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Blood-brain barrier disruption in multiple sclerosis

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The blood-brain barrier (BBB) is a complex organization of cerebral endothelial cells (CEC), pericytes and their basal lamina, which are surrounded and supported by astrocytes and perivascular macrophages. Collectively these cells separate and form the compartments of the cerebral vascular space and the cerebral interstitium under normal conditions. Without the BBB, the 'interior milieu' of the central nervous system (CNS) would be flooded by humoral neurotransmitters and formed blood elements that upset normal CNS functions and lead to vascular/neural injury. Dysregulation of the BBB and transendothelial migration of activated leukocytes are among the earliest cerebrovascular abnormalities seen in multiple sclerosis (MS) brains and parallel the release of inflammatory cytokines/chemokines. Mechanisms for breakdown of the BBB in MS are incompletely understood, but appear to involve direct effects of these cytokines/chemokines on endothelial regulation of BBB components, as well as indirect cytokine/chemokine-dependent leukocyte mediated injury.

Unique endothelial structural features of the BBB include highly organized endothelial tight junctions, the absence of class II major histocompatibility complex, abundant mitochondria and a highly developed transport system in CEC. Exposure of endothelium to proinflammatory cytokines (IFN-γ, TNF-α and IL-1β) interrupts the BBB by disorganizing cell-cell junctions, decreases the brain solute barrier, enhances leukocyte endothelial adhesion and migration as well as increases expression of class II MHC and promotes shedding of endothelial 'microparticles' (EMP). In this review we examine interactions between cytokines/chemokines, activated leukocytes, adhesion molecules and activated CEC in the pathogenesis of BBB failure in MS.
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The blood-brain barrier in health

Several unique structural and functional properties of cerebral endothelial cells (CEC) create the solute impermeable barrier of the blood-brain barrier (BBB) (Figure 1). The low level of BBB exchange necessitates that CEC maintain multiple transporters for glucose and amino acids; CEC also express cholinesterases, monoamine oxidase, alkaline phosphatase and aromatic decarboxylases for catabolizing humoral transmitter substances; γ-glutamyl transpeptidase (GGT) also appears to be another marker specific for CEC.[1] In addition to these surface enzymes, CEC share common endothelial markers, such as low density lipoprotein (LDL) and insulin receptors.

The permeability barrier formed by CEC is clearly supported by trophic factors secreted by other cells in the BBB, including pericytes, glial cells, but most importantly, astrocytes.[2,3] Astrocytes appear to be central in maintaining the BBB phenotype. The abluminal surface of the CEC is $>90\%$ covered by astrocytes and contact/communication between CEC astrocytes is necessary to maintain BBB markers, e.g., GGT.[4] Astrocytes also induce MnSOD expression in CEC, which may further protect the BBB against oxidants.[5]

The loss of this contact will abolish the BBB phenotype.[6] Several trophic factors which induce BBB in CEC include transforming growth factor-β1 (TGF-β_1), glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and possibly steroids.[2] cAMP mobilizing agents also help CEC establish maximal barrier *in vitro*.[7] CEC also modulate the astrocytes phenotype by secreting factors, e.g., Leukemia Inhibitory Factor (LIF). Interestingly, in multiple sclerosis (MS) patients, a 17-kDa gliotoxin has been described in the cerebrospinal fluid (CSF), urine, and plasma, which injures astrocytes and may promote BBB disruption in MS.[8,9]

In the BBB, the tight and adherens junctions are the subcellular structures that maintain the restrictive properties of the BBB (Figure 2). In the BBB, tight junctions are highly organized, pericellular structures that appear ultrastructurally as multilaminar, usually continuous strands containing several integral membrane proteins that seal between adjacent endothelial cell membranes. Tight junctions create distinct membrane domains that confine the numerous transport systems to apical and basal surfaces and block paracellular solute and cell flux.[10–13]

In CEC, adherens junctions are continuous cellular contact zones that contain cadherins (e.g., VE-cadherin) and a submembranous zone of catenins which bind

