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Review

The blood–brain barrier: an overview Structure, regulation, and clinical implications

Praveen Ballabh,* Alex Braun, and Maiken Nedergaard

Departments of Pediatrics, Anatomy and Cell Biology, and Pathology, New York Medical College and Westchester Medical Center, Valhalla, NY 10595, USA

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The blood–brain barrier (BBB) is a diffusion barrier, which impedes influx of most compounds from blood to brain. Three cellular elements of the brain microvasculature compose the BBB—endothelial cells, astrocyte end-feet, and pericytes (PCs). Tight junctions (TJs), present between the cerebral endothelial cells, form a diffusion barrier, which selectively excludes most blood-borne substances from entering the brain. Astrocytic end-feet tightly ensheath the vessel wall and appear to be critical for the induction and maintenance of the TJ barrier, but astrocytes are not believed to have a barrier function in the mammalian brain. Dysfunction of the BBB, for example, impairment of the TJ seal, complicates a number of neurologic diseases including stroke and neuroinflammatory disorders. We review here the recent developments in our understanding of the BBB and the role of the BBB dysfunction in CNS disease. We have focused on intraventricular hemorrhage (IVH) in premature infants, which may involve dysfunction of the TJ seal as well as immaturity of the BBB in the germinal matrix (GM). A paucity of TJs or PCs, coupled with incomplete coverage of blood vessels by astrocyte end-feet, may account for the fragility of blood vessels in the GM of premature infants. Finally, this review describes the pathogenesis of increased BBB permeability in hypoxia–ischemia and inflammatory mechanisms involving the BBB in septic encephalopathy, HIV-induced dementia, multiple sclerosis, and Alzheimer disease.

Keywords: Blood–brain barrier; Tight junction; Germinal matrix; Astrocyte; Pericyte

Introduction

The blood–brain barrier (BBB) is a diffusion barrier essential for the normal function of the central nervous system. The BBB endothelial cells differ from endothelial cells in the rest of the body by the absence of fenestrations, more extensive tight junctions (TJs), and sparse pinocytic vesicular transport. Endothelial cell tight junctions limit the paracellular flux of hydrophilic molecules across the BBB. In contrast, small lipophilic substances such as O_2 and CO_2 diffuse freely across plasma membranes along their concentration gradient (Grieb et al., 1985). Nutrients including glucose and amino acids enter the brain via transporters, whereas receptor-mediated endocytosis mediates the uptake of larger molecules including insulin, leptin, and iron transferrin (Pardridge et al., 1985; Zhang and Pardridge, 2001). In addition to endothelial cells, the BBB is composed of the capillary basement membrane (BM), astrocyte end-feet ensheathing the vessels, and pericytes (PCs) embedded within the BM (Fig. 1A). Pericytes are the least-studied cellular component of the BBB but appear to play a key role in angiogenesis, structural integrity and differentiation of the vessel, and formation of endothelial TJ (Allt and Lawrenson, 2001; Balabanov and Dore-Duffy, 1998; Bandopadhyay et al., 2001; Lindahl et al., 1997). It is believed that all the components of the BBB are essential for the normal function and stability of the BBB.

The building blocks of the BBB

Tight junctions

Junction complex in the BBB comprises TJ and adherens junction (AJ). The TJs ultrastructurally appear as sites of apparent fusion involving the outer leaflets of plasma membrane of adjacent endothelial cells (Fig. 1B). Freeze fracture replica electron micrographs depict TJs as a set of continuous, anastomosing intramembranous strands or fibrils on P-face with a complementary groove on the E-face. The number of TJ strands as well as the frequency of their ramifications is variable. Adherens junctions are composed of a cadherin–catenin complex and its associated proteins. The TJ consists of three integral membrane proteins, namely, claudin, occludin, and junction adhesion molecules, and a number of cytoplasmic accessory proteins including ZO-1, ZO-2, ZO-3, cingulin, and others (Fig. 1C). Cytoplasmic proteins link membrane proteins to actin, which is the primary cytoskeleton protein for the maintenance of structural and functional integrity of the endothelium.

Claudins

Claudins-1 and -2 were identified as integral component of TJ strands in 1998 (Furuse et al., 1998). So far, at least 24 members

* Corresponding author. Neonatal Intensive Care Unit, 2nd Floor, Main Building, Westchester Medical Center, Valhalla, NY 10595. Fax: +1-914-594-4453.

E-mail address: pballabh@msn.com (P. Ballabh).

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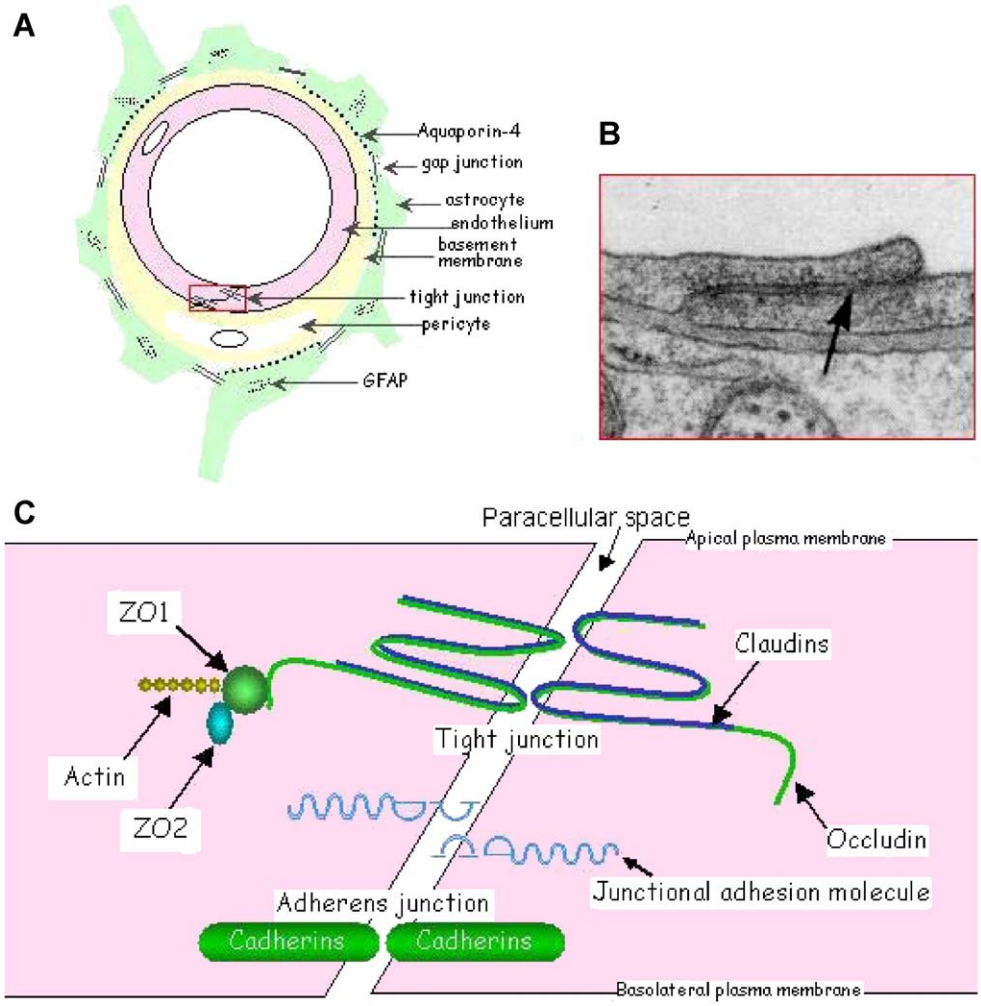

