was discovered in the plasma membranes of several different cell types. There are 11 subtypes of aquaporin located throughout the body and six have been localized to the brain (63). Aquaporin 4 (AQP4), one of the first subtypes to be discovered, was found to be highly expressed in the brain (63). In fact, AQP4 expression has been found on the astrocyte foot processes, which are adjacent to endothelial cells of the BBB (63,64). Additionally, it has been determined that endothelial cells themselves express low levels of AQP4 localized to both the abluminal and luminal membranes (23).

The importance of AQP4 in the regulation of cerebral fluid homeostasis during normal physiology and stroke are only now being recognized. It has been shown that AQP4 mRNA levels increase following ischemia (65). Recent studies have demonstrated a reduction in edema formation following ischemic stroke in AQP4 null mice compared to mice with normal AQP4 expression (66). Other studies have shown that there is a mislocalization of AQP4 away from the astrocyte foot processes but not the endothelial cells. In these models of water intoxication and hyponatremia, this mislocalization delays edema formation (23). These data suggest that AQP4 on the astrocyte endfeet processes contributes to edema formation following stroke. Furthermore, drugs targeting AQP4 may limit edema formation after stroke. However, the function of AQP4 in BBB endothelial cells needs to be further investigated in order to fully understand how AQP4 contributes to infarct damage following stroke.

2.3.2. Glutamate

During ischemic stroke, there is a large release of glutamate from neurons, which leads to excitotoxicity by binding to N-methyl-d-Aspartate (NMDA) receptors. This excess glutamate is one factor, which is responsible for cyto-toxic edema of neurons (67). While this process in neurons has been well established, the effect of glutamate on endothelial cells of the BBB has not been fully investigated. Studies have shown that BBB endothelial cells express both NMDA and metabotropic glutamate receptors (67–69). Additionally, studies have demonstrated that circulating inflammatory mediators can stimulate a release of glutamate, which disrupts the BBB via metabotropic receptors (70). More recently, in vitro studies showed that cytotoxic levels of glutamate reduce BBB integrity by binding to the NMDA receptor, while activation of metabotropic receptors increased BBB electrical resistance suggesting a tightening of the BBB (67).

The exact mechanisms by which glutamate causes these changes are unknown. However, based on our knowledge of the glutamate receptor and the BBB there are several favored hypotheses. Upon receptor binding by glutamate, the NMDA receptor acts as an ion channel for Ca²⁺. K⁺ and Na^+ , with a preference for Ca^{2+} (67). As discussed previously, increases in intracellular Ca²⁺ have been shown to alter TJ organization, cell morphology and endothelial cell tension (48,67). One way that glutamate may be altering BBB permeability is by increasing the levels of intracellular Ca^{2+} , thus activating intracellular signaling pathways to alter TJ structure. In that manner, glutamate may be causing toxicity similar to the toxicity in neurons. In addition to altering Ca²⁺ flux, prolonged NMDA receptor activation leads to increases in reactive oxygen species (ROS). The ROS are a major contributor to BBB dysfunction by altering TJ structure both directly and indirectly. Based on this knowledge, the authors investigated the role of intracellular Ca²⁺ and ROS on the NMDA receptor-mediated changes in BBB function. These studies demonstrate the role of both intracellular Ca^{2+} and ROS in the BBB alterations due to glutamate (67).

2.3.3. Exchangers and Transporters at the BBB

The maintenance of ion concentration, pH and cell volume at the BBB is extremely important during both normal physiology and pathophysiology. One mechanism that is used by the cell is regulation of the concentration of ions in the cell and the transport of ions such as Na^+ across the cell membrane. It is the activity of exchangers and transporters such as the Na^+/H^+ exchanger (NHE), Na^+/K^+ ATPase, and $Na^+/K^+/Cl^-$ cotransporter, which contribute to maintaining ion balance in the cell. During stroke, osmotic and ion balance are altered and this can lead to activation of ion transporters and exchangers.

Our understanding of how these exchangers and cotransporters contribute to neurovascular damage during stroke is minimal. However, current research has started to investigate the effects hypoxia/ischemia has on their expression and function at the BBB. For example, recent in vitro studies have shown that hypoxia/aglycemia decreases Na^+/K^+ ATPase activity and increases $Na^+/K^+/2Cl^-$ cotransporter activity (24). These results suggest that stimulation of the $Na^+/K^+/2Cl^-$ cotransporter may aid in removing excess K^+ from brain extracellular fluid, potentially as a neuroprotective mechanism (24).

Regulation of pH in the brain and endothelial cells is also of utmost importance. In vivo and in vitro studies have demonstrated that inhibition of the NHE reduces ischemic injury by attenuating both edema formation and increases in Na⁺ concentration (17,71–73). More importantly, it has been discovered that inhibition of NHE blocked endothelial cell injury and prevented disruption of the BBB. Thus, the protective effects of NHE inhibition may be partially due to maintaining BBB function (71).

3. POTENTIAL STROKE THERAPIES AND THE BBB

To date, most therapies for stroke victims focus on reducing the degree of neuronal damage following a stroke. While decreasing neuronal damage is of utmost importance in improving patient clinical outcome, it is also important to realize that a dysfunctional BBB contributes to this central nervous system damage. One of the major consequences of stroke is vasogenic edema, due to a disrupted BBB. This edema contributes to neuronal damage. Therefore, it is important to consider the changes in the BBB, which are contributing to infarct size and neurological damage.

3.1. Growth Factors and Neurotransmitters

An example of the importance of understanding how the BBB is altered following stroke is in the use of growth factors for post-stroke therapy. Growth factors from both the circulation and the "neurovascular unit" can have prophylactic and/or deleterious effects on the brain following ischemic insult. For example, it has been shown that VEGF is neuroprotective (74). However, as discussed previously, it has been demonstrated that there is an increase in VEGF, which leads to an increase in BBB permeability (42–45). Additionally, studies have shown that animal subjects treated with exogenous VEGF following ischemia had an increase in BBB permeability, which contributed to an increase in vasogenic edema and hemorrhage in the brain (44). So while VEGF is neuroprotective, its ability to alter the BBB and cause other brain damage makes VEGF a questionable therapeutic agent.

Molecular Modulation of the Blood–Brain Barrier

It is this conundrum that has led researchers to investigate the potential therapeutic benefits of other growth factors. A recent study has investigated the effects of hepatocyte growth factor (HGF) on ischemic injury (75). In these studies, the HGF gene was delivered to the brain prior to MCAO. Following MCAO, the effect of increased HGF expression in the brain was determined. Increases in HGF reduced infarct volume and behavioral deficits following stroke indicating a neuroprotective function of HGF. More importantly, HGF did not increase BBB permeability and in fact, attenuated the increase in BBB permeability seen following stroke (75). The exact mechanism by which HGF exerts these effects needs to be investigated. Additionally, it will need to be determined how treatment with HGF following the ischemic event affects neurological and BBB deficits. In a unique approach, the investigators used a viral gene delivery system in order to express HGF in the brain (75). This system required delivery of the gene several days prior to the MCAO in order for the gene to be expressed. Since most stroke patients are not treated until after having a stroke, it is very important to investigate the effects of HGF treatment on ischemic injury when the HGF has been delivered after the stroke. This will help to determine the true therapeutic value of HGF.

In contrast to VEGF, the fact that glutamate is a major contributor to neuronal cell death during stroke and also contributes to vasogenic edema by increasing BBB permeability presents a unique target for drug therapy following stroke. By developing a drug that can either regulate the release of glutamate or can scavenge the glutamate following release, one may be able to prevent neuronal cell death and BBB disruption. Both of these effects would be beneficial to the patient and may help improve clinical outcome. This treatment may also prevent potential side effects that occur with increased delivery of drugs across the BBB.

3.2. BBB Effects on Drug Delivery

In addition to understanding how BBB changes can affect choice of therapies, it is also important to understand how an altered BBB can affect the delivery and efficacy of a particular drug. Risks accompany the use of all drugs and potential therapies. For example, many stroke victims undergo thrombolytic therapy to help improve clinical outcome following an ischemic event. This therapy can involve administration of intravenous recombinant tissue plasminogen activator (rtPA) (59). However, rtPA therapy has an increased risk for hemorrhage and a higher mortality rate (59,76). What is causing this increased risk of hemorrhage and even death? Could the fact that the BBB is disrupted contribute to these risks?

In studies where MMPs were inhibited, thus causing a decrease in vascular permeability, there was an improvement in the mortality rate of rats, which had been treated with rtPA following ischemia/reperfusion (76). These studies suggest that the increased opening of the BBB which can occur with ischemia, and in particular with post-ischemic reperfusion, affects the delivery of therapeutic agents and thus affects the clinical outcome (neurological damage and survival) of stroke patients. Furthermore, these studies demonstrate the importance of understanding the changes, which are occurring at the level of the BBB. Treatments that decrease BBB permeability may directly improve clinical outcome by decreasing edema and thus neurological damage, but they may also improve clinical outcome by decreasing the risk for hemorrhage and increased edema that comes with other post-stroke therapies.

4. CONCLUSIONS

Stroke is an event that affects the whole brain (including the "neurovascular unit") and is not an event, which only affects neurons. During stroke there is an increase in vasogenic edema due to disruption of the BBB. This disruption can involve alteration in the expression and activity of TJs, channels or transporters. Understanding the changes that occur at the BBB during stroke may help us prevent or reduce damage from stroke by targeting treatments, which would prevent BBB disruption. Additionally, there are a variety of cellular mediators, which cause these changes in the BBB. Some of these cellular mediators are activated to induce BBB disruptions, while others may be stimulated to prevent disruption of the BBB. Either way, an increase in the knowledge we have regarding the intracellular mechanisms which are activated during stroke will help us develop therapies which can then improve the outcome of patients. Improved knowledge of the BBB during stroke will also help us to understand how therapeutic drug kinetics may be altered at the BBB following ischemia/reperfusion. As we have already established, a compromised BBB leads to changes in brain uptake of therapeutic drugs and this can have a dramatic effect on stroke victims.

There is a vast array of molecular changes, which are occurring at the BBB during ischemic stroke. This chapter highlights just a few of these changes. It cannot be said that one molecular alteration is more important or contributes to neurological damage more than another. It is the composite of these changes, which leads to BBB disruption. Each of these molecular pathways is a potential target for therapies to treat stroke. Our knowledge of the role of each of these changes in BBB disruption will help us to potentially prevent stroke and better treat stroke victims in the future.

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Apolipoprotein E, the Blood–Brain Barrier, and Alzheimer's Disease

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1. CEREBROVASCULAR ASPECTS IN AD

1.1. Vascular Risk and AD

Alzheimer's disease (AD) is the most common cause of dementia. It is characterized by a progressive loss of higher cognitive functions. The strongest risk factor for sporadic AD is old age, which also coincides with the increased occurrence of cerebrovascular lesions (Table 1). Additional risk factors associated with AD vascular alterations include some forms of heart disease, atherosclerosis, high plasma cholesterol, increased fat intake, history of hypertension, diabetes mellitus, head injury, stroke, systemic inflammation and ApoE e4 (1,2). The ApoE genotype is the prime susceptibility factor for sporadic AD (3). The ApoE genotype has been linked to an increased risk and reduced age of onset of AD. The ApoE has also been associated with VaD, mild cognitive impairment (MCI) (4), impaired recovery after brain trauma, amyotrophic lateral sclerosis and Pick's disease and, more recently, also with the risk and age of onset of Parkinson's disease (5). The ApoE4 was found to interfere with cognitive functions in non-diseased
Age
Family history of dementia
Transient ischemic attacks (TIA)
Strokes
Atherosclerosis and coronary heart disease
Increased markers if peripheral vascular disease-homocysteine, cholesterol
Deregulation of blood pressure-hypertension or hypotension
Diabetes type-II
Smoking
Presence of Apolipoprotein E-E4 allele

The odds ratios for the various factors have been determined to be 1.7–17.0. *Source*: From Refs. 1, 9, and 43.

adult men carrying ApoE4 in comparison with non-carriers (6,7). Memory deteriorates in ApoE4 carriers before the symptomatic occurrence of MCI, prior to age 60 (8). It remains to be clarified how ApoE4 affects cognitive functioning and neurodegenerative processes. Nevertheless, the ApoE genotype may be a leading aspect in the modification process of cerebrovas-cular functions during ageing and the increase of the predisposition to the pathogenesis of AD (9).

1.2. Cerebrovascular Changes in AD

The neuropathological hallmark of AD comprise extracellular deposits of amyloid- β in form of amyloid plaques and cerebral amyloid angiopathy (CAA), as well as the intracellular accumulation of hyperphosphorylated tau, which characterizes neurofibrillary tangles. Other features include substantial loss of synapses, a decline in cholinergic transmission, increased markers of oxidative stress and low-grade inflammatory responses (10). However, the patients with Alzheimer's lesions may also show evidence of cerebrovascular pathology. While these cases may be diagnosed as mixed dementia or VaD (with predominant CAA), it is increasingly evident that similarities in pathological, symptomatic and neurochemical features as well as cholinergic deficits between AD and VaD exist (11). Indeed, more than 30% of AD cases may exhibit cerebrovascular pathology, which involves microvessels and the cellular entities that compose the BBB. Profound morphological and biochemical changes of the microvasculature have been observed in brains of late-onset AD subjects. These entail degenerative changes in endothelial and vascular smooth muscle cells, variable degrees of macro and microinfarction and white matter rarefaction related to small vessel disease (Table 2). The CAA involves the degeneration of the larger perforating arterial vessels as well as the cerebral capillaries as seat of the

 Table 2
 Vascular Lesions and Small Vessel Disease in Alzheimer Type of Dementia

Presence of Aβ-CAA and CAA-related cerebral hemorrhages
Degeneration and intracellular changes, e.g., mitochondria, tight junctions, in the
cerebral endothelium
Basement lamina thickening and collagen accumulation
Small vessel disease including hyalinosis, fibroid necrosis and perivascular changes
Localization of inflammatory mediators and cell adhesion molecules
Ischemic white matter lesions
Microinfarcts and lacunes
Presence of lobar and intracerebral hemorrhages

Source: From Refs. 1, 9, 11, 133, and 216.

BBB. The CAA is also associated with an increased susceptibility to intracerebral hemorrhages. Moreover, changes were observed in the expression of glucose transporters, in Na^+/K^+ ATPase, in adhesion molecules, such as the intercellular adhesion molecule-1, in collagen components, in perlecans, in carnitine acetyltransferase, in α -Actin and in amyloid- β of the cortical microvasculature in late-onset AD patients. Moderate changes in other components associated with BBB functioning have also been identified and includes dys-regulation in alkaline phosphatase, γ -Glutamyl transpeptidase, acetylcholinesterase and butrylcholinesterase. While the BBB abnormalities may be induced by pathological changes within the brain parenchyma, the BBB appears particularly vulnerable in AD patients who exhibit peripheral vascular abnormalities attributed to cardiovascular disease, hypertension, and diabetes (1). Thus, cerebrovascular incongruities underscore the role of the BBB in the pathogenesis of AD. These vascular anomalies may relate to the long-term peripheral influences associated with cardiovascular disease or peripheral vascular disease.

1.3. The Blood-Brain Barrier in Health and AD

The cerebral capillary endothelium is the anatomical substrate of the BBB, isolating the brain neuropil from the systemic circulation. The cerebral endothelium lining the blood vessel lumen consists of a single layer of cells joined together by tight intercellular junctions. This layer of cells is supported by a basement membrane, which is the laminar structure formed by the fusion of the endothelial and glial vascular basement membrane (VBM). The end feet of astrocytes make up a discontinuous sheath at the abluminal surface of the VBM. Pericytes, likely of macrophage lineage, wrap around endothelial cells and are embedded in the VBM. They play an essential role in the structural stability of the vessel wall (12). The brain capillary endothelium is a crucial element in the supply of oxygen, glucose

and other vital nutrients that are instrumental in maintaining a stable internal milieu. Brain endothelial cells are also important for the catabolic outflow of CNS waste products (13,14). Disturbances in these processes may result in chronic restrictions in capillary blood flow or disrupt the BBB to contribute to the progression of AD (15). This is supported by the facts that microvascular changes in AD are located in the densely vascularized layers of the brain that correspond to areas with a high metabolic rate, such as the hippocampus and temporo-parietal areas (16–18).

2. APOLIPOPROTEIN E (ApoE) AND AD

2.1. ApoE: Gene and Protein

The ApoE gene is a member of the apolipoprotein gene family, which comprises several genes that regulate functions related to lipoprotein metabolism. The gene is located at chromosome 19q13.2 and is closely linked to the APO C-1/C-II gene complex. The DNA sequence consists of four exons and three introns spanning 3597 nucleotides. The transcripts of the three common alleles (1156 bp) exist as three isoforms, E2, E3, and E4, each with 299 residues. The ApoE2 differs from E3 by a single cysteine substituted for an arginine at position 158. The E4 differs from E3 by an arginine substitution for a cysteine residue at position 112 (19). An overview of the putative transcription factor sites in the ApoE promoter was recently provided by Lahiri et al. (20). The e4 allele of the ApoE gene is considered to be the most important genetic factor in non-familial AD. The allele has moderate specificity for AD with estimates ranging from 0.75 to 0.81. However, ApoE status is a strong predictor of outcome once the patients have been diagnosed with memory impairment. The mechanisms underlying the effect of this allele in AD and CAA pathogenesis (22) are being intensively investigated. However, both in vivo and in vitro evidence suggest the interaction between ApoE and amyloid-beta causes peptide conformation conversion and increased cellular toxicity that also pertains to the cerebral vasculature (21–23).