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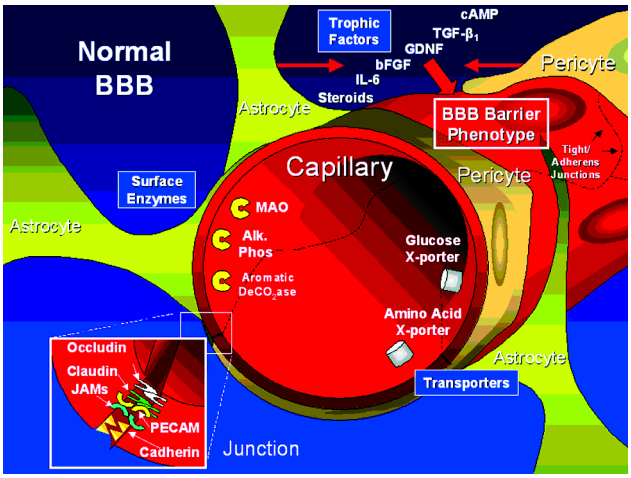

Figure 1 *Normal components of the blood brain barrier. The BBB is formed by the association of cerebral capillary endothelial cells, pericytes their basal lamina, and supporting astrocytes, which collectively separate the central nervous system from peripheral circulation. The cerebral endothelial permeability barrier is induced and supported by trophic factors secreted by nonendothelial cells in the BBB including pericytes and particularly astrocytes, which are essential for maintenance of BBB properties. The anatomic BBB is created by several junctional components in the tight and adherens junctions, which include occludin, claudins, JAMs, PECAM-1 and cadherins (see inset).*

cadherins to actin microfilaments.[14] In the BBB, adherens junctions may act as both a structural support and barrier forming element that cooperates with the tight junctions in maintaining the barrier.

MS and the BBB

MS is an inflammatory demyelinating disease of the central nervous system (CNS) that develops in genetically susceptible individuals after exposure to unknown environmental trigger(s).[15] Relapsing–remitting MS (RRMS) presents in $>80\%$ of patients and shows a female:male dominance of $\sim 2{:}1$. The age of onset for RRMS varies between 10 and 60 years, the mean in the second or third decade. The bases for MS are unknown but are strongly suspected to involve immune reactions against autoantigens, particularly myelin proteins. The most widely accepted hypothesis suggests that dialogue mediated by T cell receptors (TCR) on CD4+ T lymphocytes, with myelin antigens, presented by class II major histocompatibility complex (MHC) expressed on macrophages/microglia, astrocytes, and CEC leads to an immune attack on the myelin-oligodendrocyte complex. These interactions between active CD4+ T cells and myelin antigens apparently provoke a massive destructive inflammatory response and promotes continuing proliferation of T and B cells and macrophage activation, which sustains secretion of inflammatory mediators, e.g., cytokines/chemokines. Thus, the immune dysregulation in MS ultimately represents a failure of the immune system to anergize T cells to myelin sheath components, and an imbalance between pro- and anti-inflammatory cytokines.

Clinically, the relapsing–remitting course of MS is characterized by disturbances in cognitive, cerebellar, motor, sensory, bladder and bowel, brainstem, optic nerve, and cognitive functions. Neuropathologically, MS manifests with development of multiple demyelinated plaques throughout the neuroaxis, which show a predilection for optic nerves, periventricular white matter, brain stem, cerebellum, and spinal cord. Acute MS lesions show focal loss of myelin and a hypercellular background. The inflammatory infiltrate of acute demyelinating lesions consists of lipid and myelin-debris filled macrophages, monocytes, lymphocytes, and 'active' astrocytes. Invading CD4+ T lymphocytes and macrophages causes intense perivenular 'cuffing'. (MS plaques typically surround medium-sized venous vessels.) Besides demyelination, active MS lesions may show significant axonal damage and loss.[16,17] Lastly, acute relapses of MS are associated with expression of class II MHC by endothelial cells and imply a possible role of CEC as antigen presenting cells at least in the initial stages of T-cell responses in acute MS.[18]

Besides loss of the BBB solute barrier, enhanced transendothelial leukocyte migration into the CNS is observed early in the course of MS, and likely contributes to disturbances in neural integrity.[19–21] Correlative magnetic resonance imaging (MRI) neuropathological studies of acute MS lesions have shown that these focally enhanced areas consist of active lesions, characterized by severe inflammation, loss of barrier, perivascular cuffing, and often extensive infiltration of tissue by monocytes.[22–26] Disruption of the BBB is demonstrated *in vivo* in acute and chronic active MS lesions using contrast enhanced MRI (GdGTPA MRI; Figure 3).[27–30] The presence of enhancing lesions on MRI is an important marker for development of new lesions and expansion of existing inflammatory lesions in RRMS.