Fig. 1. Blood–brain barrier and the tight junction. (A) Schematic drawing of the blood–brain barrier in transverse section showing endothelium, basement membrane, pericytes, astrocytes, and tight junctions. The localization of gap junction, GFAP, and aquaporin-4 are shown. (B) Electron micrograph of mammalian blood–brain barrier showing endothelial tight junction. [Adapted from: The Blood–Brain Barrier Cellular and Molecular Biology. Pardridge, W.M. (Ed.). Raven Press]. (C) Schematic representation of protein interaction associated with tight junctions at the blood–brain barrier. Claudin, occludin, and junction adhesion molecule are the transmembrane proteins, and ZO-1, ZO-2, and ZO-3, cingulin, and others are the cytoplasmic proteins. Claudins are linked to actins through intermediary cytoplasmic proteins.

of claudin family have been identified in mouse and human, mainly through database searches (Morita et al., 1999a). They are 22 kDa phosphoprotein and have four transmembrane domains. Claudins are the major components of TJ and are localized exclusively at TJ strands as revealed by immunoreplica electron microscopy. Claudins bind homotypically to claudins on adjacent endothelial cells to form primary seal of the TJ (Furuse et al., 1999). Carboxy terminal of claudins binds to cytoplasmic proteins including ZO-1, ZO-2, and ZO-3 (Furuse et al., 1999). In brain, claudins-1 and -5, together with occludin, have been described to be present in endothelial TJs forming the BBB (Liebner et al., 2000a; Morita et al., 1999b). Fig. 2 depicts expression of claudin-5 in cerebral blood vessels of a term newborn. Claudin-11, also known as oligodendrocyte protein (OSP), is a major component of CNS myelin. Loss of claudin-1, but not claudin-5, from cerebral vessels was demonstrated under pathologic conditions such as tumor, stroke, inflammation (Liebner et al., 2000a,b; Lippoldt et al., 2000), as well as in vitro (Liebner et al., 2000b).

Occludin

Occludin was identified in 1993 as the first integral protein localized at the TJ by immunogold freeze fracture microscopy in chickens (Furuse et al., 1993) and then in mammals (Ando-Akatsuka et al., 1996). It is a 65-kDa phosphoprotein, significantly larger than claudin. Occludin shows no amino acid sequence similar to the claudins. Occludin has four transmembrane domains, a long COOH-terminal cytoplasmic domain, and a short NH_2-terminal cytoplasmic domain. The two extracellular loops of occludin and claudin originating from neighboring cells form the paracellular barrier of TJ. The cytoplasmic domain of occludin is directly associated with ZO proteins. The expression of occludin has also been documented in rodents (Hirase et al., 1997) and adult human brain (Papadopoulos et al., 2001) but not in normal human newborn and fetal brain. Occludin expression is much higher in brain endothelial cells compared to nonneural tissues. Occludin appears to be a regulatory protein that can alter paracellular permeability (Hirase et al., 1997).

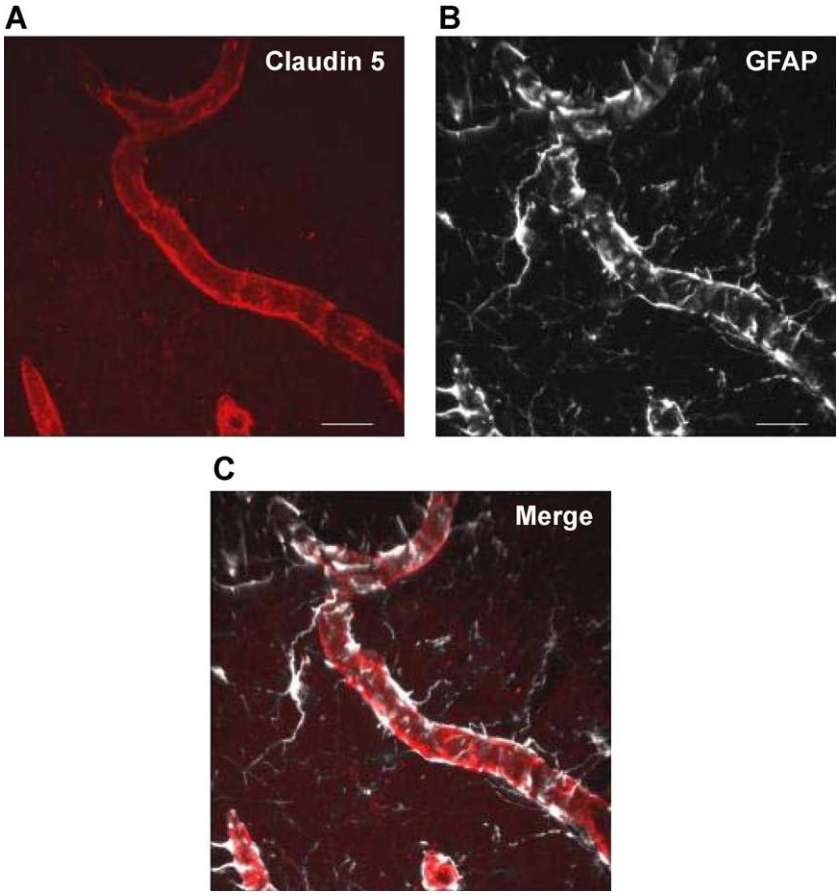

Fig. 2. Claudin-5 (red in A) and GFAP (white in B) immunolabeling of cerebral blood vessels of a term newborn. This infant with cardiomyopathy died in our neonatal intensive care unit on day 2 of life. Coronal section of frontal cortex was immunostained with claudin-5 and GFAP in our laboratory. Claudin-5 is strongly expressed in blood vessels. Astrocyte end-feet, visualized by GFAP-staining, closely cover the blood vessels. Colocalization by double-labeling of claudin-5 and GFAP (Fig. 3C). Scale bar = 20 μm.