The ApoE is a key player in the distribution of cholesterol throughout the body. The plasma concentration ranges 40–60 mg/L and the protein is associated with several classes of lipoproteins; chylomicrons, very low-, intermediate-, and high-density lipoproteins (VLDL, IDL, and HDL). The ApoE mediates their interaction with cellular receptors, including the low-density lipoprotein (LDL) and the VLDL receptors, and the LDL receptor-related protein (LRP) (24–29). Its absence induces increased cholesterol in the circulation and atherosclerosis in humans and in mice (30). In addition to its role in lipid transport, ApoE appears to be involved in a number of processes, including the deposition and clearance of amyloid- β , the aggregation of tau, various inflammatory processes, the removal of cellular debris, neuroendocrine- and oxidative functions, signal transduction and even in apoptosis (31).

2.2. ApoE Functions, Cholesterol, and the Brain

The ApoE is produced by nearly all cell types in the body (32). Apart from the liver, the brain is the largest source of ApoE. The ApoE is predominantly synthesized by astrocytes but there is evidence to suggest that microglia, endothelial cells, and pericytes in all regions of the brain may also produce it (27). It is thought that under normal conditions neurons do not synthesize ApoE. However, recent reports suggest that cortical and hippocampal neurons express ApoE, which may be up-taken (see below) under diverse physiological and pathological conditions (33,34). In normal adult subjects, the cerebrospinal fluid (CSF) concentration of ApoE is about 10 mg/L with CSF-serum ratio of 1:5.

The ApoE within the brain is separated from the extracerebral pool by the BBB. This is corroborated by the observation that subsequent to liver transplantation with a different ApoE genotype, patients exhibit a change in ApoE isoform in the circulation but not in the CSF (35). This suggests that all ApoE in the brain is synthesized locally and is not necessarily derived from the circulation. Conversely, there is no empirical evidence suggesting that the ApoE molecules originating from the brain end-up in the peripheral circulation. Within the brain, ApoE plays a major role in the re-distribution of cholesterol, and possibly phospholipids, during regenerative processes after brain injury and in synaptic plasticity (24). An altered cholesterol metabolism may be central in the pathogenesis of AD (36). Alterations in cholesterol metabolism affect the production of amyloid- β , which is thought to have a significant influence in the pathophysiology of AD. The activity of the enzymes responsible for the cleavage of amyloid. β - and γ -Secretases, which reside in cholesterol-rich lipid domains within the cell membrane, is sensitive to membrane cholesterol content (37).

The brain contains almost 23% of all free unesterified cholesterol retained within the body, while it only represents 2% of all body mass. Cholesterol is synthesized locally with minimal if any derivation from the circulation (38). Brain lipids are primarily present in cell membranes where they are, contrary to initial thoughts, constantly being replaced. In man, the daily turnover of brain cholesterol is estimated to be in the order of 6 mg. This corresponds to nearly 1% of the turnover in the rest of the body. Since cholesterol cannot be degraded compared to other lipids, the excess is secreted (27). About 40% of the cholesterol is secreted in the form of 24S-hydroxycholesterol (39,40) and the rest through another yet undefined mechanism (41,42). The ApoE is thought to modulate this process (43). The regulation of the cholesterol flux is important as excess cholesterol can form solid crystals, which are toxic, as is the formation of oxysterols.

To date there is meagre information on cholesterol metabolism in the brain, especially during ageing and progression of AD. However, alterations or defects in cholesterol metabolism or trafficking underlie several severe neurological disorders including Niemann-Pick C1 (44), Smith-Lemli-Opitz syndrome (45) and Cerebrotendinous Xantomatosis (46). Surprisingly, a viable transgenic cholesterol-free mouse was generated recently, indicating that a lack of cholesterol is not necessarily fatal (47). Increased levels of 24S-hydroxycholesterol as one of the main metabolites were reported in plasma and in CSF of patients with AD and VaD (48-50). However, the brain concentrations of 24S-hydroxycholesterol were reduced in AD compared to normal controls. Interestingly, patients with a defective BBB display markedly increased up to 10-fold absolute levels of 24S-hydroxycholesterol (51). Furthermore, a polymorphism in the cholesterol 24Shydroxylase (CYP46) gene was reported to be associated with AD. The CYP46 is the enzyme that catalyzes the conversion of cholesterol into 24S-hydroxycholesterol and is largely expressed in neurons (50,52,53). Clinical evidence suggests that high plasma cholesterol levels and/or a high fat intake are associated with an increased risk of AD. However, contradictory results have been reported in studies relating to cholesterol, HDLcholesterol and LDL-cholesterol levels in AD (54,55) that may be explained by changes during disease progression. It is unclear if changes in plasma cholesterol and other lipids have any direct link in the modulation of brain cholesterol metabolism (56).

Astrocytes provide both structural and metabolic support to neurons. They supply neurons with cholesterol to form new membranes. Astrocytes secrete small, primarily discoid HDL-like particles that contain a core lipid, with both ApoE and apolipoprotein J (apoJ) on their surface (57). The lipoprotein particles differ from those detected in the CSF by their larger size and their content of apolipoprotein A (apoA-I) (58). These HDL-like lipoproteins secreted by astrocytes interact with ApoE-binding receptors, such as the LDL receptor, LRP (59) or the heparan sulphate proteoglycans (HSPG) or perlecans (60,61). Similar to that in macrophages, the secretion of HDL-like particles by astrocytes are affected by the ApoE genotype (62). Moreover, ApoE expression and secretion by astrocytes was found to be facilitated by the liver X-receptor and retinoid X-receptor heterodimer (63). Experiments using ApoE4 and ApoE3 knock-in mice have revealed that ApoE3 generates lipid particles with fewer numbers of ApoE3 molecules than in ApoE4 mice (62). If the secretion of cholesterol and ApoE from glial cells is prevented, the growth stimulating effect of a glial cell-conditioned medium on axon (neuritic) extension is also prevented (64). The introduction of glia-derived lipoproteins containing ApoE to distal axons, but not to cell bodies, enhances the axonal outgrowth. Interestingly, ApoE enhances the outgrowth of neurites in an isoform-specific manner in the presence of a source of lipids (60,61,65,66). While ApoE4 inhibits neurite growth, ApoE3 strengthens it.

Since the LDL receptor may be involved in the uptake of the ApoEcontaining lipoproteins, the divergent interaction of ApoE4 and ApoE3 with the LDL receptor may explain these disparities. Alternatively, a variation in the interaction with HSPGs may be involved (67).

It has been demonstrated that ApoE can be re-secreted from cells after being endocytosed (68) and the re-cycling of ApoE is accompanied by a cholesterol efflux. In hepatocytes, a significant amount of ApoE4 was found to be retained within the cells after endocytosis while ApoE3 is re-secreted (Heeren J, personal communication). This may also apply to neurons. The increased levels of ApoE in neurons after CNS injury or pathological lesions may therefore be due to increased uptake of ApoE, amplified retention or augmented synthesis.

The ApoE levels in the brain are up-regulated as a consequence of almost any type of CNS injury. It is also increased in a number of degenerative diseases including amyotrophic lateral sclerosis, schizophrenia, Niemann-Pick (69-72). Recently, Harris et al.(73) provided evidence suggesting that certain factors secreted by astrocytes regulate ApoE expression in neuronal cells. It is known that specific alterations in the periphery can also modulate ApoE levels in the brain. High fat diet can lead to upregulation of ApoE levels (30) whereas chronic changes in total plasma cholesterol concentrations, as a result of dietary or pharmacological intervention, may alter ApoE mRNA levels in the brain (74). Secretion of ApoE by astrocytes is regulated in a downward fashion by statins, which inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the cholesterol-synthesis pathway (75), but the cholesterollowering drug probucol boosts ApoE production in the hippocampus (76). Epidemiological studies indicate that stating reduce the risk of AD (77) and the amyloid burden in the brains of transgenic mice over expressing the amyloid precursor gene to model AD (78). This may be possibly achieved by reducing the levels of membrane cholesterol (78). Statins not only decrease plasma cholesterol levels but they also influence cholesterol and ApoE levels in the brain albeit in guinea pigs. On the other hand statins did not affect cholesterol levels in the brain of ApoE-knockout mice, suggesting that the therapeutic effect of simvastatin depends on the presence of a functional ApoE (79).

2.3. ApoE, HSPGs, and Alzheimer Pathology

ApoE is variably localized in amyloid deposits characteristic of both cerebral and systemic amyloidosis (80). In AD, ApoE immunoreactivity has also been detected in cerebrovascular amyloid- β deposits and neurofibrillary pathology (81–83). ApoE is a common constituent of amyloid fibrils and may facilitate different types of amyloidosis by reducing the solubility of amyloid and stabilizing the β -pleated structure (84–86). It may therefore also affect the clearance of amyloid.

In the absence of other specific protein interactions with amyloid- β , ApoE, and dimeric soluble amyloid- β complexes have been observed in supernatants of neurons from brain tissue from AD patients (87). Binding studies suggest ApoE binds avidly to amyloid- β peptides and enhances the formation of amyloid- β fibrils (84–86,88–90). ApoE3 binds amyloid- β with greater affinity than ApoE4, which may have consequences for both its deposition and clearance. Whereas there is a strong association between the ApoE e4 allele and CAA, it is not clear if its product affects the extent of CAA atherosclerosis or myelin loss (91). It is plausible that ApoE may indirectly influence amyloid- β fibril formation or deposition through a modulatory effect on cerebrovascular functions or via brain cholesterol metabolism.

The HSPG or perlecans have been suggested to play a central role in the deposition of amyloid- β by promoting fibrillogenesis (92). Both HSPG and perlecans are a major component of the VBM of the cerebral vessels. HSPGs similar to ApoE, are usually detected in amyloid deposits of any type of amyloidsis. They can bind both ApoE and amyloid and are known to be involved in the clearance of lipoproteins by the liver.

They may also play a role in the ApoE-mediated effect on neurite outgrowth. Thus like the other lipid receptors do HSPGs influence the mobilization of ApoE and amyloid?

2.4. ApoE e4 as a Vascular Risk Factor for AD

The hypothesis that the modifying effect of ApoE e4 on the vascular system is related to the increased risk of AD is supported by the observation that other vascular factors linked to atherosclerosis increase the risk of AD. These include hypertension, diabetes, and stroke (1,93,94). All of these are potential modifiers of the vasculature. It is thought that atherosclerosis in extracranial vessels causes sustained hypoperfusion in the brain and may thereby indirectly contribute to the progression of AD (1). Patients with diabetes and hypertension may exhibit reduced blood flow to the brain (95). In support of this, an increased prevalence of cerebral senile plaques was observed in the brains of cognitively intact individuals with hypertension or critical coronary artery disease when compared to age-matched controls without heart disease (96). Epidemiological evidence indicates that atherosclerosis may also represent a risk for CAA (97).

The ApoE e4 is currently viewed as a compounding factor in the development of cardiovascular disease because of its association with increased plasma cholesterol levels and with atherosclerosis (30), which may also attract amyloid- β within the atherosclerotic plaques. Moreover,

hypertension results more often in white matter lesions in ApoE e4 carriers than in non-carriers (98). This implicates an interaction between ApoE e4 and atherosclerosis in the etiology of AD. Thus, ApoE e4 may affect AD through a modulatory effect on the vascular wall. The ApoE4 combined with hypertension, atherosclerosis, peripheral vascular changes or diabetes presumably further increases the risk for cognitive dysfunction. Alternatively, similar mechanisms may underlie the increased susceptibility of individuals with the ApoE4 genotype to vascular diseases and to AD. The ApoE e4 allele or ApoE4 may modulate the pathogenesis of AD through more than one pathway.

The role of ApoE in cerebrovascular disease, which may exhibit CAA but not AD phenotype, is however not clear (99). A meta analysis revealed significantly higher ApoE e4 allele frequencies with more than sixfold greater risk in patients diagnosed with ischemic cerebrovascular disease compared to age and gender-matched controls. These findings suggest a role for ApoE genotype in the pathogenesis of cerebrovascular disease (100). Frisoni et al.(101) had previously implicated comparably high ApoE e4 allele frequencies in cerebrovascular disease associated with dementia but subsequent clinical reports have not confirmed this finding. Indeed, pathologically confirmed studies showed that e4 allele frequencies did not differ between Binswanger's disease and other forms of vascular dementia (102). However, the ApoE e4-allele frequency may increase the risk of dementia in stroke-survivors and that e4 homozygotes exhibit extensive hypoperfusion related to lesions in the deep white matter than those with other genotypes (103). The latter is, however, not a consistent finding. An interaction between arterial disease and ApoE e4 was also suggested by the 9-fold increase in cardiac ischemia in e4 homozygotes (104) compared to those with e3. These observations appear in accord with the notion that the e4 allele or its product may exert its effects in tandem with hypoperfusion. Above all evidence for a direct role through pathological alterations in the vascular wall rather than by secondary mechanisms via cardioembolic or thrombotic changes is a strong epidemiological study showing that the effect of ApoE gene in dementia is not dependent on atherosclerosis, but on yet unknown factors (22,105).

3. CEREBROVASCULAR CHANGES IN MOUSE MODELS OF AD

3.1. ApoE-Knockout Mice

The ApoE-knockout mice develop severe atherosclerosis (106) but controversy abounds concerning the AD like behavioral and neuropathological anomalies (107,108) suggesting the involvement of other genetic and/or environmental factors. The ApoE-knockout mice also display impaired long-term potentiation, which may be linked to the impaired learning and memory functions (109–111). In accord with this, in vitro studies demonstrate that astrocytes from ApoE-knockout mice do not secrete HDLlike particles (112). In addition, aging knockout mice show progressively reduced number of presynaptic boutons compared to wild mice. However, no alterations were found in the number of presynaptic boutons in transgenic mice that express ApoE4 or ApoE3 in astrocytes. Only upon environmental enrichment do ApoE4 transgenic mice fail to produce increases in synapse number whereas ApoE3 transgenic mice do not. Similarly, in double transgenic mice expressing ApoE4 and an APP mutant, reduced number of synapses was observed in comparison with mice expressing the ApoE3 and the same APP mutant (113). Whereas over-expression of ApoE4 in astrocytes does not cause severe neurodegeneration, over-expression of ApoE4 in neurons results in axonal degeneration with gliosis (114).

ApoE has been implicated in the maintenance of BBB integrity during ageing (115,116). In accord with this, ApoE protects against cerebral lesions induced by a high fat diet. Moreover, when ApoE-knockout mice were fed a western type diet for a period of 10 months, dramatic immunopositive staining for IgG was observed in the brains indicating breach of the BBB. Additionally, the brain volumes were appreciably reduced compared with wildtype mice kept on the same diet. However, high plasma cholesterol levels and atherosclerosis were not considered to be the causative factors. It is not yet known whether these effects are due to lack of ApoE within the brain or systemic organs, or if there is an indirect effect of chronic inflammatory process in ApoE-knockout mice.

The lack of ApoE also appears to induce extracerebral endothelial dysfunction. Compared controls, ApoE-knockout mice have higher nitric oxide synthase (NOS) activity levels (117). This suggests an increased nitric oxide (NO) production with consequent vasodilatation. The ApoE-knockout mice fed on a western diet exhibit impaired endothelium-dependent relaxation responses to acetylcholine action in aortic vessels (118). D'Uscio et al. (119) reported that this impairment of aortic endothelial function was due to increased O2-levels resulting in significantly reduced endothelial NOS activity and cGMP in the ApoE-knockout mice. Interestingly, NO production in macrophages and microglial cells is also influenced by the ApoE genotype (120). In monocyte-derived macrophages from AD patients carrying the ApoE e4 allele significant increases in NO production were observed as opposed to AD patients with ApoE e3 allele or age-matched controls.

Recent evidence suggests that ApoE may modulate angiogenesis as well. Pola et al. (87) have identified compromised cerebral angiogenesis subsequent to ischemia in ApoE-knockout mice. This was linked to lower post-ischemic induction of vascular endothelial growth factor. Consistent with this, larger infarct volumes were evident in ApoE-knockout mice subjected to focal stroke (121). Not surprisingly, overexpression of human APP751 in the ApoE-knockout mice further augments the infarct volume. This could be attenuated by the co-expression of a human ApoE isoform, possibly due to inhibition of microgliosis (122). However, treatment with a peroxisome proliferator-activated receptor- α activator (123) reduced the propensity of stroke in ApoE-knockout mice.

3.2. Amyloid Precursor Protein-Transgenic (APP) Mice

Several transgenic mice over-expressing single or double APP mutant genes have been generated. As in AD, these mice develop cerebral amyloid- β deposits, which are ApoE positive (124). Changes in learning and memory processes have been documented in some models (125–128). Some APP transgenic mice have also been shown to exhibit compromised BBB. Aliev et al. (129) reported mitochondrial DNA deletions in endothelial and perivascular cells juxtaposed to regions with high amyloid deposition in APP transgenic mice, as well as in AD brains. Changes in the integrity of the BBB were noted in transgenic mice carrying the Swedish APP mutation (APP23) (130). Poduslo et al. (131) described structural alterations at the BBB, but no differences in the permeability of the BBB to human amyloid- β 1–40 in APP transgenic mice. The increase in BBB permeability was found to precede amyloid plaque formation in the Tg2576 transgenic mice (132).