CEC activation in MS: role of cytokines and chemokines

Part of the MS inflammatory cascade may be triggered by the penetration of the BBB by activated leukocytes, which in turn initiates the production of cytokines and chemokines within the cerebral interstitium. Many cells in active lesions release Th1 cytokines, including active T cells, macrophages/microglia, and astrocytes. The major Th1 cytokines involved in pathogenesis of relapses of MS are interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), and IL-6. Elevated plasma levels of proinflammatory cytokines in MS patients precede a surge of disease activity. Levels of Th1 cytokines are dramatically increased during periods of active disease and decrease during remission. In models of MS (experimental autoimmune encephalomyelitis, EAE), Th-1 cytokines (IFN-γ, TNF-α, IL-1β, IL-6, IL-12, and IL-18) initiate and sustain inflammatory responses in the CNS, including BBB defects as well. In both MS and EAE, these inflammatory reactions seem to be normally held in

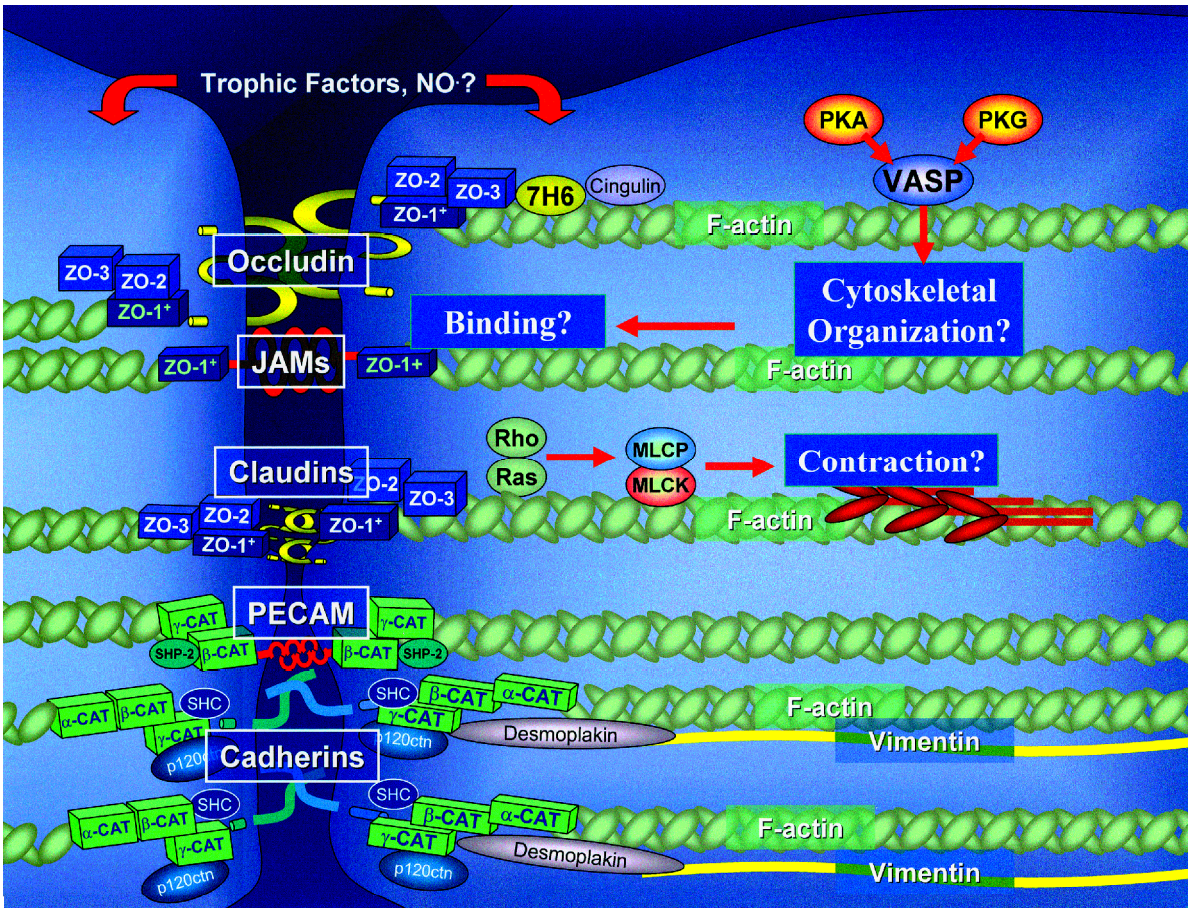

Figure 2 *Molecular structure of the endothelial barrier of the BBB. Two major divisions of the endothelial barrier are tight and adherens junctions. Tight junctions seal intercellular clefts and contain several protein components, e.g., occludin, claudins, ZO-1, ZO-2, ZO-3, cingulin, and 7H6. The junctional adhesion molecules (JAMs) are also located at tight junctions. Cerebral endothelial adherens junctions are formed mainly by cadherins (mainly VE-cadherin), which interact with catenins and desmoplakins to bind the cytoskeleton. PECAM also binds to the cytoskeleton via catenins and may be regulated by SHP-2 interactions.*