Occludins and claudins assemble into heteropolymers and form intramembranous strands, which have been visualized in freeze-fracture replicas. These strands have been proposed to contain fluctuating channels allowing the selective diffusion of ions and hydrophilic molecules (Matter and Balda, 2003). Breakdown of the BBB in tissue surrounding brain tumors occurs with concomitant loss of a 55-kDa occludin expression (Papadopoulos et al., 2001). Together, claudins and occludins form the extracellular component of TJs and are both required for formation of the BBB (Sonoda et al., 1999).

Junctional adhesion molecules

The third type of TJ-associated membrane protein, junctional adhesion molecules (JAM; approximately 40 kDa), has recently been identified (Martin-Padura et al., 1998). They belong to the immunoglobulin superfamily. They have a single transmembrane domain and their extracellular portion has two immunoglobulin-like loops that are formed by disulfide bonds. Three JAM-related proteins, JAM-1, JAM-2, and JAM-3, have been investigated in rodent brain sections. It was observed that JAM-1 and JAM-3 are expressed in the brain blood vessels but not JAM-2 (Aurrand-Lions et al., 2001). The expression of JAM in human BBB is yet to be explored. It is involved in cell-to-cell adhesion and monocyte transmigration through BBB (Aurrand-Lions et al., 2001; Bazzoni et al., 2000). However, our knowledge on function of JAM is incomplete, and more investigations are required to unfold its function in the BBB.

Cytoplasmic accessory proteins

Cytoplasmic proteins involved in TJ formation include zonula occludens proteins (ZO-1, ZO-2, and ZO-3), cingulin, 7H6, and several others. ZO-1 (220 kDa), ZO-2 (160 kDa), and ZO-3 (130 kDa) have sequence similarity with each other and belong to the family of proteins known as membrane-associated guanylate kinase-like protein (MAGUKs). They contain three PDZ domains (PDZ1, PDZ2, and PDZ3), one SH3 domain, and one guanyl kinaselike (GUK) domain. These domains function as protein binding molecules and thus play a role in organizing proteins at the plasma membrane. The PDZ1 domain of ZO-1, ZO-2, and ZO-3 has been reported to bind directly to COOH-terminal of claudins (Itoh et al., 1999). Occludin interacts with the GUK domain on ZO-1 (Mitic et al., 2000). JAM was also recently shown to bind directly to ZO-1 and other PDZ-containing proteins (Ebnet et al., 2000). Importantly, actin, the primary cytoskeleton protein, binds

to COOH-terminal of ZO-1 and ZO-2, and this complex cross-links transmembrane elements and thus provides structural support to the endothelial cells (Haskins et al., 1998).

Adherens junctions

These junctions consist the membrane protein cadherin that joins the actin cytoskeleton via intermediary proteins, namely, catenins, to form adhesive contacts between cells. AJs assemble via homophilic interactions between the extracellular domains of calcium-dependent cadherin on the surface of adjacent cells. The cytoplasmic domains of cadherins bind to the submembranal plaque proteins β- or γ-catenin, which are linked to the actin cytoskeleton via α-catenin. AJ components including cadherin, alpha-actinin, and vinculin (α-catenin analog) have been demonstrated in intact microvessels of the BBB in rat. TJ and AJ components are known to interact, particularly ZO-1 and catenins, and influence TJ assembly (Matter and Balda, 2003).

Brain structures lacking a BBB

The BBB is present in all brain regions, except for the cirumventricular organs including area postrema, median eminence, neurohypophysis, pineal gland, subfornical organ, and lamina terminalis. Blood vessels in these areas of the brain have fenestrations that permit diffusion of blood-borne molecules across the vessel wall. These unprotected areas of the brain regulate autonomic nervous system and endocrine glands of the body.

Notably, choroid plexus epithelial cells possess both TJ and AJ. Claudins-1, -2, -11, occludin, and ZO-1 are present in epithelial TJs of choroid plexus, whereas claudins-1, -5, -11, occludin, and ZO-1 form the TJ of the BBB (Wolburg, 2001). Thus, the difference in molecular composition of TJ between choroid plexus (blood–CSF barrier) and the BBB is with respect to claudins-2 and -5.

Role of astrocytes in the formation of the blood–brain barrier

A number of grafting and cell culture studies have suggested that the ability of CNS endothelial cells to form a BBB is not intrinsic to these cells, but CNS environment induces the barrier property into the blood vessels. Avascular tissue was transplanted from 3-day-old quail brain into the coelomic cavity of chick embryos; and it was observed that the chick endothelial cells vascularizing the quail brain grafts formed a competent BBB (Stewart and Wiley, 1981). In contrast, when avascular embryonic quail coelomic grafts were transplanted into embryonic chick brain, the chick endothelial cells that invaded the mesenchymal tissue grafts formed leaky capillaries and venules. Cultured astrocytes implanted into areas with normal leaky vessels have induced tightening of endothelium (Janzer and Raff, 1987). Blood vessels from solid CNS and peripheral tissues grafted to brain sustained and maintained their morphologic and permeability characteristics (Broadwell et al., 1990). However, peripheral neural and nonneural tissues not possessing BBB properties did not acquire such characteristics on transplantation to the CNS. Direct contact between endothelial cells and astrocytes was deemed necessary to generate an optimal BBB (Rubin et al., 1991). High transendothelial resistance can be reintroduced in human or bovine endothelial cell monolayers that are cultured in astrocyte-conditioned media, suggesting that an astrocyte-derived soluble factor may be responsible for induction of BBB characteristics in endothelial cells (Neuhaus et al., 1991).

However, subsequent investigators criticized the culture and transplantation experiments on methodologic grounds and also disagreed with the view that mature astrocytes play a significant role in the initial expression of the BBB (Holash et al., 1993). It was reported that intact neuronal or glial cells were not necessary for the maintenance of the BBB properties (Krum et al., 1997). They induced neuronal and glial injury by injecting immunotoxin OX7-SAP and the ribosome-inactivating protein saporin into the adult rat striatum. The microvasculature was noted to be intact, allowing a qualitative immunohistochemical analysis of several BBB markers at time points ranging from 3 to 28 days postinjection (Krum et al., 1997). These contradictions may be resolved by additional experiments using host animals of different ages, standard grafting methodology, and systematic analysis of grafts after vascularization. The role of astrocytes in the formation of the BBB is of great interest to scientists and may have therapeutic implications.