In AD, patients carrying the ApoE e4 allele had a greater risk of CAA and amyloid- β deposition, when compared to patients with the ApoE e3 (133). In accord with this, ApoE also promotes the formation of CAA and vessel damage in mice. In other studies (134) APP transgenic mice exhibiting brain amyloid deposits treated with antibodies against amyloid were more prone to bleeds compared to untreated mice. Interestingly, agedependent CAA associated with microhemorrhaging in APP transgenic mice could be substantially reduced if mice were exposed to an ApoEdeficient environment (135). The ratio of amyloid- β 1–40 to amyloid- β 1– 42 and total amyloid- β were significantly reduced in the absence of ApoE in these mice that also exhibited impediments in learning abilities and memory functions. This suggests that the interaction of ApoE with amyloid-B induces a loss of functional ApoE. Thus, these studies strongly support a role for ApoE, both in the deposition of amyloid- β and in its secretion from the brain into the circulation (43). It has been hypothesized that amyloid- β is removed, possibly in association with HDL, from the brain parenchyma to the microvascualture where it binds to, e.g., HSPG in the vascular basement membrane subsequently to be internalized by pericytes via the LRP. The intracellular accumulation of amyloid- β in pericytes may result in a loss of vessel stability, making them more vulnerable to hemorrhages.

4. A PLAUSIBLE CENTRAL ROLE FOR HDL

Collective evidence suggests HDL plays a substantial role in the pathogenesis of AD (43). The HDL is a key element in diabetes, inflammation (136),

Gene	Allele(s)	Association	Product(s)	Expression in brain
ApoE	ε4, ε2	Positive	ApoE4, apoE3	Astrocytes, EC, neurons
APOAI	Various	None	ApoA	Nc
APOB	Various	Unclear	ApoB	Glial cells
APOCI	H2 insertion	Mostly positive	ApoC	Astrocytes
APOCIII	Exon 4	None	ApoC	Nc
CYP46	2 sites	Not confirmed	C24SH	Neurons, glia
LDLR	H2	Not confirmed	LDLR	Astrocytes, EC
LRP	Exon 3	Unclear	LRP	Neurons, EC, pericytes
LPL	H1, H2	Unclear	LPL	Hippocampus, EC

 Table 3 Currently Reported Associations Between Late-Onset AD and Polymorphisms in ApoE and Other Genes Involved in Lipid Metabolism and Transport

Polymorphisms in *HMG-CoA* have also been reported to be weakly associated with AD. *Abbreviations*: C24SH, cholesterol 24S-hydroxylase; EC, endothelial cells; nc, not clear; other abbreviations see main text.

Source: For original data see Refs. 3, 30, 51, 96, 133, 162, 163, 167, 173, 174, 192, 199, 202, and 203.

and cardiovascular disorders, which are all considered to be risk factors for AD. Brain HDL may originate from at least three different sources. While small HDL may be derived from the circulation across the BBB (137,138), astrocytes and cerebral endothelial cells may be the other two sources. In addition to ApoE, astrocytes synthesize various enzymes involved in the metabolism of HDL. One of these is cholesteryl ester transfer protein (CETP), a glycoprotein that facilitates the transfer of cholestervl esters, phospholipids and triglycerides between lipoproteins and regulates plasma HDL levels. The CETP has previously been identified in astrocytes localized in the white matter. However, in AD subjects CETP-positive reactive astrocytes were detected in the grey as well as white matter (139). The lecithin: cholesterol acyltransferase (LCAT), an enzyme that is involved in the esterification of cholesterol in HDL and in the remodeling of HDL, has also been reported to be present in the CSF. Moreover, astrocytes synthesize several other proteins (Table 3) involved in HDL metabolism including apoA-I (140), apoCI, and LPL and HDL-binding receptors, including the low-density lipoprotein receptor (LDLR), the LRP, scavenger receptor class B, type 1 (SR-B1), and CD36.

4.1. Apolipoprotein A-I and AD

The apoA-I is the major protein component of HDL, predominantly synthesized in the liver and intestine (141). Together with ApoE, apoA-I is a major lipoprotein of HDL in CSF (27). While the origin of apoA-I in the CSF remains to be determined, some reports suggest that apoA-I may be produced by the cerebral endothelium (142). There is evidence to validate the notion that lipid-free apoA-I or that HDL lacking ApoE but associated with apoA-I can be transported across cerebral endothelium (143,138). The apoA-I activates LCAT, thereby facilitating the transport of excess cholesterol from tissues. High LCAT activities have been found in rat brains (93) and in the cerebellum and cerebral cortex of baboons (144). The LCAT activities were found to be reduced by as much as 50% in CSF of AD patients compared with aging controls (58).

The apoA-I along with small HDL typically facilitates the efflux of cholesterol from tissues and induce the translocation of cholesterol from intracellular membranes to the cell surface (145). Not surprisingly, apoA-I also promotes the secretion of ApoE by macrophages (146). The ATPbinding cassette transporter (ABCA1) plays a major role in the elimination of tissue cholesterol, because it enables the apolipoprotein-dependent transfer of intracellular cholesterol and phospholipids to lipid-free apoA-I (147). The ABCA1 and apoA-I appear to bear a distinct role in the basolateral efflux of cholesterol. The ABCA1 expression, as well as the secretion of apoA-I, generally associated with HDL, from the basolateral compartment of cultured endothelial cells can be induced by 24S-hydroxycholesterol (148), a ligand for the nuclear liver X receptor. This receptor dimerizes with the retinoic acid receptor and boosts transcription of proteins important for cholesterol and fatty acid metabolism (149). Alternatively, the basolateral compartment may be involved in efflux of phospholipids alone. It has been suggested that endothelial cells reassemble and secrete either intracellular or ABCA1-dependent HDL-like lipoproteins. Interestingly, variants of ABCA1 may increase the risk of AD (150).

The ABCG2 too has been detected in cells associated with the BBB (151). Other ATP-binding cassette transporters involved in lipid metabolism that have been detected in the brain include ABCG1/ABCG4 (see Chapter 7 from Zhang et al.), which mediates the efflux of cholesterol to HDL. Furthermore, ABCA2 was found to regulate LDLR and HMGCoA synthase expression, and ABCA7 to mediate the efflux of phospholipids, but not cholesterol (152,153). Since both apoA-I and ApoE fulfill important roles in the transfer of cholesterol from cells to HDL, it is plausible that this also occurs in the CNS (27,154,155).

Several other functions for apoA-I have been implicated. These include the binding of lipopolysaccharides (LPS), engendering of antiviral activity, nerve regeneration (156) and regulation of the complement system (157). Furthermore, apoA-I may directly interact with endothelial NOS (158) to increase its activity through multisite phosphorylation changes in endothelial cells. An apoA-I mimetic peptide was found to suppress the infection-induced trafficking of macrophages into arteries (159).

Like amyloid- β , the amyloid protein product of apoA-I causes a distinct amyloidosis originating from the specific cleavage of the precursor (160,161). Point mutations or deletions in the apoA-I gene cause a very rare disease known as hereditary non-neuropathic systemic amyloidosis, which involves deposition of polymers of apoA-I (162,141). Although there is no clear evidence for APOA-I gene to be associated with risk of AD (163), a previous study (164) reported apoA-I immunoreactivity in endothelial cells, hippocampal pyramidal neurons, astrocytes and also in cortical amyloid- β plaques in AD subjects. Whether these cellular localizations bear any influence on the decreased serum HDL cholesterol and apoA-I concentrations found to be correlates with severity of AD (165) is unclear. However, this appears consistent with the finding that individuals who reach a very old age in relatively good health exhibit significantly higher levels of HDL cholesterol and apoA-I compared to age-matched diseased subjects (166).

4.2. Apolipoprotein CI in AD

The ApoE e4 allele is considered to be in disequilibrium with the H2 allele of APOCI, which is localized immediately downstream from the ApoE gene, and results in 50% increased expression of apoCI (167). The H2 allele of APOCI may be an independent or an additional risk factor for AD (168,169) (Table 3). The ApoCI is primarily expressed in the liver, while low expression was reported in lung, skin, testes and spleen (170), and it has been detected in astrocytes. The ApoCI blocks the interaction of ApoE with all of its known receptors and, consequently delays the clearance of ApoE-containing lipoproteins (171). The ApoCI may also activate cholesterol esterification via LCAT (172) and it is a known inhibitor of CETP (173).

The ApoCI may play a role in the pathogenesis of AD but the underlying mechanisms have yet to be defined. ApoCI could retard uptake of the ApoE-containing HDL-like lipoproteins by neurons and affect delivery of cholesterol required for the outgrowth of synapses. However, it was recently reported that apoCI protein levels are elevated, while those of ApoE4 are reduced in the brains of AD patients carrying an ApoE e4 allele (174). Additionally, the effect of APOCI polymorphism on hippocampal volumes, memory and frontal lobe function in subjects with age-associated memory impairment (175,176) has been described.

5. HDL RECEPTORS AND THE CEREBRAL ENDOTHELIUM

5.1. Low-Density Lipoprotein Receptor

In the brain, the LDLR has been predominantly detected in astrocytes. Other cell types, including neurons and endothelial cells also express this receptor (27,59,177–179). In contrast to non-CNS endothelium, its

expression in brain endothelial cells remains robust during ageing. The LDLR enables the cellular interaction and internalization of ApoE- and/or apoB100-containing lipoproteins (180). Malfunctioning of this receptor in humans gives rise to familial hypercholesterolemia and causes cardiovascular disease (181). Patients with familial hypercholesterolemia that carry the ApoE e4 allele are further disadvantaged (182), since they develop xanthoma in the brain (183–185).

Although the non-CNS functions of the LDLR have been previously investigated, very little is known with respect to the extent of its influence on the CNS. The primary ligand for the LDLR present in brain is ApoE, as apoB100 does not cross the BBB and does not appear to be synthesized within the brain (186). Another ligand for the LDLR in the brain is likely the lipoprotein lipase (LPL) (187). Lipases are involved in the formation of endogenous canabinoids that modulate learning and memory abilities (188). The hippocampus, and to a lesser degree cerebral microvessels are the main sites for expression of LPL (189,190). The LPL was also found to be involved in synaptic remodeling (191). Its expression in the hippocampus, relative to other brain regions is regulated by severe diet restriction (192). Furthermore, LPL has been shown to affect the uptake and transcytosis of LDL across BBB endothelium in vitro (193). An association between LPL polymorphism and AD was reported (194) but could not be confirmed by Fidani et al. (195) or others (Kalaria et al., unpublished observations).

The LDLR is an important receptor for ApoE in the brain and actively facilitates the distribution of lipids (59). Consistent with the occurrence of small HDL-like lipoproteins the interaction between ApoE and LDLR increases with small particle size (196). A clear indication of the potential role for the LDLR in the ApoE-mediated distribution of cholesterol is the observation that both LDLR-deficient and ApoE-deficient mice display an altered distribution of cholesterol in brain synaptic plasma membranes with a higher percentage in the exofacial leaflet (197). Furtheromore, LDLR-knockout mice were shown to display impaired memory and a reduced number of presynaptic boutons in the hippocampus CA1 (198). Retz et al. (199) have reported a trend toward the association between an LDLR polymorphism and AD (Table 3).

5.2. Low-Density Lipoprotein Receptor-Related Protein

In addition to the LDLR, the LRP is a multifunctional endocytic receptor expressed predominantly in neurons. However, it has also been detected in capillaries (200) and pericytes. It has been reported that the tissue-type plasminogen activator induces opening of the BBB via interaction with the LRP (201). Several studies have tested associations between the LPR

gene and late-onset AD exist but there is no clear indication for a correlation between LRP exon 3 polymorphism and AD or brain LRP levels (202,203).

5.3. Scavenger Receptor Class B, Type I

The SR-BI is detectable almost exclusively at the apical membrane of cerebral endothelial cells. It is also expressed by astrocytes (204,205), however. The SR-BI, a member of the CD36 superfamily, is a high affinity receptor for HDL stimulating selective uptake of cholesteryl esters and the efflux of unesterified cholesterol from cells (196). The HDL cholesteryl ester uptake has been demonstrated in all tissues with the exception of the brain (206). Therefore it is likely that SR-BI has an important role in the efflux of cholesterol from the brain. Such effect on free cholesterol efflux presumably occurs independently from binding to HDL. The SR-BI may in effect modulate membrane free cholesterol domains to provoke cholesterol flux between cells and HDL (207). The SR-BI was also found to stimulate NO production in cerebral endothelium indicating a positive effect on the vasculature (208). Interestingly, the efflux of 24S-hydroxycholesterol from the apical surface of endothelial cells in the presence of HDL is enhanced by over-expression of SR-BI (148). However, so far no abnormalities in the CNS have been detected in SR-BI-deficient mice (209).

5.4. Cd36: A Scavenger Receptor

Similar to SR-BI, CD36 is another scavenger receptor. Both SR-BI and CD36 have common ligands. However, the specific function of CD36 is to facilitate the uptake of long chain fatty acids (208). The CD36 is strongly expressed in normal brain capillary endothelium (210). The CD36 also binds HDL, most likely via apoAII (211). Notably, small dense ApoE-free HDL3 seems to be involved in brain entry of polyunsaturated fatty acids, which can be used for membrane synthesis (212). This is compatible with the HDL3 enhanced conversion of phosphatidylethanolamine into phosphatidylcholine by stimulating phospholipase A2. However, CD36 was originally identified as a receptor for oxidized LDL. Although normally the larger lipoproteins do not pass the BBB, oxidized LDL has been detected in astrocytes surrounding cerebral infarcts (213). Oxidized LDL also stimulates the secretion of interleukin-6 from astrocytes. The CD36 is apparently localized in macrophages and microglia. The interaction of amyloid-ß with CD36 bearing macrophages was found to trigger H₂O₂ production (214). Not surprisingly, high levels of CD36 were found in AD, as well as in cognitively normal subjects with diffuse amyloid plaques. Expression of CD36 correlated with amyloid- β deposition, but not with AD (215).

6. CONCLUSIONS

Recent advances indicate the pathology of AD includes cerebrovascular abnormalities, which may modify the mobilization, metabolism and storage of brain lipids and lipid carriers. The specialized localization within reactive glia and endothelial cells indicates the brain retains its own repertoire of proteins and receptors linked to lipid transport for handling lipids and proteolipids during brain injury and repair. Since blood to brain traffic of lipids is restricted, impairment of the BBB has implications on mobilization and sequestration of lipids during injury and impact upon neuronal repair processes. While variable associations have been reported in polymorphisms of genes for lipid metabolic enzymes and receptors the e4 allele of the ApE gene appears the most robust in increasing the burden of disease. Geneknockout and transgenic mice models suggest a variety of ligands and receptors associated with lipid transport or metabolism are vital for neuronal growth and maintenance, and have implications in the pathogenesis of AD.

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18

Techniques for Measuring the Blood–Brain Barrier Integrity

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1. INTRODUCTION

Dysfunction of the blood-brain barrier (BBB) is a significant pathological event in many neurological diseases. Many techniques have been developed to study the integrity of the BBB. Histological measurements are used in animal models of neurological diseases. With imaging modalities like single photon emission computed tomography (SPECT), positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI) it is possible to measure the integrity of the BBB in vivo, in animals and humans. This chapter will review the most commonly used methods to qualitatively and quantitatively measure the integrity of the BBB, with a special emphasis on in vivo imaging techniques.

For passage across the intact BBB, different routes are available. Passage may be passive (across the lipophilic cell membrane), carrier mediated, or brought about by endocytosis and transcytosis. Paracellular pathways are also available through the hydrophilic tight junction. Transport of a specific molecule depends on the size, lipophilicity, ionization, and availability of specific carrier mechanisms. Permeability of the BBB may be altered by damage of tight junctions, or due to a decline in the production of barrier inducing factors. Generally, measurement of the integrity of the BBB relies on pathological leakage of intravascular contrast agents across the damaged BBB. Consequently, an adequate contrast agent has to remain intravascular if the BBB is intact, should accumulate into the brain parenchyma where the BBB is damaged and should be readily detectable. Most exogenously applied contrast agents are therefore relatively large in size, and non-lipophilic. Molecular weights between 100 and 1000 Da seem to be ideal. The techniques described in this chapter rely on passive diffusion of these relatively large molecules over the damaged BBB.

2. EX VIVO BASED TECHNIQUES

The concept of the existence of a functional BBB was first shown by Ehrlich (1). He injected specific dyes into the blood stream of an animal, and the stain could be histologically demonstrated in any organ but the brain parenchyma. He concluded that there should be a special barrier present in the brain that prevents leakage into the brain parenchyma. In 1913 one of his students (Edwin Goldmann), showed the presence of this barrier in a similar experiment. He injected comparable dyes into the cerebrospinal fluid (CSF) of animals, and showed that they remained in the brain, and could not be detected in any other organ (2). Researchers nowadays still use similar approaches to determine the integrity of the BBB. In a typical experiment, a contrast agent is intravenously administered to an animal, which after a given time is sacrificed. The brain is dissected and deposition of the agent in the brain is determined qualitatively or quantitatively with various techniques. A major disadvantage is that an animal has to be sacrificed in order to obtain information about leakage of the BBB. However, spatial resolution is superior to the in vivo detection techniques discussed later in this chapter.