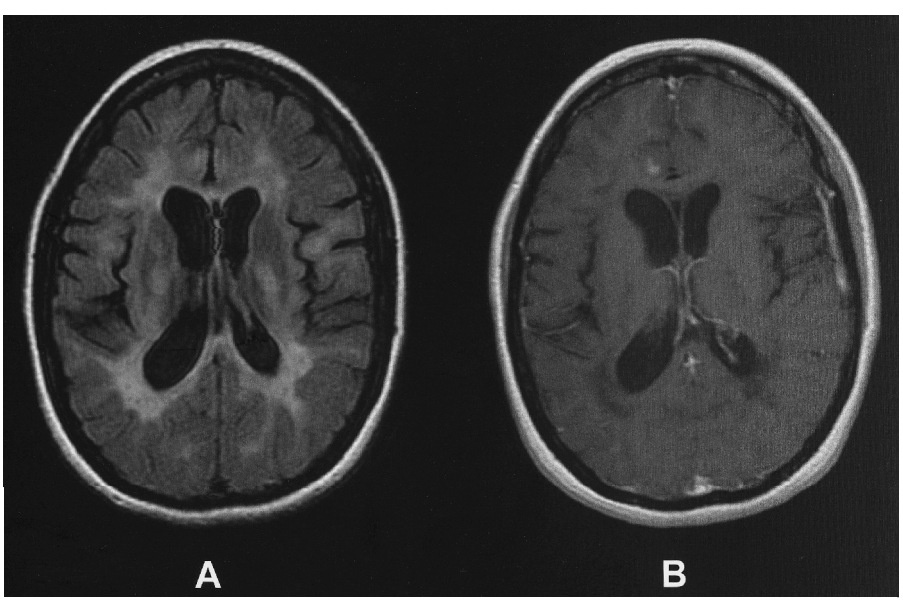

Figure 3 *MRI scans of the brain of a 32-year old woman with RRMS. An axial FLAIR (fluid-attenuated inversion recovery) image reveals multiple hyperintense lesions in the periventricular white matter. (B) A gadolinium-enhanced axial T1-weighted image of the same patient reveals an enhanced lesion, which is indicative of BBB disruption during active inflammation.*

check by anti-inflammatory cytokines, like IL-10 or IFN-β.[31,32]

CEC activation by Th1 cytokines can modulate the BBB phenotype by induction of several inflammatory genes. Cytokines induce expression of endothelial cell adhesion molecules (ECAMs), including ICAM-1, VCAM-1, E-selectin, and PECAM-1.[33,34] Elevated levels of ICAM-1 have been described in MS during relapses and are associated with enhancing lesions on MRI.[35–37] Conversely, ICAM-1 expression decreases in MS during remission, or following treatment with high-dose corticosteroids.[38] Similarly, soluble forms of ECAMs are also increased in MS sera/CSF (soluble E-selectin, VCAM-1, and PECAM-1)[33,39–41] and correlate with disease activity. Elevated plasma levels of insoluble PECAM-1 also have been reported in patients with RRMS (Figure 4).[42] Since PECAM-1 (CD31) is a guide molecule in leukocyte emigration, the release of PECAM-1 might limit leukocyte binding and extravasation, but this has yet not been examined.