Astrocyte–endothelial interaction and signaling pathways

Intercellular signaling: inductive influence of astrocytes on endothelial cells

Numerous efforts have been directed on defining agents that mediate the induction and maintenance of the BBB. We are reviewing only selected studies here. It has demonstrated in astrocyte–endothelial coculture experiments that TGF-β produced by astrocytes is responsible for the down-regulation of tissue plasminogen activator (tPA) and anticoagulant thrombomodulin (TM) expression in cerebral endothelial cells (Tran et al., 1999). It is plausible that TGF-β secreted by astrocytes may have a role in protecting the brain against intracerebral bleeding in children and adults and against intraventricular hemorrhage in premature infants by decreasing the levels of these anticoagulant factors. In another experiment involving TGF-β, the influence of astrocytes and TGF β on differentiation of endothelium and PCs was studied in an in vitro culture mode (Ramsauer et al., 2002). This study suggested that a close association of astrocytes and endothelium was required for the induction and organization of endothelial cells into capillary-like structure (CLS). In contrast to the influence of astrocytes, TGF-β1 led to the formation of a defective CLS, which lacked PCs, recruited fewer endothelial cells, and was shorter in length. Thus, astrocytes have a significant influence on the morphogenesis and the organization of the vessel wall, and the effect of TGF-β1 is different from the astrocytic effect. Glial cell-derived neurotropic growth factor (GDNF), a member of TGF-β family, seems to be involved in postnatal maturation of the BBB (Utsumi et al., 2000). Cerebral endothelial cells are a major source of adrenomedullin, which regulates the cerebral circulation and BBB function (Kis et al., 2003). Other chemical agents that have been shown to differentiate BBB are interleukin-6 (IL-6), hydrocortisone (Hoheisel et al., 1998), and basic fibroblast growth factor (bFGF) (Sobue et al., 1999).

In summary, due to technical limitations associated with live tissues, only culture methods have been utilized so far to describe agents that are involved in BBB maturation. A number of factors

have been shown to induce formation of CLS in culture studies, but it is not known whether similar mechanisms apply in vivo.

Intercellular signaling: inductive influence of endothelial cells on neuronal precursors and astrocytes

Intriguingly, there are also reports about the inductive influence of endothelial cells on astrocytes and on neuronal precursors. It appears that astrocytes and endothelial cells cross talk with each other and regulate each other's function. Endothelial cells seem to be the primary source of leukemia-inhibiting factor (LIF), which helps to induce astrocyte differentiation in vivo (Mi et al., 2001). Changes in the morphology of neonatal mouse cortical astrocytes following their coculture with mouse brain capillary endothelial cells (bEnd 3) have been observed. bEnd 3 cells altered the morphology of astrocytes by transforming them from confluent monolayers into a network of elongated multicellular columns (Yoder, 2002). In addition, astrocytes in cocultures showed increased Ca^{2+} responsiveness to bradykinin and glutamate. Furthermore, the glial–endothelial partnership has been shown to up-regulate aquaporin-4 expression in astrocyte end-feet (Rash et al., 1998) and increase synthesis of antioxidant enzymes in both astrocytes and endothelium (Schroeter et al., 1999).

It has been observed that dividing neuronal cells are found in dense clusters associated with the vasculature, and roughly 37% of all dividing cells are immunoreactive for endothelial markers. This suggests that neurogenesis is intimately associated with active vascular recruitment and subsequent remodeling (Palmer et al., 2000). Recent observations suggest that there is a causal interaction between testosterone-induced angiogenesis and neurogenesis in the adult forebrain (Louissaint et al., 2002). This study has demonstrated that testosterone up-regulates vascular endothelial growth factor (VEGF) and its endothelial receptor in the higher vocal center of adult canaries, which leads to angiogenesis. Angiogenic stimulation induces synthesis of brain-derived growth factor, which stimulates neurogenesis. Hence, these studies indicate that there is an instructive role of endothelial cells on neurogenesis, gliogenesis, and CNS development.

Calcium signaling between astrocyte and endothelium

Calcium waves that propagate in an astrocyte network have been demonstrated in primary cell culture experiments, hippocampal slices, and in isolated retina. However, only few studies have addressed the issue of dynamic signaling between endothelium and astrocyte. This astrocyte–endothelium calcium signaling mechanism has been investigated in two in vitro coculture models: (1) rat cortical astrocytes with ECV304 cells and (2) rat cortical astrocyte with primary rat brain capillary endothelial cells (Braet et al., 2001; Paemeleire, 2002). They have demonstrated that intercellular calcium waves mediate bidirectional astrocyte–endothelial calcium signaling in both culture models. Their experiments suggest that two signaling mechanisms are involved. First, astrocytes and endothelial cells can exchange calcium signals by an intracellular IP3- and gap junction-dependent pathway. Second, pathway involves extracellular diffusion of purinergic messenger. However, in situ, the basement membrane is interposed in between the endothelium and astrocytes. Furthermore, PCs are embedded in the basement membrane and thereby not in direct contact with either endothelium or astrocyte. These findings therefore need confirmation in brain slices. Interestingly, a recent elegant study performed on rat cortical slices has shown that dilatation of arterioles triggered by neuronal activity is dependent on glutamate-mediated $[Ca^{2+}]$ oscillations in astrocytes (Zonta et al., 2003). Inhibition of calcium responses resulted in impairment of activity-dependent vasodilatation. In addition, direct astrocyte stimulation triggered vasodilatation and astrocyte-mediated dilatation was mediated by cyclooxygenase (COX) product. In conclusion, neuron–astrocyte signaling is central to the dynamic control of brain microcirculation. Since up-regulation of COX expression leads to increase in prostaglandin and since prostaglandin may influence BBB permeability, we speculate that neuron–astrocyte signaling may be a mechanism in regulation of BBB permeability.