2.1. Histological and Immuno-Histochemical Techniques

Endogenously BBB impermeable plasma proteins may be used as tracers for BBB leakage. The most commonly used are the macromolecular proteins albumin [molecular weight (mw): 66–69 kDa], immunoglobulin G (mw: 150 kDa), fibronectin (mw: 200 kDa), and fibrinogen (mw: 340 kDa). Compared to exogenously applied tracers, these proteins are large in size, which might theoretically compromise diffusion. However, because of the permanent presence of plasma proteins, leakage through the BBB is relatively time-independent. So even with little leakage and slow diffusion, measurable amounts may be present on examination. The detection of plasma proteins of different sizes may be used to determine the severity of BBB damage. However, the use of plasma proteins as markers of BBB damage

is restricted, since the concentration of these proteins in the blood is largely influenced by all kinds of physiological and pathological processes like kidney-damage, malnutrition, or hemostasis.

Exogenously applied tracers are less influenced by physiological processes, although kidney and liver functions still have effect on intravascular concentrations. Frequently used markers are horseradish peroxidase (mw: 40 kDa) and Evans Blue (EB, mw: 950 Da), which strongly binds to plasma albumin (mw EB-albumin complex: 66–69 kDa). These are still regularly used as a marker in models of neurological disorders like stroke (3,4), hypertension related cerebral damage (5), brain tumors (6), MS (7,8), neuroinflammation (9,10), Alzheimer's disease (11), Parkinson's disease (12), and surgical stress (13). The combinations of both agents may show different patterns of BBB leakage (6).

The contrast agents used in histology are often combined with specific tracers in the methods that we describe below. Since these markers are larger than most of the other commonly used contrast agents, care should be taken when comparing the results of the different methodologies.

2.2. Radioactive Techniques

Quantitative autoradiography is a technique, which uses the distribution pattern of radioactive markers in the brain to quantify the permeability of the BBB. In a characteristic experiment, a radio-active tracer is given intravenously, and plasma radioactivity is sampled periodically. The animal is sacrificed after several minutes, and the brain is prepared for measurements of radioactivity. If the concentration of the radio-active tracer in blood is known, leakage over the BBB may be quantified by the use of pharmoco-kinetic models (14). The permeability surface area (PS) product for example, is a typical parameter that quantitatively describes BBB leakage. This parameter depends on the surface area of the cerebral capillaries (in healthy humans approximately 100 cm² g⁻¹) and the permeability of the capillary wall for the given tracer.

¹⁴C-α-Aminoisobutyric acid (mw: 103 Da) is commonly used as a radioactive tracer to determine PS (or related) products, in animal models of neurological disorders (15). It has recently shown its value in models of stroke (16), brain tumors (17), neuro-inflammation (18), and epilepsy (19). Also ³H/¹⁴C labeled sucrose (mw: 342 Da) is frequently used (20). In addition to these low molecular weight tracers, high molecular weight tracers, like ³H/¹⁴C labeled inulin (mw: 5kDa),³H/¹⁴C labeled dextran (mw: >10kDa), and ³H/¹²⁵I labeled albumin (mw: 66–69kDa) are available. Combinations may be used to demonstrate size dependency of BBB damage in different neurological disorders (17,21–26).

3. IN VIVO BASED TECHNIQUES

The above-mentioned procedures require tissue dissection, which makes them unsuitable for serial measurements and clinical diagnosis. Other methods have been developed, which are less- or non-invasive. Especially neuroimaging modalities have made it possible to qualitatively or quantitatively image the permeability of the BBB in vivo, in animals or humans.

3.1. Non-Imaging Techniques

3.1.1. Plasma Protein Determination in CSF

Normally, the presence of BBB impermeable plasma proteins in CSF is restricted. In different pathological conditions an increase of these proteins in CSF may be used as a measure of BBB leakage. Especially albumin measurements have been used, and CSF/blood ratios can be calculated to account for variations of blood levels. For example, albumin levels have been determined in patients with Alzheimer's disease (27,28), vascular dementia (28,29), and MS (30). The relevance of the parameter may be limited, since leakage may also occur if the BBB is intact, especially via the CSF producing choroid plexus. Furthermore, as mentioned above, levels of plasma proteins may vary considerably.

3.1.2. Microdialysis

For microdialysis, a probe is implanted into a specific area of the brain and samples are withdrawn in which concentrations of exogenously applied molecules or endogenous metabolites are measured. Samples collected are small, which makes the sensitivity of the used analytic technique an essential issue. Moreover, detection is restricted to the volume directly surrounding the sampling probe. Microdialysis is mainly used in animal studies and humans to determine the uptake of specific drugs, or the presence of metabolites (14,31). However, it may also be used to study leakage of plasma proteins or exogenously applied compounds, such as the radioactive tracers discussed before, through the BBB into the brain parenchyma (32–34).

3.2. Imaging Techniques

In the last decades SPECT, PET, CT, and MRI have revolutionized the ability to examine brain pathology in animals and humans in vivo. Various protocols have been developed to explore the integrity of the BBB. Generally, a specific tracer (for SPECT and PET a radio-pharmaceutical, for CT and MRI specific contrast agents) is injected intravenously, and leakage of this tracer over the damaged BBB into the brain parenchyma is measured. Leakage is mostly measured qualitatively, which means that only its presence or absence is determined.

Quantitative analysis of BBB leakage is challenging. For these experiments, the temporal distribution of a tracer in a given brain volume, and the concentration of the tracer in a blood compartment (like the sagittal sinus) are determined by sequential imaging. Imaging starts before injection of the tracer, and is continued until a steady state is reached or until washout from the tissue occurs. Over this period (approximately 20 to 30 minute after the tracer injection), images are obtained with a temporal resolution of several seconds to minutes. Thus curves that represent tracer dynamics may be created. From the initial part of such curves hemodynamic parameters may be calculated, like cerebral blood flow (CBF) and cerebral blood volume (CBV). The middle and final parts of the curves may be used to calculate the flux rate of contrast agent from the intravascular to extravascular compartment. Ideal tracers should remain in the extracellular space after diffusion into the brain parenchyma, as intracellular uptake would complicate pharmocokinetic calculations. Specific calculations may reveal a PS product (in mL min⁻¹ per g or mL tissue) or other quantitative measures of BBB leakage like the volume of the extracellular extravascular space (35).

Pharmacokinetic models used for describing the uptake of the tracer range from flow-limited models (in which uptake is restricted by blood flow, and permeability is nearly unlimited) to permeability-limited models (in which blood flow and tracer concentration are constantly high enough, and uptake is determined by the permeability of the endothelium). In most quantitative analyses of the BBB, permeability-limited models are used, as permeability of the BBB is low and blood flow is high enough to keep tracer concentrations constant. A general description of the flux of contrast agent across the BBB in this situation is given by, $dC_t/d_t = PS(C_p - C_t/V)$, where V is the volume fraction of the extravascular extracellular space, C_t the concentration of the tracer in tissue, and C_p the concentration of the tracer in plasma (35). However, for some types of pathology, blood flow may be obstructed (like in cerebral ischemia), and other pharmocokinetic models should be used. Next to quantification problems, PS related products obtained with the different techniques, cannot be compared freely. Still quantification might be useful for follow-up of the severity of BBB damage over time (within subjects), for example to measure the effect of therapeutic interventions.

3.2.1. Nuclear Imaging Techniques

Nuclear imaging techniques use exogenously applied radio-pharmaceuticals to image functional properties of tissue in vivo. The two most commonly used types of nuclear imaging techniques are SPECT and PET. SPECT studies utilize radio-pharmaceuticals, which emit photons while decaying, whereas PET studies utilize radio-pharmaceuticals that emit positrons (which dissolve into photon pairs).
Although nuclear imaging has an important place in functional imaging of brain metabolism, its place in BBB measurements is mainly historical, and currently it has been taken over by CT and MRI. Still SPECT and PET are sometimes used to study the integrity of the BBB qualitatively or quantitatively. Typical radio-pharmaceuticals used in SPECT to measure the integrity of the BBB have ^{99m} technetium (^{99m}Tc, physical half life decay of radioactivity is 6 hour) incorporated (36,37).^{99m}Tc-pertechnetate (mw: 163 Da) (38–40),^{99m}Tc-diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA, mw: 492 Da) (41-45), and ^{99m}Tc-glucoheptonate (mw: 226 Da) (46-49) are commonly used tracers. Another SPECT radio-pharmaceutical is ²⁰¹thallium chloride (²⁰¹TlCl, physical half life decay of radioactivity is 73 hour), which is mainly used in patients with brain tumors. However, part of the high sensitivity of ²⁰¹TlCl in tumor uptake is a result of cellular uptake, making it less suitable for quantitative measurements of BBB permeability (37,50). In PET studies both ⁶⁸gallium-ethylenediaminetetraacetic acid (mw: 356 Da: physical half-life decay of radioactivity 68 minute) (51,52) and ⁸²rubidium chloride (physical half-live decay of radioactivity 1.25 minute) (53–55), a potassium analog which like ²⁰¹TlCl is partially incorporated intracellularly, have been used to determine the integrity of the BBB both in animals and humans.

3.2.2. CT

With CT, images can be obtained within several seconds. Ultrafast CT techniques are available, with multi-slice detectors that provide information on the dynamic distribution of exogenous contrast agents by repetitive imaging, a procedure called dynamic contrast enhanced (DCE) imaging. The most frequently used CT-contrast agents to study the integrity of the BBB are labeled with iodine. Iodine has a high x-ray attenuation coefficient, providing a hyperdense signal and contrast compared to surrounding low attenuated tissue (56). Examples of non-ionic contrast agents are the tri-iodinated monomeric benzoic acids iopamidol (mw: 777 Da), iopromide (mw: 791 Da), and iohexol (mw: 821 Da).

CT has a higher spatial resolution than PET and SPECT, and a comparable resolution to MRI. The relationship between the presence of contrast agent and attenuation changes on the CT image is linear, making CT based quantification of BBB permeability straightforward, and more accurate than MRI (57). With DCE-CT, BBB dysfunction has been determined quantitatively in animals with brain tumors (58) and patients with different neurological diseases, such as brain infarcts (59), brain tumors (60–64), and neuroinflammatory diseases (65–68). CT is less expensive and readily available. CT is therefore routinely used as a qualitative diagnostic and prognostic tool for BBB leakage. For example, in patients with brain infarcts, contrast enhancement can predict hemorrhagic transformation (69). Major disadvantages are the inherent risk of the radiation used to generate the images. Furthermore, the rate of side effects of the CT-based contrast agent (such as severe allergic reactions) is higher than that of MRI contrast agents.

3.2.3. MRI

MRI has evolved over the last decades into the most versatile neuroimaging modality. MRI can provide information on anatomy, structure, physiology, and function. Contrast enhanced MRI is extensively used to measure qualitatively the integrity of the BBB. Quantification is very well possible with DSC techniques. Like in CT, DCE-MRI measures the change of signal intensity in a given brain volume during the passage and distribution of a contrast agent. However, these signal changes are less straightforward in MRI.

MRI is based on the principle of nuclear magnetic resonance and there are many good reviews available describing the theoretical background of MRI. In clinical and biomedical MRI, the MR signal from protons from tissue water is spatially encoded to generate an image. The magnitude of the proton MRI signal depends primarily on the proton density and the relaxation times T_1 and T_2 (the latter is called T_2^* if there are any disturbances in the local magnetic field). In addition, the MR signal is affected by molecular self-diffusion and flow. Importantly, specific contrast on MR images can be achieved by explicit sensitization of the MRI experiment to these processes. For example, MR images can be made proton, T_1 , T_2 , or diffusion weighted.

Both proton density and diffusion are reflection of the properties of the tissue. They cannot be altered by an exogenously applied tracer. However, T_1 and $T_2^{(*)}$ relaxation times of protons may be changed by the presence of MR contrast agents. MR contrast agents are molecules with unpaired electrons and are called paramagnetic or super-paramagnetic depending on their potency in changing relaxation times. Most contrast agents used for measurements of BBB integrity have gadolinium (Gd) or iron (Fe) incorporated in their structure. These ions have known strong (super) paramagnetic properties. The change in the reciprocal of the relaxation times, i.e., $1/T_1$ or $1/T_2^{(*)}$, is largely linearly dependent on the concentration of the contrast agent making pharmocokinetic modeling in principle straightforward. However, the presence of the tracer in a compartment is measured indirectly. Not the tracer itself, but the effect of the tracer on the surrounding protons is measured. This effect may cause overestimation of the permeability of the BBB, as the intravascular tracer may change the properties of protons beyond the vascular wall, even if the BBB is intact (35, 56).

Gadolinium chelates are the most commonly used MR contrast agents, because these structures form stable non-toxic complexes with the highly toxic gadolinium ion. They substantially reduce the T_1 -relaxation

time of surrounding protons. In larger concentrations they may also have a significant $T_2^{(*)}$ effect. There are many Gd-based contrast agents available, but only a few are approved for clinical use. From these, the ionic contrast agent gadopentetate dimeglumine (Gd-DTPA, mw: 590 Da) is mostly used in clinical practice. Quantitatively, Gd-DTPA based DCE-MRI has been used to obtain PS related products in many models of neurological diseases like stroke (70,71), brain trauma (72), and dementia (73). In humans, Gd-DTPA based DCE-MRI is used to quantitate BBB integrity in patients with MS (74,75), and for grading and typing different brain tumors (76,77). Gadoterate meglumine (Gd-DOTA, mw: 557 Da), which has a relatively longer plasma half-life than Gd-DTPA, may also be used. For example, Gd-DOTA-based DCE-MRI showed progressive BBB leakage in rats with gliomas (78) and in patients with MS (79). Other Gd-based agents are the non-ionic compounds gadodiamide (mw: 573 Da) (80,81) and gadoteridol (mw: 558 Da) (82). Different sized Gd-based contrast agent may be used to quantitatively determine the severity, i.e., size dependency, of BBB leakage in tumors (83).

Another class of MR contrast agents consists of particles with incorporated iron. These agents have a more pronounced $T_2^{(*)}$ reducing effect. The T_1 reducing effect may also be present, depending on size and concentration of the contrast agent (84). Many of these agents have a crystalline iron oxide core, which is coated with either dextran or siloxanes. Depending on their size they are either called superparamagnetic iron oxides (SPIO, approximately 50 nm and larger) or ultrasmall SPIO (USPIO, approximately 30 nm and smaller). Like large Gd-DTPA complexes, SPIOs are mainly used as intravascular agents for CBV and CBF determinations. Despite their relative large size, these agents have been applied to detect severe BBB leakage, in animal models of stroke (85), brain tumors (86), osmotic disruption (87,88), and trauma (89). A special consideration is that monocytes may incorporate circulating SPIOs by phagocytic processes. Infiltrated macrophages may thus induce signal changes with an intact BBB, especially if the time between tracer injection and MRI is relatively long (up to several hours). Mainly the smaller sized USPIOs may cause this effect, as they have a longer blood plasma half-life of several hours.

An interesting recent development in MRI is the concept of in vivo imaging of molecular and cellular processes. MRI based molecular and cellular imaging is in its infancy, but interesting results have already been achieved in which processes involved in BBB damage have been visualized in vivo. In many neurological disorders, leakage of the BBB is preceded by the activation of adhesion molecules at the endothelium (90). Several of these molecules have been visualized with specially designed (super) paramagnetic probes. For example, the expression of intercellular adhesion molecule-1 (ICAM-1), involved in leukocyte binding during inflammatory processes, has been shown in an animal model of MS with antibody-conjugated liposomes containing gadolinium (91). In another animal model of neuroinflammation, the up-regulation of E-selectin, also involved in leukocyte adhesion, was visualized with a gadolinium-based target agent after stimulation of the endothelial E-Selectin expression with TNF- α or IL-1 β (92). The eventual intra-cerebral infiltration of leukocytes has been visualized with MRI too. For this purpose leukocytes were labeled with paramagnetic compounds. In vivo labeling strategies use the above-mentioned capacity of monocytes to incorporate circulating USPIOs. In a typical experiment, USPIOs are introduced intravasculary, and 12-24 hr later the presence of infiltrated macrophage is measured with MRI. This method has been successfully applied in animal models of stroke (93), brain tumors (94), MS (95), and recently in patients with brain infarction (96). Cells may also be labeled ex vivo, and introduced thereafter in living systems. This strategy has been used to visualize the migration of lymphocytes into the central nervous system in animal models of MS (97). Finally, the intracerebral homing of monocytes and T-cells, has been visualized after labeling these cells in vivo with iron containing antibodies directed toward cell-specific marker molecules (98).

4. SUMMARY AND CONCLUSION

Measuring the BBB integrity is a valuable tool for the diagnosis and followup of brain pathology. If spatial resolution is an issue, histological techniques are superior to CT and MRI. However, in most studies CT and MRI have largely replaced invasive ex vivo techniques, and are the most important imaging modalities in clinical practice. With pharmocokinetic models, BBB leakage may be quantified. Recent developed molecular and cellular imaging methods may be used to characterize different molecular and cellular events underlying BBB pathology in vivo. This, together with the foreseen development of higher resolution scanners, will undoubtedly result in further advances in imaging the dynamic processes involved in BBB breakdown.

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19

Influence of Brain Trauma on Blood—Brain Barrier Properties

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According to the last assembly of the World Health Organization in Geneva nearly 1.2 million people died in 2002 from road traffic injuries, the majority in road accidents. This issue was discussed for the very first time in 30 years. Road accidents are estimated to increase and by 2020, rank third in World Health Organization's Global Burden of Disease, above HIV, malaria, and tuberculosis.

1. INTRODUCTION

Traumatic brain injury (TBI) remains the leading cause of death and permanent neurological disability of young individuals living in industrialized countries. Most of these victims are involved in road traffic accidents. The significant advancement in critical care management of the last decades has achieved a remarkable increase in the survival rate of such patients. However, prevention and attenuation of secondary brain damage remains a fundamental task in neurotrauma research in order to improve the quality of life of the survivors and reduce further the death rate.

2. CLASSIFICATION OF TRAUMATIC BRAIN INJURY

The TBI results from either external or internal mechanical forces. It impacts on permanent or temporary impairment of cognitive, physical, and psychosocial functions with an associated diminished or altered state of consciousness (1). Despite improvements in medical and surgical treatment of TBI, there are currently no neuroprotective agents available with the ability to enhance tissue repair and neurological recovery (2).

In general, TBI can be classified according to the time of onset, location and pattern of tissue injury (3). Cerebral damage resulting from TBI is commonly termed either primary or secondary injury. The first occurs at the time of the incident, whereas secondary injury is determined by a very complex number of physiological and molecular cascades that lead to further tissue destruction and consequent neurological damage (4). Activation of such processes begins at the time of the primary impact and persists for hours or days after trauma aggravating the initial damage. The time following primary brain injury offers a potential therapeutical window of intervention for reducing irreversible loss of neuronal cells and ultimately improving neurological recovery. Therefore, current scientific and medical research focuses on the development of new pharmacological strategies with the objective to neutralize selective pathways proven to be neurotoxic in experimental settings or alternatively to enhance those with protective action. Unfortunately, the recent decades have witnessed the failure of a large number of clinical trials that applied compounds previously successful in animal models of TBI. One of the reasons for this failure remains the question of whether these drugs have crossed the blood-brain barrier (BBB) and reached the therapeutical target. Therefore, the importance of studies on the function and dysfunction of the BBB resulting from TBI needs to be emphasized.

2.1. Primary Brain Injury

Primary injury to the brain causes immediate tissue deformations that directly damage vessels, axons, neurons, and glia. These morphological changes are the result of mechanical forces and manifest in either a focal or a diffuse pattern (Table 1 for classification of TBI). While focal brain injury is caused by contact forces, which directly impact the head causing skull fractures and underlying localized tissue damage, diffuse axonal injury is generated by acceleration/deceleration forces to the head and does not necessarily require a direct contact (5). Distinction between the two classifications is clinically determined by neuroradiologic means as well as physiological observations. Focal lesions are easily identified with computed tomography (CT), magnetic resonance imaging (MRI), and single proton emission photography (PET) scanning. However, the most reliable method

Time of injury	Injury by location	Pathophysiology
Primary injury	Focal	Cellular
Skull fracture	Cortical contusions	Cell swelling
Focal injury	Cortical lacerations	Cell necrosis/apoptosis
Diffuse injury		Axonal disconnection
Penetrating injury	Haematomas	Blood-brain/CSF barrier dysfunction
	Epidural	-
Secondary injury	Subdural	Molecular
Hypoxia	Intracerebral	Free radical release
Ischaemia		Excitotoxicity (glutamate)
Oedema	Diffuse	Inflammation
Neurochemical alterations	Concussion	
	Diffuse axonal injury	

 Table 1
 Classification of Traumatic Brain Injury^a

^aClassification of primary TBI can be achieved by means of computed tomography and sophisticated intensive care monitoring that allow the early detection of secondary brain damage. The table shows the morphological features of primary, focal or diffuse brain injury and their localisation within the brain. The major cellular and molecular events that characterise the pathophysiology of secondary events are also mentioned.

to detect diffuse axonal damage still remains microscopical analysis rather than the conventional imaging techniques.

Focal brain injury comprises a variety of enclosed lesions including skull fracture, vascular injuries that, depending on their localization, produce epidural, subdural, and intracerebral hemorrhages, and/or hematomas. Parenchymal contusions, lacerations, penetrating wounds, and in small proportion axonal damage are also found.

Based on the histological definition, diffuse brain injury causes axonal swelling and retraction ball formation of disconnected fibers. It particularly affects the white matter and is characterized macroscopically by general cerebral swelling and global neurological dysfunction (5). Irreversible differentiation occurs either at the time of injury (primary axotomy) or at later times, as a result of neuropathologic changes that involve neurofilament disassembly and cytoskeletal disarray, impaired axonal transport, axonal swelling, and disconnection (secondary or delayed axotomy) (6).

2.2. Secondary Brain Injury

The development of secondary brain injury strictly depends on the quality of the initial type of damage and also on systemic circulatory and respiratory alterations that perturb brain perfusion and oxygenation. Overall, the ultimate consequences of the structural and biochemical changes comprise elevation of intracranial pressure (ICP), ischemic or hypoxic insults, edema, and as a result of open fractures, infections. The most frequent features observed following focal brain injury are enlargement of contusions and focal hypoxic-ischemia, whereas following diffuse brain injury, generalized brain swelling. However, it is important to state that both types of primary damage contribute to the generation of secondary changes as most of the patients with severe head trauma present with an individual combination of focal and diffuse brain lesions.

3. CEREBRAL VASCULATURE AND AUTOREGULATION

One of the first consequences of TBI includes the alteration of cerebral blood perfusion that compromises brain regions initially intact. Following primary TBI, loss of vascular autoregulation has been described, which is the physiological ability of the vessels of contracting or dilating the lumen in response to changes in the balance of oxygen and carbon dioxide. This is an active compensatory or adaptive response aimed at adjusting cerebral blood flow (CBF) in order to maintain a homeostasis of tissue metabolism. The vascular smooth muscle responds to changes in pH in the perivascular space. Since, CO₂ can cross freely the BBB and change perivascular pH. hypercarbia induced by hypoventilation, induces vasodilation and higher CBF. It is known that vasodilation occurs early after TBI, increasing the total blood volume present in the brain tissue and thus contributing to cerebral edema and elevation of ICP. Smooth muscle relaxation is regulated by various soluble factors that are secreted in response to brain injury, such as arachidonic acid cycle metabolites and nitric oxide (NO) (7). These factors are produced by endothelial cells upon activation by different stimuli. Interestingly, vasodilatation that occurs in the small vessels seems to be a compensatory mechanism to the vasospasm observed in the large intracranial arteries in the early stage following traumatic injury. Studies on head injured patients have shown that both CBF and cerebral blood volume were significantly lower in ischemic zones, indicating that early compromise of the microvasculature is likely the cause of ischemia, rather than vasospasm of the larger conductance vessels (8). Martin et al. (9) identified three different circulatory stages after severe head injury: phase I of hypoperfusion within the first day, phase II of hyperemia, with increasing CBF and phase III from day 4 to 15, of vasospasm with fall of CBF and further increase in cerebral artery blood flow velocity and normal hemispheric index. Despite the knowledge on modulation of the cerebral vasculature in response to TBI, the mechanisms that determine the loss of autoregulation and their potential therapeutic management are largely unknown.

Systemic physiological alterations can strongly affect the structure of the endothelium and consequently the permeability of the BBB. Dramatic and rapid rise in blood pressure has often been observed in patients with severe TBI. Experimentally, proof of increased permeability of the BBB caused by hypertension has been given in a study by van den Brink et al. (10). When comparing rats with or without post-traumatic hypertensive surge, they showed that the former caused a higher permeability index of the endothelium based on the degree of extravasation of radio-labeled albumin. It is conceivable that stretch injury applied to the brain tissue in similar fashion to that provoked by acceleration–deceleration forces, may cause alterations of the endothelial membrane and therefore affect its permeability by triggering the synthesis of vasoactive mediators that contribute to the dysfunction. Such mechanisms have been somewhat reproduced in vitro on cultured endothelial cells and demonstrated immunoactivation in response to physical trauma (11).

4. METHODS TO ASSESS BLOOD-BRAIN BARRIER DYSFUNCTION AFTER BRAIN INJURY

4.1. Extravasation of Blood—Markers

It has already been mentioned in the previous chapters that the BBB represents a unique interface between the brain and the peripheral circulation that allows passage exclusively to a highly selected number of ions, amino acids, molecules, and cells in order to guarantee the homeostasis of the brain metabolism. Integrity of the BBB is generally lost in response to severe TBI by two main mechanisms: (1) early physical rupture provoked by tearing forces that generate local hemorrhages and/or hematomas immediately after trauma and (2) later on, the release of multiple soluble factors that attack the membrane of endothelial cells and cause the opening of tight junctions, allowing the diffusion of serum components in the interstitial space. Increase of water content in the brain parenchyma through the leakage of the BBB contributes together with vasodilation and increased CBF, to edema and elevation of intracranial pressure.

Various methods have been employed to assess the regional and temporal profile of BBB dysfunction after TBI. Evidence of increased BBB permeability to large molecules such as albumin (ca. 60 kDa) has been demonstrated in numerous animal models of TBI by measuring the amount of Evan's Blue extravasated in the brain following intravenous injection. This dye binds to serum proteins, mostly albumin, and can either be visualized on tissue sections by fluorescence microscopy or quantified in homogenized tissue by fluorospectrophotometry (12). Another simple method employs immunohistochemistry for detection of endogenous extravasated albumin or IgGs on brain sections. Alternatively, molecular tracers have been injected intravenously and their accumulation analyzed once diffused into the injured brain. One of the tracers most commonly used is horseradish peroxidase. These relatively simple methods have been utilized in numerous animal models of TBI, the majority of them producing focal brain lesions such as lateral fluid percussion brain injury (13–21), cortical impact (22–27) and acceleration/deceleration injury (10).

Collectively in these studies, increased permeability to labeled serum proteins in the early hours post-trauma (2 to 6 hours) was found in the contused zone of the brain hemisphere. However, it has been indicated by Schmidt and Grady (21) that the pattern of BBB damage strictly depends on the region of impact. Application of the cannula at different brain levels to deliver fluid percussion injury (central or paracentral position) resulted in BBB dysfunction of both, common areas of the lesioned hemisphere such as the cortex, hippocampus, thalamus, and brain stem, but also regional differences when the impact was localized more centrally resulting in bilateral extravasation of the tracer (21).

Differences and similarities in the extent and regional distribution of increased BBB permeability were shown by comparing fluid percussion with control cortical impact injury at two distinct severities of injury (28). Although the impact was produced exactly on the same site, moderate injury generated a stronger extravasation of IgG in the cortex and hippocampus in the cortical contusion model, whereas following mild injury, fluid percussion resulted in more abundant IgG accumulation in the hippocampus. In conjunction with regional changes, it seems that a temporal biphasic leakage of the BBB can also occur. After cortical impact injury, early BBB dysfunction within the injured cortex was present for 3 hr and was followed by a secondary leakage at the ipsilateral hippocampus at 1 and 2 days, suggesting the action of secondary mediators acting on the cerebral vasculature. Interestingly, delayed BBB damage colocalized with the region of abundant neuronal loss (26).

Post-traumatic hypoxia is a significant factor contributing to the dysfunction of the BBB. In fact, when hypoxia was added for 30 to 40 minute at 10% O₂ immediately after fluid percussion injury, BBB permeability was extended to the contralateral side and lasted up to 24 hours. Thus, hypoxic insult exacerbates and prolongs vascular damage, edema and neurological dysfunction caused by trauma (17,29). This is clearly of clinical significance since it has been documented that severe TBI is frequently associated with respiratory distress, in 45% of the patients that are at higher risk of secondary complications and death (30).

The cerebral vasculature is an extremely sensitive structure subjected to changes in response to minimal variations of the environment. Therefore, the use of specific methods for the assessment of BBB permeability needs to be taken with caution. One example was shown years ago by Povlishock et al. (31), with regard to the use of horseradish peroxidase. Extravasation of horseradish peroxidase in the brain parenchyma was shown in spite of having injected the tracer after the traumatized animal had been killed and the brain tissue perfusion-fixed. Post-mortem diffusion through the altered endothelial membrane seemed to be the mechanism involved rather than the metabolically active passage hypothesized, based on the presence of endothelial vesicles filled with the tracer, as seen using electron microscopy. This study shows certain limitations in the interpretation of results on the dynamics responsible for the passage of molecules across the BBB.

4.2. Neuroimaging Techniques for Assessment of BBB Damage

Sophisticated neuroimaging techniques routinely used in clinical practice are now being applied to animal models of TBI to study the function of the BBB. The MRI and diffuse weighted imaging (DWI) provide information on the anatomy and biophysical processes responsible for BBB damage and have been successfully used in stroke and ischemia. These techniques have revolutionized the diagnostics for the classification of the severity and type of TBI in humans, and in particular for the detection of diffuse axonal damage, known to be more difficult to assess (32). The DWI detects changes in water diffusion movements based on random kinetic changes of molecules. This technique presents various advantages: (1) it is a sensitive indicator of early pathological changes, (2) it can be applied repeatedly at multiple time intervals on the same animal to monitor changes in brain structures, (3) it facilitates an overall scanning imaging along multiple planes of the whole brain, and (4) can discriminate traumatic from ischemic lesions (33).

4.3. BBB Dysfunction and Post-traumatic Brain Edema by MRI

In general, focal brain injury seems to generate a more profound and prolonged damage of the BBB as compared to diffuse brain injury. MRI has been used to better define the features of the dysfunction of the BBB caused by diffuse axonal injury in a rat model. This technique also allows the detection of post-traumatic brain edema and the distinction between vasogenic and cellular edema (34,35). Comparison between various models of focal and diffuse TBI with the use of both MRI and Evan's Blue methodology, clearly showed that in response to axonal injury there is a rapid and brief opening of the BBB (from 15 to 30 minute) with a delayed increase of hemispheric water content, a parameter used to quantify edema. Conversely, BBB dysfunction after controlled cortical impact injury generated a BBB opening in both hemispheres for up to 4 hr, although edema was shown exclusively in the injured hemisphere. Altogether this study supports the view that BBB dysfunction is not a major factor for edema formation, although maintaining its role as a potential contributor (36). An elegant study by Albensi et al. (37) showed a strong correlation of the neuroimaging findings using MRI with histological and neurological alterations in rats following fluid percussion injury. Interestingly, increased apparent diffusion

coefficient (hyperintensity), corresponding to augmented BBB permeability, was observed at 24 hours and 2 weeks post-injury particularly in the cortex and hippocampus, where edema and BBB dysfunction had been previously identified (15). In addition, simultaneous hypointensity was observed early in the corpus callosum and at the junction between the cortex and hippocampus, which may reflect the presence of hemorrhage and tissue destruction. Similar results were published by Assaf et al. (38), in a model of closed head injury that similarly to fluid percussion injury generates a cortical contusion. Hyperintensity at 24 hours corresponded to highest brain edema and resolved by 1 week, whereas regions of hypointensity correlated with the infarcted area identified by histology. This study also proved the higher sensitivity of diffusion weighted MRI versus T2-weighted MRI in capturing a stronger and earlier signal of cerebrovascular pathology.

More importantly, neuroimaging techniques like MRI, together with other clinical or molecular parameters, allow the assessment of the efficacy of preclinical administration of neuroprotective agents, providing a good measure for the development and resolution of edema, hemorrhage and tissue damage in the traumatized animals (39).

In head trauma patients, a restricted number of studies on the dysfunction of the BBB are available as the methods existing for such analyses are more limited than techniques used in animal models of TBI. In spite of these limitations, opening of the BBB to molecules normally excluded by the intact barrier has been demonstrated by intravenous injection of gadolinium later visualized by MRI (40,41). Increased tissue accumulation of gadolinium was observed at the pericontusional edema site as early as 2 hours and lasted for the early days post-injury, suggesting that edema may be partially of vasogenic nature. Clearly, in brain injured patients these pathological changes follow a prolonged time course as compared to the animal models of TBI.

5. CELLULAR AND MOLECULAR CONTROL OF BBB AFTER BRAIN INJURY

5.1. Role of Reactive Oxygen Species and Nitric Oxide

The pathophysiological sequelae of secondary brain damage is very complex and comprises a myriad of molecular cascades that are set in motion immediately after primary injury and maintained for a long time period, with the result of compromising the entire metabolism of the brain. Neurochemical disbalance exerts pathogenic effects on blood flow and BBB integrity, consequently exacerbating neuronal dysfunction.

Only the most relevant cascades that involve the participation of reactive oxygen species, NO and inflammatory mediators will be mentioned in this chapter (4). Post-traumatic reduction of CBF is responsible for the development of what is defined as ischemia-reperfusion injury (4). The initial vasoconstriction and the resulting dramatic reduction in O_2 supply is rapidly followed by abundant re-introduction of O_2 once perfusion has been re-established, leading to the release of reactive O_2 species. The O_2 free radicals such as hydroxyl (OH^{*}) and superoxide (O_2^*) have been implicated in traumatic cerebral vascular injury and neuronal cell death. These highly reactive free radicals can cause peroxidative damage to membrane phospholipids and the oxidation of intracellular proteins and nucleic acids. Generation of O_2 radicals occurs via different cascades including the activation of glutamate receptors (NMDA) that result in dramatic increase of intracellular Ca^{++} , as well as through the metabolism of arachidonic acid and inflammation (42).