Signaling pathways associated with tight junctions

There are two principal types of signal transduction processes associated with TJ: (1) signals transduced from the cell interior towards TJ to guide their assembly and regulate paracellular permeability and (2) signals transmitted from TJ to the cell interior to modulate gene expression, cell proliferation, and differentiation (Matter and Balda, 2003). The mechanism of signal transduction is not completely understood. Multiple signaling pathways and proteins have been implicated in the regulation of TJ assembly including calcium, protein kinase A, protein kinase C, G protein, calmodulin, cAMP, and phospholipase C (Balda et al., 1991; Izumi et al., 1998). Calcium acts both intracellularly and extracellularly to regulate TJ activity, and several of the molecules modulating BBB permeability seem to act by alteration of intracellular calcium. Intracellular calcium plays a role in increasing transendothelial resistance as well as in ZO-1 migration from intracellular sites to plasma membrane and thus restoring the TJ assembly (Stevenson and Begg, 1994). Raising extracellular calcium triggers a series of molecular events, which increases resistance across the membrane and decreases the permeability (Stevenson and Begg, 1994). These events are mediated through heterotrimeric G protein and protein kinase C (PKC) signaling pathways. Furthermore, tyrosine kinase activity is necessary for TJ reassembly during ATP repletion, and the tyrosine phosphorylation of occludin, ZO-2, and p130/ZO-3 has roles to play in TJ reformation (following TJ disruption) (Tsukamoto and Nigam, 1999). Based on the studies done so far, it appears that ZO and occludin molecules are primary regulatory proteins of TJ that modulates BBB permeability. However, the role of claudins in regulation of TJ has yet to be explored.

Pericytes and the BBB

Pericytes (PCs) are cells of microvessels including capillaries, venules, and arterioles that wrap around the endothelial cells. They are thought to provide structural support and vasodynamic capacity to the microvasculature. Importantly, PC loss and microaneurysm formation in PDGF-B-deficient mice have been observed (Lindahl et al., 1997). This suggests that PCs play a key role in the structural stability of the vessel wall. Metabolic injury to PCs in diabetes mellitus is associated with microaneurysm formation in the retina (Kern and Engerman, 1996), and PC

degeneration is seen in hereditary cerebral hemorrhage with amyloidosis (Verbeek et al., 1997). This evidence supports the view that PCs play an essential role in the structural integrity of microvessels. PCs express a number of receptors for chemical mediators such as catecholamines (Elfont et al., 1989), angiotensin II (Healy and Wilk, 1993), vasoactive intestinal peptides (Benagiano et al., 1996), endothelin-1 (Dehouck et al., 1997), and vasopressin (van Zwieten et al., 1988), indicating that PCs may also be involved in cerebral autoregulation.

The role of PCs in angiogenesis and differentiation of the BBB has been studied in an in vitro culture model (Ramsauer et al., 2002). This study suggests that PCs stabilize CLS formed by endothelial cells in culture with astrocytes by preventing apoptosis of endothelium. The fact that endothelial cells associated with PCs are more resistant to apoptosis than isolated endothelial cells further supports the role of PCs in structural integrity and genesis of the BBB. A number of experimental observations support the concept that PCs regulate angiogenesis and may play a role in BBB differentiation (Balabanov and Dore-Duffy, 1998; Hirschi and D'Amore, 1997). An ultrastructural study in embryonic mouse brain has shown that endothelial cells together with PCs start invading the neural tissues around E10 (Bauer et al., 1993). Lastly, PCs exhibit phagocytic activity and may be involved in neuroimmune functions (Balabanov et al., 1996).

Fetal brain anatomy, germinal matrix, and development of BBB

Fetal brain anatomy and germinal matrix

The wall of the fetal cerebral hemisphere consists the ventricular zone, subventricular zone, intermediate zone, cortical plate, and marginal zone, as described by the Boulder Committee (1970). A localized thickening medial to the basal ganglia in the subventricular zone, which bulges into the lateral ventricle, is referred as the germinal matrix. This periventricular germinal matrix (GM) in human fetuses, located in the region of the thalamostriate groove beneath the ependyma, is densely packed with neuroblasts and glioblasts and is richly supplied with capillaries. It undergoes progressive decrease in size from a width of 2.5 mm at 23–24 weeks to 1.4 mm at 32 weeks and to complete involution by approximately 36 weeks (Hambleton and Wigglesworth, 1976; Szymonowicz et al., 1984).

Development of the BBB in the GM has been studied in baboon and beagle pup models at the developmental stage, during which premature infants develop GM hemorrhage. Electron microscopic examination of germinal matrix capillaries in baboons at 100 days (54%) of gestational age has revealed continuous endothelium, prominent tight junctions, uninterrupted basement membrane, and clearly identifiable astrocyte end-feet (Bass et al., 1992). GM capillaries in the beagle model showed a significant increase in basement membrane area, tight juntion length, and coverage of capillary perimeter by glial end-feet (from 79% to 95%) on day 10 compared to day 1 (Ment et al., 1995). In contrast, microvessels of the white matter showed no changes in these parameters during this time period, which suggests that blood vessels in the white matter mature earlier than those of vasculature in the GM. Subsequent investigators have studied cortical plate vasculature in human fetus telencephalon of gestational age 12 and 18 weeks, and their findings are consistent with the observations made in the beagle pup model. They observed that perivascular coverage by astrocytes and radial glia was more extensive for 18-week fetuses compared to 12-week fetuses (Bertossi et al., 1999). Thus, it seems that coverage of blood vessels by astroctye end-feet, tight junction length, and basal lamina area in the GM increases as a function of conceptional and postnatal age and that their maturation in GM possibly lags behind the white matter.

Development of the BBB

The temporal development of the BBB varies with species and this has been best studied in rodents. The first blood vessels invade the outer surface of the developing neural tube at E10 in the mouse and E11 in the rat (Bauer et al., 1993; Stewart and Hayakawa, 1994). Neurogenesis in the developing mouse neocortex occurs between embryonic days 11 and 17 and up to E21 in rats (Jacobson, 1991). Gliogenesis starts from E17 in rodents and continues in subventricular zone even in the adult period (Jacobson, 1991). The invasion of blood vessels into the developing nervous tissue is therefore associated with neurogenesis rather than with gliogenesis (Rakic, 1971). The formation of the BBB starts shortly after intraneural neovascularization, and the neural microenvironment seems to play a key role in inducing BBB function in capillary endothelial cells. Fenestrations in the intraparenchymal cerebral vessels are frequent at E11, decline rapidly, and are not seen after E17 (Stewart and Hayakawa, 1994). Thus, the BBB seems to develop between E11 and E17. This also suggests that development of TJ may precede the development of astrocyte end-feet. Occludin expression has been reported to be low in rat brain endothelial cells at postnatal day 8 but clearly detectable on postnatal day 70 (Hirase et al., 1997). Development of other TJ molecules has not been investigated. At present, there is no systemic study of the development of TJ, astrocyte end-feet, and PCs in the developing human brain.

GFAP, vimentin, and aquaporin 4

In rodents, astrocytes, as assessed by glial fibrillary acidic protein (GFAP) immunoreactivity, are first detected at E16 (Liu et al., 2002). In humans, vimentin has been demonstrated in the ventricular zone at 7 weeks and older, and GFAP-positive cells start to appear at 9 weeks in the spinal cord and at 15 weeks in the cerebrum (Sasaki et al., 1988). Ependymal tanycytes are GFAP-positive with their radial processes extending into subventricular zone (SVZ) at 19 weeks (Gould and Howard, 1987). However, GFAP-positive astrocyte differentiation in GM occurred progressively only after 28 weeks, which led to dense network of fibers by 31 weeks. Hence, GFAP immunostaining is not an effective method to evaluate astrocytes in GM before 28 weeks.