The main factors involved in radical-mediated tissue damage were identified through the blockade of cyclooxygenases (COX), which reduced the number of endothelial lesions. The COX catalyses the metabolism of arachidonic acid, which is responsible for the synthesis of prostaglandins and the release of O_2 radicals. Experimental proof of this role was demonstrated with the application of scavengers, which resulted in clear reduction of endothelial damage after TBI and restoration of CBF and vascular vaso-dilator responses (43). Association of reactive hydroxyl radicals and post-traumatic BBB dysfunction was assessed by a dramatic burst of hydroxyl found to be maximal within minutes after control cortical impact injury. This phenomenon was followed by delayed lipid peroxidation (30–60 min) which was simultaneous to the extravasation of Evan's Blue dye into the parenchyma (44). In the same study, application of Tirilazad reduced both lipid peroxidation and BBB permeability.

Among the scavengers used in animal models of TBI, the most effective were superoxide dismutases (SODs), indomethacin, and Tirilazad. Unfortunately, these successful results were not sustained by randomized, controlled clinical trials on TBI patients (45). Despite the failure of clinical trials, there is a large body of experimental evidence showing the clear contribution of oxygen free radicals in the damage of cerebral vasculature after brain injury.

NO is a key reactive nitrogen radical involved in both normal and post-traumatic cerebral vascular function. NO is a potent vasodilator and its tissue levels increase transiently and decrease again below normal levels within minutes after experimental TBI (46). Interestingly, these fluctuations reflect the reduction and augmented pattern of CBF observed after TBI. Despite its potent role as regulator of vascular reactivity in physiological and pathological conditions, NO seems to be involved in the dysfunction of the BBB. Increased expression of endothelial nitric oxide synthase (eNOS), used as an indicator for NO synthesis, at times and regions corresponding to maximal breakdown of the barrier, edema and post-injury angiogenesis, suggests a potential role for NO in these processes (47). On this line of evidence, application of antioxidant compounds significantly attenuated constitutive and inducible (i) NOS and also reduced BBB permeability, edema, and tissue damage in a hyperthermic injury model (48). Besides vasodilatation, iNOS and eNOS also contribute to the inhibition of platelets and leukocyte adhesion and to the proliferation of vascular smooth muscle cells thus promoting post-traumatic angiogenesis (49). Despite the accumulating evidence for these functions, the ultimate impact of NO and of the various NO synthase isoforms on the function of the BBB needs to be further elucidated.

5.2. Impact of Inflammation on BBB Function After Traumatic Brain Injury

The literature accumulated in the last two decades clearly demonstrates that the brain is an immunologically active organ. It is generally accepted that after an insult, inflammation is initiated directly by resident cells of the brain before becoming detectable in the blood stream. Of particular importance is the role played by microglia that correspond in embryological origin, phenotype and function, to their blood-borne counterparts, the macrophages.

Pivotal in the inflammatory processes is also the role of astrocytes, not only because they represent the most abundant cell type in the brain but also since they share with microglia and macrophages most of the immunological functions (50). Once brain cells have been primed, locally secreted cytokines and chemotactic cytokines (also termed chemokines), synergistically act on the endothelium of the BBB, where they induce the synthesis of cell adhesion molecules and mediate the accumulation of peripheral leukocytes into the lesion site (Fig. 1).

By acting as the interface between the brain parenchyma and the systemic circulation, the BBB is subjected to a bidirectional exchange of immune mediators elicited by either cerebral or systemic inflammatory diseases. As septic infections can lead to encephalopathy, post-traumatic cerebral inflammation may trigger the passage of cytokines into the blood stream and influence the function of other organs. This concept seems to apply when chronic CNS pathologies like multiple sclerosis are exacerbated by independent systemic inflammatory reactions, which have been shown to trigger relapses (for review see Ref. 51). The cause of these relapses has been attributed to the release of systemic cytokines that activate blood immune cells facilitating their extravasation into the nervous system where local inflammation is re-ignited. It has also been proposed that CNS trauma itself may promote the formation of new lesions in MS patients. The opening of the BBB caused by traumatic injury may contribute to further lesion formation, since myelin components such as myelin basic protein, PLP and MOG may enter the peripheral circulation where they can activate immunocompetent cells and humoral mediators that will ultimately penetrate the CNS (for review see Refs. 52-54).



Cerebral Inflammation Impacts on BBB Function

Figure 1 Impact of inflammatory mediators on the function of endothelial cells at the BBB. Following TBI, a bidirectional passage of inflammatory mediators can occur between the brain parenchyma and the blood stream, either via passive diffusion across the dysfunctional BBB or by mechanisms of active transport mediated by cytokine receptors expressed on endothelial cells. Cytokines produced in the brain trigger the synthesis of cell adhesion molecules on the endothelium and the secretion of chemotactic factors. Consequently, these molecules act in concert for the activation, migration, adhesion, and extravasation of neutrophils and macrophages across the BBB into the lesioned brain area.

If not necessarily of autoimmune etiology, a chronic and progressive neurodegeneration has been observed in humans and rodents after brain injury (55–57). The molecular processes involved in these uncontrolled events need to be identified, however it can be presumed that prolonged BBB dysfunction and exposure of CNS specific proteins to the immune system may create the conditions for a slow and chronic inflammation.

That traumatic injury to the brain induces a profound inflammatory response derives from a multitude of studies carried out in different animal models, as well as in patients with head injury (for review see Refs. 58–60). Detection of pro- and anti-inflammatory cytokines and other immune mediators has been demonstrated in brain tissue of rodents subjected to different models of focal or diffuse brain damage (fluid percussion, control cortical impact, closed head injury, and traumatic axonal injury) as also in head



Figure 2 Peripheral and cerebral inflammatory response after traumatic brain injury. Immunoactivation has been described in both CSF and serum of patients with severe head trauma. A variety of cytokines and adhesion molecules have been detected in these fluids for several days post-injury. Comparison of the concentrations of each mediator, revealed that some of them are at higher levels in the CSF (TNF, IL-6, -8, -10) while others are higher in serum (TGF- β , soluble ICAM) (59,69). Separation of the two compartments is mediated by the BBB and the blood—CSF barrier where inflammation plays a pivotal role in modulating the permeability of endothelial cells.

trauma patients' cerebrospinal fluid and serum or microdialysates obtained from the brain parenchyma (61–72) (Fig. 2). In rodents, increase of such secreted cytokines occurs in the first hours post-trauma and usually returns to baseline concentrations within 12–24 hr whereas in humans their levels remain above control concentrations for several days or weeks (66–69).

Almost all cells found in the brain tissue have the ability to synthesize cytokines, including neurons, which also express some of their corresponding receptors (72,73). The cells participating in these complex immune responses are mainly localized in the tissue surrounding the primary lesion especially in focal damage. This area, defined as the penumbra, is initially healthy but becomes at great risk to degenerate due to the robust neurotoxic cascades activated after TBI.

In conjunction with excitotoxicity, predominantly mediated by excess of the neurotransmitter glutamate or the release of reactive oxygen species and other free radicals, a detrimental role has also been attributed to neuroinflammation since the concentrations of specific mediators and in

particular of TNF, correlated with tissue damage and local invasion of leukocytes, BBB permeability and neurological impairment. The most compelling demonstration that cytokines are involved in BBB damage is suggested by studies using cytokine antagonists in models of TBI. In experimental TBI, inhibition of TNF with the administration of general immunosuppressive agents or specific neutralizing compounds showed that these harmful events could be attenuated (72,74-76). However, more recently, the genetic deletion of TNF or TNF receptor expression in knockout mice used in focal brain injury models clearly showed that TNF displays essential neuroprotective properties and that its expression cannot be completely abolished (27,77,78). Several in vitro studies on cultured astrocytes have demonstrated that upon stimulation with cytokines including TNF, these cells express a variety of neurotrophic factors that contribute to neuronal differentiation and survival (58). As a consequence of these studies we are left with the dichotomy to resolve as to what extent cerebral inflammation is neurotoxic or rather promotes mechanisms of tissue repair.

Given these premises, let's return to the BBB and its alterations caused by cerebral inflammation after neurotrauma. Due to their position, endothelial cells are exposed to the humoral and structural changes that occur to the neighboring cells, in particular astrocytes as well as recruited blood cells. Endothelial cells have been shown to express cytokine receptors thus allowing an active passage of these molecules across the BBB (79,80). In a model of spinal cord injury, CNS penetration of TNF injected systemically, was shown via increased TNF-receptor expression at the spinal cord barrier, supporting a receptor-mediated saturable transport system into the brain (81,82). However, the same group also demonstrated that cerebral endothelium lacks the receptors for certain anti-inflammatory cytokines such as IL-10 and TGF- β proposing that their intravenous infusion for therapeutic purposes is only effective if applied at times with known opening of the BBB (83,84).

Differences in the extent of inflammatory response and BBB dysfunction are found with respect to the area of the CNS that has been traumatized. An elegant study by Schnell and others clearly demonstrated selective sequelae of inflammation elicited either by brain or spinal cord injury (85). The activation of resident cells, infiltration of leukocytes, patterns of cytokine release and increased BBB permeability were found at a greater magnitude in the injured spinal cord when compared to brain injury. To pursue this hypothesis, injection of recombinant TNF or IL-1 β into normal brain or spinal cord, independent of injury, failed to induce BBB dysfunction in the first but elicited a marked breakdown of the BBB in the second, supporting the evidence of substantial structural and functional differences between the two compartments of the CNS (86).

Following TBI, chronologically, the first cells crossing the BBB are neutrophils immediately followed by blood macrophages. The activation of these cells has been thought to contribute to the dysfunction of the BBB as they release abundant mediators of the oxidative pathway that affect the integrity of the endothelial membrane. The early release of cytokines such as TNF, IL-1, and IL-6 induces the up-regulation of specific cell adhesion molecules on the endothelium (Fig. 1). Among these, E-selectin and the cell adhesion molecule-1 (ICAM-1) have been extensively studied in animal models of TBI as well as in head trauma patients (87-89). Despite the fact that the putative role of these adhesion molecules has been largely considered, the experimental results present some temporal and functional discrepancies. In this regard, in our and in other laboratories, the up-regulation of ICAM-1 in the brain of mice with focal brain injury, as well as in traumatic axonal damage in rats, was maximal a few days (4-7) after TBI. The increase in ICAM-1 was thus subsequent to the accumulation of leukocytes that peaks at 1 day after focal injury. This suggests that other endothelial molecules such as E-selectin may play a decisive role for initiating early cell adhesion (27,90–93). In accordance with this are studies carried out on ICAM-1 knockout mice subjected to control cortical impact injury that failed to show changes in the extent of cerebral leukocyte infiltration, tissue damage, and functional outcome (94).

We have previously shown that both ICAM-1 and MIP-2 (the mouse homologue of human IL-8, a chemotactic and activating factor to neutrophils) are induced by TNF in cultured astrocytes and cerebrovascular endothelial cells (92), and that they are up-regulated after focal brain injury in brain homogenates. However, when TBI was induced in TNF/lymphotoxin- α double knockout mice, we observed a decrease in ICAM-1 and MIP-2 proteins but no differences in BBB dysfunction or amount of infiltrated neutrophils (27,95). However, although no changes in BBB breakdown were noticed between the TNF/lymphotoxin- α knockout mice and their wild types, evident differences were found when comparing two strains of mice with distinct genetic background. Namely, TNF/lymphotoxin- α knockout of a B6x129 strain, presented a milder dysfunction of the BBB as compared with the IL-6 knockout of the C57/BL6 strain. This implies that the individual genetics may predispose to the BBB dysfunction induced by TBI.

In our studies, we have been interested to verify and compare the data derived from animal models of TBI with cerebral inflammation elicited in patients with severe head injury. To estimate the dysfunction of the BBB in head trauma patients is not an easy task. However, we have used a method based on the CSF/serum ratio of albumin (QA) that takes into account the leakage of serum albumin into the CSF when the BBB/ blood–CSF barrier is disrupted. This method has been used in the diagnosis of neurological diseases but has the limitation of not distinguishing between barrier disruption and blood contamination due to hemorrhages, frequently occurring after TBI (96). In the clinical study, the concentration of various

soluble cytokines, chemokines, or adhesion molecules has been measured in CSF and serum of head trauma patients, with respect to the daily QA. We attempted to determine whether increased cytokines in the CSF were derived from intracerebral synthesis or were a reflection of BBB breakdown. Interesting results were observed on the relationship between systemic and brain elicited inflammatory processes. Every cytokine presented an individual pattern of release over time but was usually found markedly increased in the early days post-injury and returned to lower levels after the first week (69). In addition, some immune mediators were consistently higher in the CSF as compared to the parallel serum measurements (IL-6 and IL-8; 66, 97). Others on the contrary that were normally present at higher levels in control serum but not in control CSF, increased significantly in the CSF after TBI whereas serum levels remained within the normal range at most times analyzed (TGF- β and soluble (s) ICAM-1; 88,98). In order to assess the origin of these cytokines, either cerebral or systemic, we used the QA for an estimation of BBB function. When analyzed against the QA, TGF-β and sICAM-1 in the CSF strongly correlated with the dysfunction of the BBB, indicating the passage of these cytokines from the periphery into the CNS. However, the assessment of the TGF-β or sICAM-1 index, calculated as the ratio of TGF- β or sICAM-1 in CSF and serum (QTGF- β) divided by the QA, that takes into account the concentration of albumin in the CSF, suggested that partial cerebral synthesis of both immune mediators also occurred (88,98). Moreover, sICAM-1 measured in the CSF was significantly elevated in patients with large cerebral contusions estimated on computed tomograms as compared with patients without focal damage (88). Of notice is the fact that these regions are mostly subjected to the disruption of the BBB.

The opposite directional passage of cytokins from the injured brain into the blood stream across the BBB was instead proposed for the high levels of IL-6. After leaking into the blood stream, IL-6 may induce the acute phase reaction that we observed in all TBI patients included in the study (66)." This hypothesis is difficult to prove in humans; however, experimental evidence showed that: 1) IL-6 increases endothelial cell permeability in vitro and 2) IL-6 induces an acute phase response in rats when injected intracerebrally (79,99,100). This hypothesis is corroborated by the fact that IL-6 is up-regulated at the mRNA and protein level in rat brain following axonal injury and is released at higher concentrations in the CSF as compared to serum (67).

In addition to IL-6, it has been shown that direct infusion of IL-1 in the ventricular compartment elicited an increased permeability of the endothelium and the adhesion of various leukocyte subtypes (101). A well-designed study by Rothwell and coworkers (102) also indicated that IL-1 may be implicated in the pathogenesis of ischemic brain damage and is therefore relevant in TBI. The results showed that inhibition of IL-1 activity with the intraventricular application of IL-1 receptor antagonist (IL-1ra), in a model of cerebral ischemia, reduced the volume of the infracted area. Moreover, therapeutical application of IL-1ra in this model was more effective as compared to the chronic deletion of IL-1 α and - β in gene knockout mice, possibly due to compensatory mechanisms acting in the absence of their expression. In agreement with these data, the use of astrocyte-specific transgenics overexpressing IL-1ra, significantly improved the neurological outcome in a cortical contusion model. Immunological analysis showed no increases in IL-1 and IL-6 but no changes in the expression of TNF after injury (103). Further studies should investigate the role of IL-1ra in the attenuation of BBB dysfunction.

6. CONCLUDING REMARKS

The BBB is a delicate structure that works on fine-tuning the selection of molecules and cells allowed to enter the brain tissue. As such it is susceptible to systemic physiological changes as well as microenvironmental disarray that occur in neurotrauma. Its vulnerability depends on a complex network of cascades and factors that are released by the injured tissue. To date we have not completely understood how its function is regulated and how it could be re-established in pathological conditions. The understanding of these mechanisms will also be the key to improving the success of therapeutical clinical trials, the majority of which has failed in patients with head trauma, despite showing benefit in preclinical experiments on animal models of TBI.

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Viral Interactions with the Blood–Brain Barrier

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1. INTRODUCTION

Although viral infections of the central nervous system (CNS) occur infrequently these infections are tremendously important given the potential neurological damage they can cause. As the highly specialized brain tissue is exquisitely sensitive to metabolic changes and that injured brain tissue recovers slowly and often incompletely, the CNS has inherent mechanisms for restraining immune responses. The CNS represents a very appealing site for persistent viral infections. First, many CNS cells are long-lived therefore allowing pathogens to niche or to survive within the same host cells for extended period of times. Second, the immune-privileged character of the CNS, such as low basal expression of major histocompatibility complex (MHC) molecules and limited migration of immunocytes, creates a unique environment that restricts the ability of the immune system to perform its functions (1). Nevertheless, the immune system can successfully clear viruses from the CNS without causing permanent neurologic damage. Multiple RNA and DNA viruses have the capacity to infect the CNS (Tables 1 and 2). The mechanisms used by some of these viruses to reach the CNS will be reviewed with a specific emphasis on the interactions with the blood-brain barrier (BBB).
Virus	CNS disease	Pathway to CNS
Herpesviridae		
Herpes simplex virus (HSV)	Encephalitis; meningitis; meningoencephalitis	Neuronal
Cytomegalovirus (CMV)	Encephalitis (immunosuppressed and neonates)	Blood
Epstein-barr virus (EBV)	Encephalitis; meningitis, myelitis, Guillain-Barré	
Varicella-zoster virus (VZV)	Cerebellitis, encephalitis, meningitis, myelitis, zoster ophthalmicus	Blood and neuronal
Human herpes virus-6 (HHV-6)	Encephalitis, meningitis, febrile seizures	?
B virus (Herpes simiae virus) Adenoviridae	Encephalitis	Neuronal
Adenovirus	Meningitis, encephalitis	Blood
Polyomaviridae		
Polyomavirus JC virus	Progressive multifocal leukoencephalopathy	Blood

 Table 1
 Pathogenesis of Viral Infections of the CNS by DNA Viruses

Source: Adapted from Ref. 2.