Aquaporin 4 (AQP4) immunostaining is an excellent tool to evaluate astrocyte end-feet and the BBB (Fig. 3). Immunogold electron microscopy has demonstrated that AQP4 is restricted to glial membranes and ependymal cells. AQP4 is particularly strongly expressed in glial membranes that are in direct contact with capillaries (Nielsen et al., 1997). We have also shown that AQP4 expression is highly polarized and most immunoreactivity is present in astrocyte end-feet. AQP4 expression had been demonstrated on embryonic day 14 in chick embryos (Nico et al., 2001).

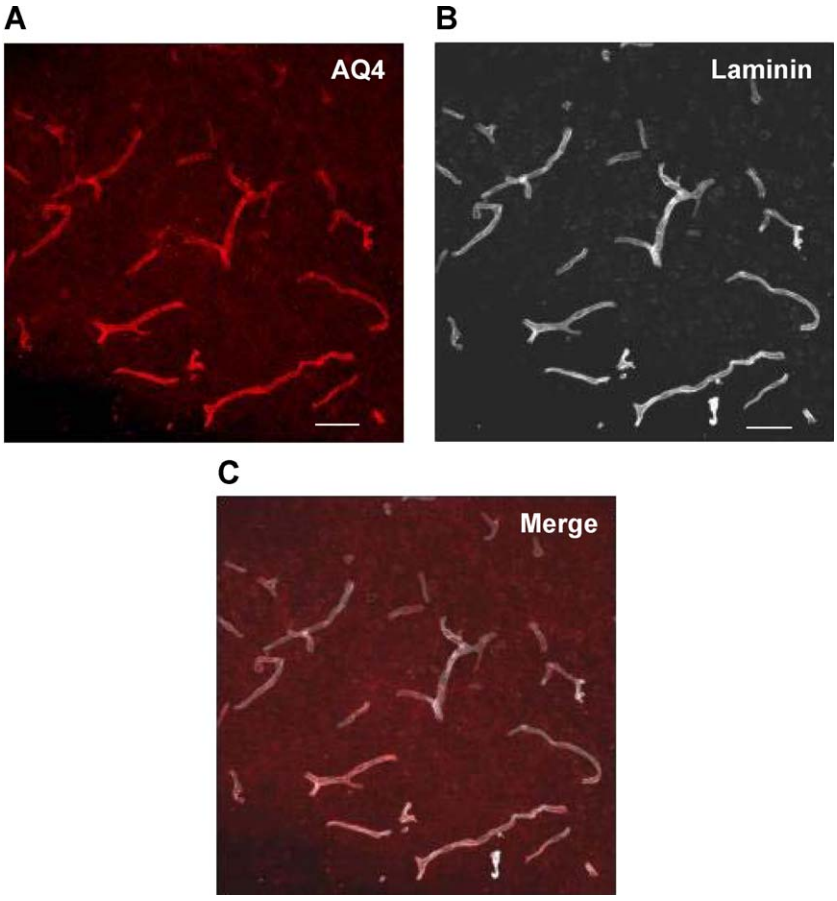

Fig. 3. Aquaporin-4 (A) and laminin (B) expression in cerebral blood vessel of a term human newborn. Coronal section of cerebral cortex (frontal lobe) immunostained in our laboratory for aquaporin-4 and laminin. (A) Aquaporin-4-staining of astrocyte end-feet seems to be continuous. (B) Laminin stains the basement membrane of the blood vessels. (C) Colocalization of aquaporin-4 and laminin. Laminin expression is on luminal side, and aquaporin-4 labels the astrocyte end-feet covering the blood vessels. Scale bar = 50 μm.

We have seen AQP-4 staining of astrocyte end-feet in premature infants as early as 23 weeks of gestation.

Clinical implications

Opening of the BBB in pathophysiology

As discussed earlier, under physiologic conditions, the BBB is relatively impermeable. In pathologic conditions, a number of chemical mediators are released that increase BBB permeability. Several of these mediators of BBB opening have been studied in both in vivo and in vitro experiments and include glutamate, aspartate, taurine, ATP, endothelin-1, ATP, NO, MIP-2, tumor necrosis factor-α (TNF-α), MIP2, and IL-β, which are produced by astrocytes (Abbott, 2000, 2002; Chen et al., 2000; Kustova et al., 1999; Magistretti et al., 1999). Other humoral agents reported to increase BBB permeability are bradykinin, 5HT, histamine, thrombin, UTP, UMP, substance P, quinolinic acid, platelet-activating factor, and free radicals (Abbott, 2002; Annunziata et al., 1998; Pan et al., 2001; St'astny et al., 2000). The source of these BBB-modulating mediators is of interest. Some of these agents are released by endothelium and endothelium itself responds to the released agents. For example, endothelin (ET-1) acts on ETA receptors. In physiologic conditions, nerve terminals of neurons running close to blood vessels release mediators, such as histamine, substance P, and glutamate, which influence BBB permeability.

Germinal matrix hemorrhage and the BBB

GM hemorrhage commonly affects premature infants and has an incidence of 1.45% of live births or approximately 5800 cases per year in the United States (Anstrom et al., 2002). Intraventricular hemorrhage (IVH) occurs when hemorrhage in the GM ruptures through the ependyma into the lateral ventricles (Fig. 4).

The etiopathogenesis of GM hemorrhage is multifactorial, and a combination of vascular and intravascular factors is considered to be responsible. Perinatal and postnatal events, such as vaginal delivery (Ment et al., 1992), chorioamnionitis (DiSalvo, 1998), hypoxia (Antoniuk and da Silva, 2000), hypercarbia (Kenny et al., 1978), pneumothorax, patent ductus arteriosus (Volpe, 1989a), seizures, and respiratory distress syndrome (Volpe, 1989b), lead to significant fluctuation in cerebral blood flow or blood pressure inside the blood vessels (intravascular) and may participate in rupture of the GM microvasculature. Vascular risk factors relate to fragility of the immature thin-walled GM vasculature. Since TJ, astrocyte end-feet, PCs, and BM potentially stabilize the cerebral blood vessels, we speculate that the reason for fragility of the GM vasculature is incomplete coverage of GM capillaries by astrocyte end-feet, poorly developed TJ joining cerebral endothelial cells and