2. VIRUS REPLICATION

Viruses are obligatory parasites of cells; they can only replicate by *hijacking* a cellular machinery to replicate their genome, produce their proteins, and finally generate virions that can infect other cells. The virus replication starts with the attachment of viral protein(s) to cellular receptor(s). Receptor binding provides a close virus-cell contact facilitating viral entry into the cells. Few virus receptors have been conclusively identified and they are hypothesized to be proteins, lipids, and/or oligosaccharides. Viruses tend to target essential and/or tightly conserved host receptor domains in order to ensure that their key to enter target cells is always operational. Viruses can use more than one mechanism for entering a cell, as well as using different receptors for entering different cell types. The number and distribution of such receptors contribute to the viral tissue tropism. Receptor presence is not the only determinant of viral tissue tropism; cells can bear the appropriate receptors but still be non-permissive for viral replication. Some viruses require the presence of certain genes and transactivating factors to successfully infect a cell (2). For example, despite the expression of a functional mouse hepatitis virus (MHV) receptor (CEACAM), replication of MHV could not be detected in vivo in brain endothelial cells although liver endothelial cells were infected (3). Following attachment to the cellular

Virus	CNS disease	Pathway to CNS
Togoviridae-alphavirus (arbovirus)		
Western equine encephalitis virus	Meningitis, encephalitis	Blood
Eastern equine encephalitis virus	Meningitis, encephalitis	
Venezualan equine encephalitis virus	Meningitis encephalitis	
Flaviviridae (flavivirus)	interningicus, encopinantis	
Japanese encephalitis virus	Meningitis encephalitis	Blood
St Louis encephalitis virus	Meningitis, enceptiantis	biood
West nile fever virus		
Murray valley virus	Encenhalitis	
Runyaviridae (arbovirus)	Encephantis	
California (La Crosse)	Maningitis ancanhalitis	Blood
camorhalitia virus	Weiningitis, enceptiantis	biood
Paquinidaa arbinimus		
Colore do tiels foren	Maninaitia anomhalitia	Dlaad
Disconstruction of the content of th	Meningius, encephanus	Blood
Picornaviriade (enterovirus)	Manina itia maalitii	D111
Poliovirus	Meningitis, myelitis	Blood and
		neuronal
Coxsackievirus	Meningitis	Blood
Echovirus	myelitis	
Paramyxoviridae		
Measles virus	Encephalitis, subacute sclerosing panencephalitis (SSPE), measles inclusion body encephalitis	Blood
Mumps virus	Meningitis, encephalitis, myelitis	Blood
Orthomyxoviridae		
Influenza viruses	Encephalitis	Olfactory system, blood?
Rhabdoviridae		
Rabies virus	Encephalitis,	Neuronal
	encephalomyelitis	
Arenaviridae		
Lymphocytic	Meningitis, encephalitis	Blood
choriomeningitis virus		
Coronaviridae		
Coronavirus	Meningitis (rare)	Blood, olfactory system?

 Table 2
 Pathogenesis of Viral Infections of the CNS by RNA Viruses

Source: Adapted from Ref. 2.

receptor, the virus will penetrate into the target cell and will shed its genome inside. Some virus replication occurs predominantly in the cytoplasm while other viruses will have a nuclear phase in their replication cycle. Once the viral genome is in the cell, it will be replicated in multiple copies and translated into viral proteins necessary for the assembly of virions. The newly formed virions will be released from the cell with or without lysing the infected cells (1).

3. VIRUS EFFECTS ON CELLS

Infected cells are not ignoring viral infections. In fact, the presence of foreign nucleic acids (i.e., double stranded RNA in some cases) and foreign viral proteins will induce changes in the target cell such as a rapid production of type I interferon (IFN) by neurons and glial cells (1). During viral infection adhesion molecules (ICAM-1, VCAM-1) are up-regulated by cerebral capillary endothelial cells (1). The CNS cells under normal circumstances do not express MHC class I molecules but endothelial and meningeal cells as well as microglia abundantly express these molecules during most viral infections. Oligodendrocytes and astrocytes can also express MHC class I molecules in some occasions, whereas neurons rarely express these molecules. The MHC class-II molecules are not constitutively expressed by cells in the brain parenchyma but can be induced quickly after initiation of infection or by trafficking of activated T cells through the CNS (1). Microglia and perivascular macrophages are the main cells expressing MHC class II.

4. CNS INFECTION

The clinical symptoms associated with CNS viral infections mainly depend on the type of cells targeted by these pathogens. Meningitis is the most common outcome of CNS infection. In this case viruses target cells of the leptomeninges that cover the brain surface. These infections are usually not critical for the host since these cells are easily renewed and that they are easily cleared by the immune system. However, when viruses infect neurons they can cause more permanent and fatal damage to the host (1). Several types of neurons have been shown to be susceptible to viral infection depending on virus tropism, route of entry into the CNS and the mechanism of spread. Some viruses, such as measles can spread within the nervous system by axonal transport and move from neuron to neuron through connecting synapses (4). Other viruses infect the supporting glial cells: oligodendrocytes, astrocytes, and microglia. Oligodendrocytes produce and maintain the CNS myelin sheath that surrounds the neuronal axons; infection of these cells could lead to demyelinating diseases as observed following infection with JC virus in humans. Astrocytes are the mechanical and metabolic support for neurons; they maintain the BBB, produce neurotropic factors, and remove toxic molecules. Indirect damage to neurons could result from astrocyte infection. Microglia, which are the resident parenchymal macrophages of the CNS as well as perivascular macrophages express MHC classes I and II molecules. They can secrete a large spectrum of immune mediators (Chapter 13) and can therefore play important roles in local immune responses. Nevertheless, the various immune factors that microglia and perivascular macrophages produce in response to infection can also be toxic to neurons and other glial cells (1).

5. CNS ENTRY

Viruses can use different routes to enter the CNS. The first path is by *neural* viral spread. Some viruses such as rabies, herpes simplex virus, and reovirus enter the CNS by infecting peripheral or cranial nerves and then pass from neurons to neurons by intra-axonal transport. Viruses can also spread *trans*-neuronally throughout the CNS and within peripheral nervous system. Rabies virus classically infects the CNS by a myoneuronal route (5). Rabies virus replicates locally in the soft tissue following a rabid animal bite (2,5). After primary replication, the virus enters the peripheral nerve (2). Experimental evidence demonstrates that acetylcholine receptor binding is used by rabies virus for myocyte entry (2,6,7). The virus then travels by anterograde and retrograde intra-axonal transport to infect neurons in the brainstem and limbic system. Viruses appear to cross the *trans*-synaptic space between neurons by passive transport rather than by receptor-mediated transport (2). Experimental evidence indicates that rabies virus enters projections in the post-synaptic neuron that extend into invaginations on the presynaptic side (2). These projections pinch off and fuse with the presynaptic membrane, allowing the virus to spread along motor or sensory neural pathway (2). The nerve shields the virus from immune regulation and allows access to the CNS.

The second potential route of entry is through the olfactory nerve. The *olfactory system* is unique among cranial nerves in that the neurons regenerate and have approximately a 1-month life span (2). The olfactory neurons are not protected by the BBB, potentially providing direct neuronal access to the brain (2). Animal studies have shown that herpes simplex virus can infect the brain through the olfactory system and the trigeminal nerve (2,8) and murine coronavirus can infect the CNS through the olfactory system (9). The initial location of early herpes simplex virus encephalitis (inferomedial temporal lobe) correlates with the anatomical connections between the temporal lobes and the olfactory bulb (2).

The third potential route to enter the CNS is via *hematogenous spread*. Studies in humans and animal models indicate that the majority of viral CNS infections are acquired from the blood (2,5). Entry from the blood to the CNS may take various forms. Viruses can pass from the blood into the brain or cerebrospinal fluid (CSF) at many anatomic sites and by several different mechanisms. Viruses migrating from blood into the stroma of the choroid plexus can potentially infect epithelial cells and then seed directly into the CSF. Alternatively, viruses can be transported via pinocytosis through the elongated epithelial cells. For example, human immunodeficiency virus has been shown to enter brain microvascular endothelia by macropinocytosis without disrupting the BBB and without productively infecting the endothelial cells (10). Once in the CSF, a virus may either linger in the meninges or enter the brain parenchyma across either ependymal cells or pia linings. For example, influenza virus has been detected in human ependymal cells (11). Viruses, such as Semliki Forest virus, can infect endothelial cells and then leak across damaged endothelium. Transport of viruses may occur in a manner analogous to that of ferritin particles, which are transported across the capillary endothelial cells in pinocytotic vesicles and then are deposited in the cytoplasm of the adjacent astrocytes. Japanese encephalitis virus is transported in endocytic vesicles across the brain endothelial cells and pericytes (12). Some viruses such as Theiler's murine encephalomyelitis virus infect the CNS vascular endothelial cells prior to infect adjacent glia and neurons. Whereas other viruses initially infect glia surrounding small intact vessels, without evidence of endothelial cell infection. In addition, viruses, such as cytomegalovirus, mumps virus, and measles virus can be carried across the CNS endothelial cells using infected leukocytes as a Trojan horse. A hamster model of mumps meningoencephalitis has suggested that viral entry into the CNS is achieved via the egress of mumps-infected mononuclear cells across the choroid plexus endothelium (13). Under normal circumstances leukocyte traffic into the CNS is limited, but activated T lymphocytes routinely cross the BBB to perform their immune surveillance (14,15). Viremia and neuronal spread to the CNS can occur concurrently and are not mutually exclusive (2). For example, poliovirus and reovirus can infect the CNS by hematogenous route, but also via peripheral neurons as shown in experimental animal models (16).

Invasion of the CNS from blood is quite rare compared to the overall number of viral infections that an individual has to cope with throughout his/her life (1,5). Viral meningitis and encephalitis in humans are relatively rare, although infections with potentially neurotropic viruses are common. Multiple events are required prior to the CNS invasion. Numerous mechanisms are in place to limit viral infections at their initial site of entry: respiratory tract, the skin, and the gastro-intestinal tract. In addition, anti-viral molecules such as IFN produced by infected cells and potent immune responses are often efficient at clearing viral infections from the peripheral circulation before they can spread to the CNS. In addition, intrinsic characteristics of the CNS as mentioned previously (such as the immune-privileged character) limit virus access. Only viruses that escape from all of these barriers will gain access to the CNS (1).

6. INFECTION OF ENDOTHELIAL CELLS

Data to document the susceptibility of endothelial cells to viral infection are accumulating mainly for non-CNS cells. Few studies have been performed to evaluate the specific susceptibility of brain endothelial cells and of the BBB to viruses. Therefore, information concerning viral infection of endothelial cells in general needs to be briefly mentioned.

The viral infection of endothelial cells could in fact represent a powerful strategy to invade multiple organs. When endothelial cells are permissive to viral infection they allow viruses to leave the circulation and initiate infection in adjacent tissues. Some viruses may lyse endothelial cells during their replication cycle, for, e.g., poliovirus produces a lytic infection of human umbilical endothelial cells (HUVEC) (17). Furthermore, the direct infection of endothelial cells, as well as the indirect effects of viral infection leading to lysis of these cells may have important clinical counterpart, such as hemorrhagic fever. In general, BBB breakdown and inflammation are common features of viral encephalitis, as demonstrated by the uptake of gadolinium on brain magnetic resonance scans (Chapter 19). In addition, viruses have been shown to up-regulate the expression of adhesion molecules (18) and MHC molecules on endothelial cells (19). Moreover, viral infection of endothelial cells has been shown to increase peripheral blood mononuclear cell binding and to enhance granulocyte adherence (20,21). Substances produced during infection or chemicals secreted by cells, such as histamine, interleukins, change the permeability of the BBB, thus modulating entry of viruses and immune cells into the CNS (22-24). Human umbilical vein endothelial cells (HUVEC) as well as human dermal microvascular endothelial cells (HMEC) have been shown to be persistently infected in vitro by coxsackie virus B (25). Moreover, the coxsackie B virus infection was shown to increase the production of IL-6, IL-8, and TNF- α by endothelial cells (26,27). Although the immune system is essential to clear viral infections, in some cases, the immune responses are instrumental to the extend of CNS tissue damage (22). In this regard, several viruses have also been shown to activate lymphocyte subsets, thus promoting their migration within the CNS parenchyma and allowing various neuro-toxic cytokines to access the fragile CNS environment.

7. SPECIFIC VIRUS INTERACTIONS

7.1. Picornaviridae: Enterovirus

Within the family of *Picornaviridae*, the enteroviruses include nearly 70 distinct serotypes: *polioviruses*, coxsackieviruses A and B, echoviruses, and the newly numbered enteroviruses. Enteroviruses are usually acquired by fecal-oral contamination and less commonly by respiratory droplet. These viruses are stable at acidic pH levels, which explain their ability to cross

the stomach prior to reach their primary site of infection in the lower gastrointestinal tract (28). Following replication in the mucosal lymphoid tissue, enteroviruses can enter the circulation and eventually find their way to secondary replication sites including the CNS, liver, lungs, and heart, dictating the patterns of symptoms caused by the infection. Enterovirus infection exhibits a wide range of clinical manifestations including meningitis, encephalitis, paralysis, but also common cold-like symptoms, eye infections, and skin disease (28,29).

Primary culture of human endothelial cells are highly susceptible to infection by various enteroviruses (coxsackievirus A13, echoviruses 6, 7, 11, 30, and poliovirus 1). Conversely, other enteroviruses, such as coxsackievirus A 9 and echovirus 1 infect only few individual endothelial cells. Moreover, infected human primary endothelial cells have increased levels of activation markers: E-selectin and intercellular adhesion molecule-1 (29). On the other hand human parechovirus 1 did not show evidence of replication in primary human endothelial cells (29).

The mechanisms used by enteroviruses to leave the blood and enter the CNS in vivo are not clear. It has been proposed that leakiness in the vessels of the choroid plexus (meningitis) and/or of the parenchyma (encephalitis) is likely responsible for virus entry into the CNS, as opposed to active transport of viral particles across the BBB (28). Endothelial cells may express enterovirus receptors in which case up-regulation of those receptors may facilitate viral entry into the CNS.

7.1.1. Poliovirus

Poliovirus, an enterovirus, is the causative agent of the poliomyelitis, which is characterized by meningitis and lytic infection of the anterior horn cells of the spinal cord leading to transient or permanent paresis of one or more extremities. The vast majority of naturally occurring poliovirus infections are imperceptible but some patients experience a minor illness characterized by fever, headache, and sore throat. Rare patients (0.1-1.0%) develop paralytic poliomyelitis as a result of neuronal destruction (28). Naturally occurring infections have been eliminated in developed countries by widespread and successful immunization. However, poliomyelitis is still occurring in African and Asia and global eradication remains a major priority for the World Health Organization (28).

Following the first multiplication cycle, poliovirus moves into the blood and circulating viral particles can then invade the CNS and replicate in neurons, predominantly the motor neurons. Given that neutralizing peripheral antibodies to poliovirus prevent the development of poliomyelitis, viremia seems necessary for the spread of virus to the CNS (28). Poliovirus has been shown can use two possible dissemination paths to enter the CNS: one is virus permeation through the BBB and the other is virus transmission via peripheral nerves (28).

Viral Interactions with the Blood–Brain Barrier

Poliovirus uses a common cell membrane attachment protein PVR (poliovirus receptor), which is a member of the Ig superfamily and is expressed in both CNS and muscle tissues, but also in non-susceptible tissues such as kidney (30,31). The PVR is widely expressed on normal cells including neuronal, epithelial, endothelial, and fibroblastic cells (31,32). Experimental infection of mice bearing the receptor (human PVR) has shown that poliovirus inoculated both intravenously or intramuscularly can enter the CNS and replicate in neurons. Intravenously inoculated poliovirus invaded the CNS mainly through permeation of the BBB (31.33) whereas following intramuscular inoculation, poliovirus reached the CNS through the neural pathway (31.32). Similar distribution of the virus has been observed in brain tissues obtained from experimental mice bearing the human PVR and the wild type mice (not expressing the human PVR) suggesting that specific distribution of poliovirus is not caused by expression of human PVR. Moreover, poliovirus migration rate in the CNS was at least 100 times higher than that of albumin, which is not thought to permeate the BBB via specific transport system, and only three times lower than that of a monoclonal antibody to transferrin receptor, which is a potential candidate for CNS drug delivery (31–33). Thus, some host cell molecules other than human PVR must be involved in the BBB permeability of poliovirus.

7.2. Arenavirus

Arenaviruses, such as Lassa virus, Junin virus, and lymphocytic choriomeningitis virus (LCMV) are small RNA viruses that are maintained in nature by chronic infection of rodents. Human infections with arenaviruses occur when humans come in contact with infected rodents or their excreta (34.35). The human infection results in a wide spectrum of clinical manifestations from asymptomatic, flu-like, and gastrointestinal symptoms to CNS disease or severe hemorrhagic fever depending on the virus and host factors. Lassa virus has been associated with neurologic complications such as confusion, tremor, convulsion, and coma. Lassa virus could not be always isolated from serum and CSF of these patients but the virus was present in the CSF and not in the serum of a patient having fever, disorientation, seizures, and BBB dysfunction (35–37). Lassa virus may thus persist in the CNS (37). Similar to Lassa virus, Junin virus is also associated with a hemorrhagic shock syndrome in human. Several members of the arenavirus family use α -dystroglycan, which is ubiquitously expressed, as a cellular receptor (38,39). Therefore, multiple cell types could be targeted by arenaviruses.