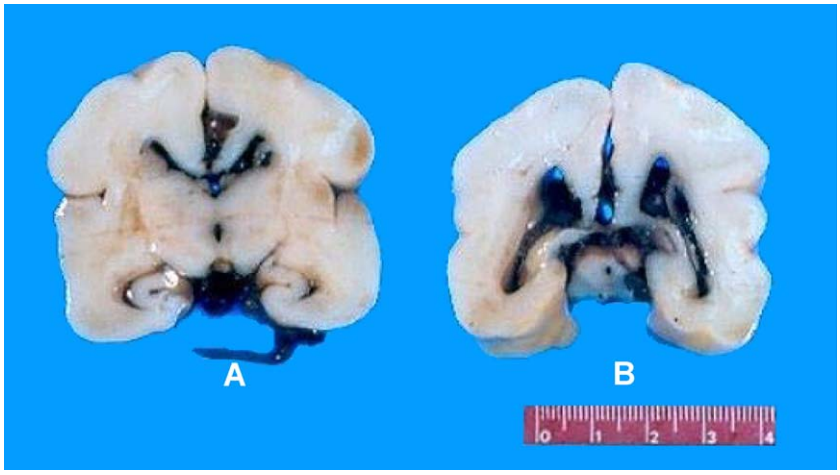

Fig. 4. Intraventricular hemorrhage. Coronal sections of the brain of a premature newborn of 26 weeks of gestational age who died in our neonatal intensive care unit. Lateral ventricles are filled with blood and are mildly dilated (A and B).

immaturity of BM, and/or PCs. Interestingly, a recent investigation has suggested that GM hemorrhage is primarily venous in origin (Ghazi-Birry et al., 1997). However, this report based on postmortem study of brain from four premature infants with IVH needs confirmation by studying larger group of subjects.

The GM hemorrhage may lead to hydrocephalus and other long-term sequelae. Hemorrhage in GM presumably destroys neuron and glial cell precursors that are destined to populate layers II to VI of cerebral cortex. Infants with a history of IVH have a higher incidence of seizures, neurodevelopmental delay, cerebral palsy, and death. Understanding the reason for vulnerability of GM microvessels to hemorrhage will definitely help in developing therapeutic strategies.

Hypoxic–ischemic insult of the BBB

The effect of hypoxia–ischemia on the BBB has been extensively investigated. Hypoxia–ischemia sets in motion a series of events, which leads to disruption of TJ and increased BBB permeability. These events seem to be mediated by cytokines, VEGF, and NO.

Elevated levels of proinflammatory cytokines, IL-1β, and TNF-α have been demonstrated in animal brains after focal and global ischemia (Feuerstein et al., 1994) and in cerebrospinal fluid of stroke patients (Tarkowski et al., 1997). In an in vitro model of the BBB consisting human cerebrovascular endothelial cells and astrocytes, it has been observed that simulated ischemia stimulates IL-8 and MCP-1 secretion from endothelial cells and astrocytes (Zhang et al., 1999). In a further study, the same group of investigators provided evidence that human astrocytes subjected to in vitro hypoxia release inflammatory mediators that are capable of up-regulating genes of IL-8, ICAM-1, E-selectin, IL-1β, TNF-α, and MCP-1 in human cerebrovascular endothelial cells (Zhang et al., 2000). Increased cytokines and subsequent up-regulation of endothelial and neutrophil adhesion molecules lead to transmigration of leukocytes across the endothelium and the BBB. Blood vessels associated with neutrophil recruitment display increase in phosphotyrosine staining, loss of TJ molecules including occludin and zonula occludens, and apparent redistribution of adherens junctions protein vinculin (Bolton et al., 1998). Thus, leukocyte recruitment seems to trigger signal transduction cascades that lead to disorganization of TJ and BBB breakdown.

Hypoxia induces permeability in porcine brain microvascular endothelial cells via VEGF and NO (Fischer et al., 1999). VEGF enhances transcytosis and gap formation between endothelial cells and induces fenestration in unfenestrated human and porcine endothelial monolayers in vitro (Hippenstiel et al., 1998). Subsequent studies have shown that hypoxia-increased release of VEGF led to decreased expression, dislocalization, and increased phosphorylation of ZO-1 (Fischer et al., 2002). In another study, hypoxia induced a 2.6-fold increase in [(14) C] sucrose, a marker of paracellular permeability, increased expression of actin, and changes in occludin, ZO-1, and ZO-2 protein localization in primary bovine brain microvessel endothelial cells (Mark and Davies, 2002). Interestingly, astrocytes protect the BBB against hypoxia-induced disruption of tight junction protein, zonula occludens, and paracellular permeability changes by decreasing VEGF expression in porcine brain microvascular endothelial cells (Fischer et al., 2000). Other investigators have made similar observations (Kondo et al., 1996).

In conclusion, these investigations suggest that increased BBB permeability induced by hypoxia–ischemia involves a cascade of events in which cytokines, VEGF, and NO are the main players and astrocytes appear to play a protective role. Most of these conclusions based on in vitro experiments need further confirmation by performing experiments in vivo and in intact tissues.

Break down of BBB in septic encephalopathy

The pathophysiology of septic encephalopathy including decreased cerebral blood flow and oxygen extraction by the brain, cerebral edema, and breakdown of the BBB may be related to several reasons—the effect of inflammatory mediators on the cerebrovascular endothelium, abnormal neurotransmitter composition of the reticular activating system, impaired astrocyte function, and neuronal degeneration (Papadopoulos et al., 2000). A variety of evidence demonstrates that the BBB is compromised in septic encephalopathy. Colloidal iron oxide (Clawson et al., 1966), ^{14}C amino acid (Jeppsson et al., 1981), and ^{125}I-albumin (Deng et al., 1995) have been shown to enter brain parenchyma from the circulation in rodents. In addition, elevated CSF protein has been observed. Cellular pathology underlying this blood–brain disruption has been reported by several investigators including (1) increased pinocytosis in cerebral microvessel endothelium and

swelling of astrocytes in rabbit with endotoxemia (Clawson et al., 1966); (2) perivascular edema, swollen astrocyte end-feet with ruptured membranes, and detachment from microvessel walls in septic pigs (Norenberg, 1994); and (3) dark shrunken neurons in pigs 8 h after inducing peritonitis (Papadopoulos et al., 1999). The adrenergic system has been implicated in the inflammatory response to sepsis (Tighe et al., 1996). β_2 adrenoreceptor stimulation appears to be suppressed, and α_1 adrenoreceptor stimulation seems to induce an inflammatory response and hence influence BBB permeability.

Disruption of BBB in brain tumor

The BBB is poorly developed in brain tumor leading to increased vascular permeability (Groothuis et al., 1991). Investigations have shown that there is opening of interendothelial TJ in human gliomas (Long, 1970) and metastatic adenocarcinoma (Long, 1979). The expression of the TJ protein claudin-1 is lost in the microvessels of glioblastoma multiforme, whereas claudin-5 and occludin are significantly down-regulated and ZO-1 expression is unaffected (Liebner et al., 2000a). A loss of 55 kDa occludin expression in microvessels, observed in astrocytoma and metastatic adenocarcinoma, may also contribute to endothelial TJ opening (Papadopoulos et al., 2001).

The explanation for loss of TJ molecules in brain tumor microvessels is not clear. However, VEGF, cytokines (de Vries et al., 1996), and Scatter factor or hepatocyte growth factor (Lamszus et al., 1999) secreted by astrocytoma and other brain tumors may be involved in down-regulating TJ molecules leading to TJ opening, increased vascular permeability, and cerebral edema. It is also possible that poorly differentiated neoplastic astrocytes do not release factors necessary for BBB function.

Since cerebral edema is an important consequence of brain tumor, water channel molecule, AQP4, has been examined in brain tumor by several investigators. AQP4 is massively up-regulated in astrocytoma and metastatic adenocarcinoma and this correlates with blood–brain barrier opening assessed by contrast-enhanced computed tomograms (Saadoun et al., 2002). Mice deficient in AQP4 have a much better survival than wild-type mice in a model of brain edema caused by acute water intoxication. Up-regulation of AQP4 has also been noted in rat models of ischemia (Taniguchi et al., 2000) and brain injury (Vizuete et al., 1999). Thus, it seems that breakdown of the BBB associated with brain tumors and other forms of brain injury increases the expression of AQP4. However, the exact mechanism of AQP4 up-regulation in different clinical situations is not known.

Inflamed BBB: HIV-induced dementia, multiple sclerosis, and Alzheimer disease

In normal brain, highly specialized cerebral endothelial cells limit entry of leukocytes and circulating substances into the brain. Neurologic conditions including HIV-associated dementia, multiple sclerosis, and Alzheimer disease alter the integrity of the BBB with consequent migration of leukocytes into the brain (Lou et al., 1997; Minagar et al., 2002). Leukocyte migration into brain has been shown to trigger signal transduction cascades leading to loss of TJ molecules including occludin and zonula occludens and BBB breakdown (Bolton et al., 1998).

Astrocytes and microglia play a significant role in host defense as well as in the pathogenesis of infectious and autoimmune diseases of CNS. They ordinarily protect the CNS but in pathologic circumstances can amplify inflammation and mediate cellular damage. Interaction of astrocyte, microglia, and immune system leads to an altered production of neurotoxins and neurotropins by these cells, which have roles to play in the pathogenesis of HIV-induced dementia, multiple sclerosis, and Alzheimer disease (Minagar et al., 2002). HIV encephalitis is associated with immune activation of astrocytes and macrophages. HIV-infected macrophages or microglia and astrocytes release cytokines, chemokines, reactive oxygen species, and a number of neurotoxins, which impair cellular functioning, modify transmitter action, and cause leukoencephalopathy and neuronal loss (Sharer, 1992). The neurotoxins include TNF-α, arachidonic acid, nitric oxide, platelet-activating factor, and quinolinic acid. TNF-α is released by HIV-infected macrophages, which particularly affect oligodendrocytes (Johnson, 1988). Nitric oxide is synthesized by macrophages, endothelial cells, and neurons, which react with superoxide anion to produce peroxynitrite (Boven, 1999). In addition, nitric oxide is associated with NMDA-type glutamate-induced neurotoxicity. Quinolinic acid also plays a major role in pathogenesis of neuronal damage in HIV-induced dementia.

In Alzheimer disease, microglia and astrocyte are activated by β-amyloid protein and related oligopeptides, leading to a cascade of events producing toxic molecules, neuronal damage, and synaptic dysfunction (Giulian et al., 1995). Reactive macrophages or microglia closely associated with neuritic and β-amyloid plaque, and interaction of macrophages and astrocyte possibly leads to release of interleukin-1 β (IL-1 β), TNF-α, transforming growth factor-β, neurotropic factors such as NGF and bNGF, and reactive oxygen species (Klegeris, 1997a,b). In addition, it has been shown that β-amyloid stimulates NF-κB that induces transcription of TNF-α, IL-1, IL-6, monocyte chemoattractant protein-1, and nitric oxide synthetase (Akama, 1998). The details of the signal transduction pathway that mediates neurotoxicity in Alzheimer disease are not known.

Most investigators believe that multiple sclerosis is an autoimmune disease in which reactive T cells recognize and destroy myelin sheath and the underlying axons (Wekerle, 2003). An antigen-specific T cell receptor called Hy.2E11 has been isolated from a T cell line from a patient with multiple sclerosis (Lang, 2002). This receptor recognizes two peptides, one derived from myelin basic protein, which is bound to HLA-DR2b, and other derived from the Epstein–Barr virus, which is bound to HLA-DR2a. The reactive T cell interacts with the antigen presented by macrophages- or microglia-expressing HLA-DR2a and HLA-DR2b. Activated macrophages synthesize and secrete nitric oxide and cytokines including interferon-γ, TNF-α, and IL-3, which damage oligodendrocytes causing interference with myelination and myelin gene expression (Chao et al., 1995; Merrill et al., 1993). Disruption of the BBB is one of the initial key steps in multiple sclerosis, which follows massive infiltration of T cells and the formation of demyelinative foci.

In conclusion, the pathogenesis of HIV-associated dementia, multiple sclerosis, and Alzheimer disease involves activation of inflammatory mechanisms, production of toxins and neurotropins, and the breakdown of the BBB.

Future directions

Rapid progress in the BBB research has led to a better understanding of BBB morphology and physiology. However, several key questions related to normal human development of the BBB are unanswered. Data on expression of claudins-1, -2, -5, and -11 in brain endothelial cells in human fetuses, preterm, and

term infants are lacking. Morphology of the gliovascular interface of germinal matrix compared to other areas of human brain cortex has not been adequately studied. This may be critical to understanding why the blood vessels of GM are fragile and prone to bleeding in premature infants. In addition, a number of puzzles with respect to functional property of the BBB are yet to be resolved: How do TJ molecules assemble? How are they regulated in different physiologic conditions? How do they interact with several mediators, neurotransmitters, and medications? How do they alter in disease conditions? Over the coming years, emerging information on the mechanism of BBB disruption may help in formulating strategies to protect BBB and to prevent and treat BBB-related pathologies.

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