Given the hemorrhagic fevers caused by arenaviruses such as Junin virus and Lassa virus, studies have been performed to evaluate the susceptibility of endothelial cells to these viruses. These viruses can replicate in vitro in HUVECs (40). Moreover, persistent but not acute infection by LCMV enhances the expression of MHC class-I glycoproteins on the brain endothelial cells of mice cultured in vitro. In addition, following intracerebral inoculation of LCMV, which induces a fatal CD8+ T-cell mediated meningitis, expression of ICAM-1 and VCAM-1 is up-regulated on the endothelial cells in immuno-competent mice and dysfunction of the BBB could be detected (41,42). The BBB dysfunction is immuno-mediated in this animal model as CD8+ T cells are necessary to observe these effects on the brain endothelial cells (42).

7.3. Influenza

Influenza virus infections can cause a broad array of respiratory illnesses and occasional CNS disorders, such as encephalopathies and encephalitis (43). Although rare and mostly diagnosed in children, Reye's syndrome and acute necrotizing encephalopathy are characterized by coma, vomiting, convulsions, and cerebral edema. Both encephalopathies can be the consequence of influenza infection (43). Acute necrotizing encephalopathy presents with multifocal brain lesions in the thalamus, brainstem, periventricular white matter, and cerebellum, often associated with variable level of brain edema (43).

The pathogenesis of these neurologic syndromes of influenza infection have not been completely elucidated. In fatal cases, congestion and hyperemia of the brain without inflammatory cell infiltration have been detected suggesting that the BBB is specifically targeted by this infection (11,43,44). Influenza virus has been detected in human CSF, indicative that the virus can be neuroinvasive. Moreover, influenza viral antigens have been detected in ependymal cells of immunosuppressed patients (11) and in cerebellar Purkinje cells and neurons of pontine nuclei in one child who died from influenza encephalopathy (44).

Animal models have demonstrated that influenza virus can use the neuronal pathway via the olfactory and trigeminal nerve system and infect specific areas of the brain (45,46). Moreover, given that the influenza viremia is rather uncommon in humans, the hematogenous route might not be favored by this pathogen to gain access to the CNS (43). Since the presence of neurologic symptoms are suggestive of BBB involvement, it is possible that cytokine secreted by CNS infected cells cause the breakdown of the BBB. In addition, influenza virus can infect and cause the release of cytokines [IFN-inducible protein-10 (IP-10), monokine induced by IFN-gamma (Mig) and IL-6] by HUVECs and may potentially perform the same effects on human brain endothelial cells (47,48). The described CNS symptoms could also be associated with an abortive but still damaging infection of cerebral capillary endothelial cells by influenza virus; although viral genome and proteins could be detected in ECs, no infectious particles were found to be released by ECs in experimental models (49).

7.4. Arbovirus

Arboviruses are viral pathogens that are transmitted by arthropod vectors. Numerous viruses have been identified worldwide having distinct seasonal and geographic characteristics determined by the biological patterns of the particular vector (the arthropod) and the animal reservoirs (50). The arboviruses are classified in four viral families: Togaviridae, Flaviviridae, Bunyaviridae, and Reoviridae and a small subset of these viruses are detrimental for humans (50). Among the Flaviviruses that are causing human diseases, St. Louis encephalitis virus is the most common vector-transmitted cause of aseptic meningitis. West Nile virus appeared in North America quite recently (U.S. in 1999). The California encephalitis group of viruses includes members of the Bunyaviridae family (La Crosse, Jamestown Canavon, and Snowshoe hare viruses). These have been associated with aseptic meningitis. Encephalitis is the most clinically significant and commonly recognized neurologic manifestation of these infections, but certain viruses also cause meningitis or meningoencephalitis as part of their disease spectrum (50). Following inoculation via the bite of a blood-sucking mosquito or tick, arboviruses replicate in the skin and local lymph nodes, causing a viremia before entering the CNS. Invasion of target organs appears to depend in part on the extent of viremia but also on other invasive viral characteristics. The mechanisms of virus entry into the CNS may include infection of or transport across vascular endothelial cells, and infection of olfactory neurons or of choroid plexus epithelial cells (50). Although the cellular immunity and the inflammatory response are important in most infections, some patients die from virally induced neuronal cell death before there is evidence of a cellular immune response and inflammation (50).

7.4.1. Dengue Virus

Dengue virus is a flavivirus associated with hemorrhagic shock syndrome in human. Viral RNA has been detected in brain microvascular endothelial cells from a fatal case of dengue hemorrhagic fever (51). Dengue virus can replicate in vitro in both rabbit and human endothelial cells (40,52,53). Dengue virus genome and anti-dengue IgM could be detected in the CSF of patients supporting the notion that the virus gains access to the CNS (54).

Dengue virus infects HUVECs and other endothelial cell lines by binding one of its glycoprotein to the cell surface of these endothelial cells (53). The infected endothelial cells produce high levels of IL-8 as well as IL-6 (52,55). Dengue virus would target microvascular endothelial cells in several tissues where plasma leakage is believed to occur (56,57). Human dermal microvascular endothelial cells (HMEC-1) can be infected by Dengue virus and the confluent monolayers formed by these cells show alterations in their permeability following such infection, as well as actin cytoskeleton rearrangements and displacement of occludin from the tight junction complex (58). The dengue virus effects on these HMECs could be reproduced by addition of IL-8 to uninfected cells and were partially inhibited by neutralizing antibodies to IL-8 (58). The gene expression pattern in HUVECs induced by dengue virus infection in vitro has shown broad functional responses including stress, defense, immune, cell adhesion, wounding, inflammatory, and antiviral pathways and more specifically inhibitor of apoptosis-1, 2'-5' oligoadenylate synthetase (OAS), galectin-9, myxovirus protein A (MxA), regulator of G-protein signaling, endothelial, and smooth muscle cell-derived neuropilin-like protein, and phospholipid scramblase 1 (59,60). These dengue virus effects on cellular gene expression of HUVECs have not been tested on human brain endothelial cells but we can hypothesize that at least part of these responses could be reproduced, since a breakdown of the BBB is observed in infected mice (59).

7.5. Coronavirus

Coronaviruses are enveloped positive-stranded RNA viruses that have been associated with up to one-third of common colds in humans (Myint, 1994). Since their discovery, other human pathologies have occasionally been associated with these viruses such as pneumonia, meningitis, and more recently the severe acute respiratory syndrome (SARS) (61). Accumulating evidences from animal models and from studies in humans suggest a neurotropic potential for these viruses (62–65). Neurotropic strains of MHV, the murine coronavirus, induce extensive inflammatory cell infiltration and serve as animal models for virus-induced encephalitis and demyelination.

Coronaviruses use multiple strategies to get access to the CNS. Neurotropic strains of MHV can invade the CNS following an intranasal inoculation in mice (9), and could potentially also gain access to the CNS via the hematogenous and/or lymphatic systems in mice (66). Following intracerebral, intranasal, and ocular inoculation of coronavirus in non-human primates viral RNA and antigens could be detected in the brains of infected animals (67,68). The detection of viral products has been predominantly in blood vessels and perivascular regions, supporting the theory that hematogenous spread through the endothelium is important for coronavirus entry into the CNS, at least in animals (68). Furthermore, human coronavirus variants can reach the CNS after intranasal inoculation in mice demonstrating neuroinvasive properties (69). Given that human coronaviruses are respiratory viruses, they might also invade the CNS following a primary infection of the upper respiratory tract. At least one SARS infected patient has develop tonic-clonic convulsion and had detectable levels of the SARS human coronavirus RNA in the CSF, suggesting that the SARS-coronavirus may cause a CNS infection (70). Moreover, human coronavirus can infect macrophages (71,72) and at least one strain (229E) may infect human brain endothelial cells (73), which are possible alternative routes for CNS invasion.

The receptors used by various coronaviruses are expressed on endothelial cells. The MHV uses murine carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) as a sole receptor (74,75). In vitro MHV infection of primary culture of mouse brain endothelial cells obtained from susceptible (expressing the susceptible allele of CEACAM-a) mouse strains caused a down-regulation of MHC class I whereas the cells obtained from resistant strains (expressing the resistant allele of CEACAM-b) led to an up-regulation of MHC class-I molecules. In addition, susceptible mouse endothelial cells secreted significant amount of interleukin-6 (IL-6) after infection with MHV in vitro (76).

A number of coronaviruses (i.e., human coronavirus 229E), require the zinc metalloprotease aminopeptidase N (CD13) for entry into their target cells (77–79). The CD13/aminopeptidase N is expressed on the endothelial cells of angiogenic, but not normal vasculature; its expression on activated blood vessels is induced by angiogenic signals (80,81). The functional receptor used by the SARS coronavirus has been identified as being angiotensin-converting enzyme 2, a metallopeptidase which is expressed in testis, renal and cardiovascular tissues, gastrointestinal system, and moderately in CNS and lymphoid tissues (82). An additional molecule can serve as a human receptor CD209L, a C-type lectin (also called L-SIGN) (83), which is expressed on multiple endothelial cell types (84). As most coronavirus receptors could be expressed at some level on endothelial cells the potential infection of such cells remains plausible but yet to be tested.

Intraocular coronavirus inoculation results in a retinal disease in susceptible mice characterized by an acute inflammatory response followed by retinal degeneration. Blood retinal barrier breakdown is observed during the first phase of the disease and is primarily due to the inflammation rather than to retinal cell destruction (85). Also, following intracerebral injection of neurotropic strains of MHV, neutrophils are the first infiltrating cell populations within the CNS and these cells have been shown to contribute to the loss of BBB integrity possibly via MMP-9 secretion as well as through other mechanisms (86).

7.6. Cytomegalovirus

Cytomegaloviruses (CMV) are significant opportunistic viruses with a very high prevalence in humans. Infection, usually acquired early in life, remains latent in immunocompetent individuals. However, immunocompromised patients can develop severe clinical disease from either a new primary CMV infection or reactivation of a latent infection (87,88). The CMV is a common secondary pathogen of AIDS patients infecting more than 90% of this population. Disseminated CMV disease is characterized by infection across virtually all organs, leading to mononucleosis, severe respiratory infection, liver and kidney damage, intestinal disease, and CNS damage. In healthy adults, CMV infection of the CNS is uncommon; however, as the population of immunosuppressed adults is rising, the incidence of neurotropic CMV infection is now significant. The CMV is thought to be a cofactor of AIDS dementia. Clinical manifestations of CMV infection of adult CNS may include retinitis, encephalitis, myeloradiculitis, subcortical dementia, obtundation, and other significant neurological deficits, with potentially fatal outcomes (2).

Using mouse CMV (mCMV) as an animal model, it has been demonstrated that CMV infects the brain only after a prolonged period of peripheral infection (88). Moreover, the mCMV infection starts as random small foci of different types of cells scattered through the brain and only in immunodeficient and not in immunocompetent hosts (88). However, neither breakdown of the vascular system, nor transport of virus via the olfactory nerve, nor axonal transport have been observed in these experimental models. Also, there is usually no important viremia, supporting that mCMV does not infect directly the CNS as free particles. In fact, infected endothelial cells and monocytes have been suggested as vectors for viral dissemination (88-90). Because disseminated asymmetric foci of CMV infection were identified throughout the brain, with no apparent interrelationship to one another, this pattern suggests multiple independent seedings of the CNS from a circulatory source. Mouse mCMV infects endothelial cells in the brain (91) indicating that mCMV may infect CNS vessels prior to target the parenchymal CNS. In humans, CMV is frequently detected in various cell types including glia, neurons, and human brain capillary endothelial (HBCE) cells (92–95). In addition, CMV antigens can be detected after in vitro infection in HBCE cells (96). Therefore, available data strongly support CMV transmission from the periphery to the CNS by infected leukocytes and direct infection of CNS endothelial cells.

7.7. Measles

Measles virus is a highly contagious human pathogen causing primarily an acute disease characterized by fever, coryza, cough, conjunctivitis, exanthematous rash, photophobia, and headache. After an acute infection of the upper respiratory tract, measles virus is transported to draining lymph nodes where it establishes a systemic infection and spreads to different organs. Measles virus replicates primarily in endothelial cells, epithelial cells, and monocytes/macrophages. Endothelial cells of dermal capillary and small vessels throughout the body show clear evidence of MV infection. Measles infection causes severe CNS complications, including acute post-infectious measles encephalitis, measles inclusion body encephalitis (MIBE, occurring in individuals with an impaired immune response) and subacute sclerosing panencephalitis (SSPE) (97). The SSPE usually occurs several years after the initial childhood infection. This CNS complication

demonstrates the potential detrimental effects of a persistent infection in humans by an RNA virus. Multiple reports have shown that measles virus RNA is found in inflammatory cells present within perivascular cuffs in SSPE brains (98–100). Brain endothelial cells and capillary endothelium of both lymph nodes and thymus have been found to be infected in fatal cases of acute measles (101). In SSPE patients, brain endothelial cells appear to be infected as well as various neural cells (102–104).

It has been suggested that measles virus could cross the BBB by hiding in infected leukocytes. In fact, measles infects leukocytes during the primary infection. It is also possible that measles virus enters the CNS by direct infection of the endothelial cells at the BBB causing up-regulation of adhesion molecules on these cells and then allowing activated leukocytes to further migrate within the CSN (97). Moreover, infectious viruses are produced by infected cerebral endothelial cells in vitro (97). Therefore, infection of endothelial cells at the BBB could provide an additional opportunity for measles virus to penetrate the CNS. Increased expression of endothelial adhesion molecules, following virus infection at the BBB, may be an important mechanism for inducing inflammatory infiltration of the CNS in SSPE (97).

Two receptors have been identified for measles virus: CD46 and SLAM (signaling lymphocytic activation molecule). Only CD46 has been detected on primary HUVECs. However, even in the presence of CD46 blocking antibodies, measles virus has been taken up by human endothelial cells suggesting that another receptor could be used to infect these cells specifically (105).

7.8. JC Virus

The majority of adults have developed antibodies and cellular immunity for the human polyomavirus JC without any detectable clinical symptoms (106). The virus remains latent in the vast majority of individuals in the kidney and perhaps in the brain. In rare occasions, almost exclusively in immuno-suppressed individuals such as AIDS patients and transplant recipients, the virus JC produces a lytic infection of oligodendrocytes, the cells responsible for the myelin sheath formation in the CNS. The disease observed is a progressive multifocal leukoencephalopathy (PML). The JC virus has been detected in the kidney and in the CNS, as well as in multiple other organs (heart, spleen, lung, colon, and liver) (107). The JC virus can infect multiple cell types: CD34⁺ hematopoietic progenitor cells, kidney cell lines, and primary cultures of vascular endothelium and amnion cells (106,108). In the CNS, a high density of infected cells are found surrounding blood vessels (107) and JCV-induced PML lesions are detected throughout the brain white matter. The hematogenous route is the most probable dissemination to the CNS. B lymphocytes are a potential carrier of the virus given that these cells are positive for the virus. T lymphocytes are probably not involved, as JC virus cannot infect them nor bind to their membranes. During the dissemination of JC virus, the virus may be carried to the brain through viremia, but this has not been demonstrated (106,108).

7.9. Prions

Prion diseases include scrapie in sheep, bovine spongiform encephalopathy in cattle and Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) disease and fatal familial insomnia in humans (109). These disorders are rare in humans but their course is fatal. The clinical manifestations of prion diseases, also called transmissible spongiform encephalopathies (TSE), usually appear after a long latency period from the initial time of infection (5). The clinical symptoms include dementia, tremors, ataxia, and sensory involvement (dysesthesias) (5). The transmissible agent of these diseases remains infectious after treatments that would normally inactivate viruses or nucleic acids (detergent, formalin, ioninzing radiation, nucleases). In fact, the infectious agent is principally abnormal (scrapie) forms (PrP^{sc}) of a normal protein called the cellular prion protein (PrP^c); PrP stands for *protease resistant protein*. The PrP^{sc} are thought to propagate by recruitment and autocatalytic conformational conversion of the PrP^c to PrP^{sc}(109). The PrP^{sc} accumulates into insoluble aggregates and forms amyloid (109,110). In vitro studies suggest that mammalian ribonucleic acids may enhance the efficiency of the PrPsc amplification (111). The neuropathology of prion disease is characterized by neuronal cell death without obvious inflammation (110).

Although neurodegeneration is the main outcome for prion disease, the major route for prion entry is extra-CNS. For some TSE, such as bovine spongiform encephalopathy and vCJD, ingestion has been hypothesized as being the main route (109). Animal models have demonstrated that prion could be transmitted via the blood (112,113). Regardless of the initial site of infection, the final destination for prion is the CNS. The exact mechanisms used by prion for its neuroinvasion are still unclear. Follicular dendritic cells are a key mediator of prion pathogenesis but they are probably not the mediator facilitating their CNS invasion (109). Experimental models have suggested that the autonomic nervous system might transport prions. The sympathetic nerves appear to play a role since sympathectomy slows the onset of prion disease whereas sympathetic hyperinnervation enhances prion replication in the spleen and then neuroinvasion after peripheral inoculation (114).

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about the book ...

This reference analyzes the cellular and molecular biology and mechanisms of the blood-brain barrier (BBB) and presents the most recent studies on the role of the BBB in the development and initiation of a wide range of physiological and pathological conditions affecting the central nervous system.

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