CEC exposure to Th1 cytokines, particularly IFN-γ, can alter the architectural organization of the tight and adherens junctions of the CEC. Two of the major structural proteins in tight and adherens junctions are occludin and vascular endothelium-cadherin (VE-cadherin), respectively. We have observed that chronic exposure of several types of endothelial cells to Th1 cytokines, such as IFN-γ.[43] While cytokines like TNF-α and IL-1β may induce inducible nitric oxide synthase (iNOS) (which promotes injury to the BBB), the role of these cytokines in MS is complex and they may also aid in repair processes, such as remyelination.[44]

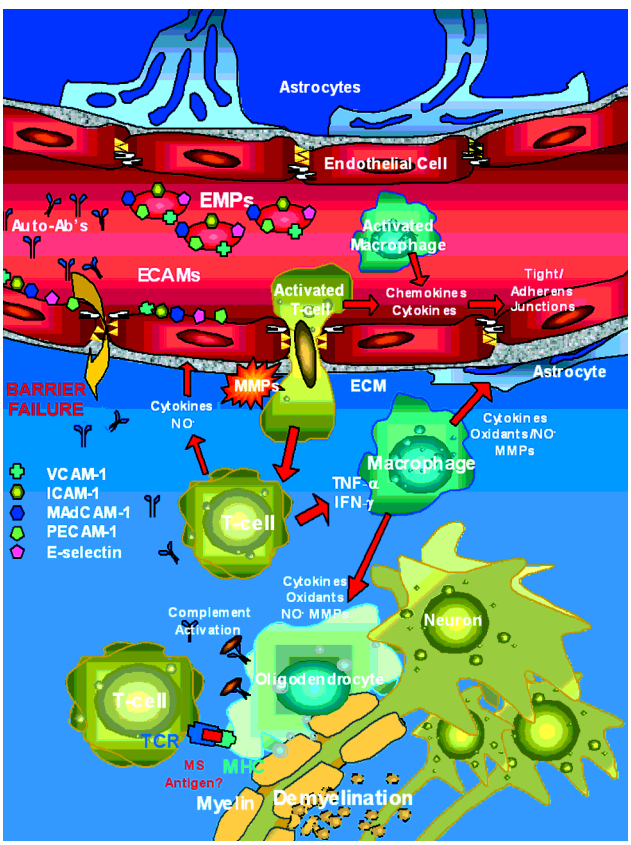

Figure 4 *Possible mechanisms leading to BBB disruption in MS. Following exposure of genetically susceptible individual to as yet unknown environmental factor(s), leukocytes are activated against CNS antigens. Activated leukocytes, mainly CD4 T lymphocytes and macrophages set in motion cerebral endothelial activation and injury by releasing proinflammatory cytokines (e.g. IFN-γ and TNF-α) and chemokines. Expression of endothelial cell adhesion molecules (ECAMs) such as VCAM-1, ICAM-1, MAdCAM-1, E-selectin, and PECAM-1 by activated cerebral endothelial cells is upregulated. Some ECAMs are shed as vesicles from endothelial membranes carrying ECAMs from the parent EC known as 'endothelial microparticles' (EMP). The molecular organization of the tight/adherens junctions of the BBB is also possibly altered by effects of proinflammatory cytokines (particularly IFN-γ) on expression of occludin and other junctional elements. Activated leukocytes also secrete matrix metalloproteinases (MMPs) which may promote transendothelial migration of these cells into the CNS by degrading extracellular matrix macromolecules. Binding of putative MS antigen(s) within the CNS to CD4 T lymphocyte receptor and MHC (trimolecular complex) further promotes the inflammatory cascade against bound antigen(s).*

Chemokines

Chemokines are small (8–10 kDa) chemotactic peptides released by activated macrophages, microglia,[45–47] astrocytes and inflammatory cells.[45,46,48] Chemokines direct leukocyte chemotaxis and adhesion. In MS, proinflammatory cytokines, e.g., IFN-γ, IL-1β, and TNF-α, may be the main stimuli for chemokine production.[49,50] The chemokines affecting CEC interactions with activated leukocytes belong to two families: α-chemokines (CXC), chemotactic for neutrophil/T cells, and β-chemokines (CC), which are chemotactic for monocyte/macrophages. The two major chemokine groups involved in MS pathogenesis are the CXC and CC. Elevated levels of these two subfamilies, which include CCL2, -3, -4, -5, -7, -8, and CXCL10, have been described in MS.[51] During active MS inflammatory responses, chemokine signals convert low affinity, selectin-mediated interaction of leukocytes with CEC into the higher affinity, integrin-mediated interaction that leads to transendothelial migration of lymphocytes, monocytes, and macrophages.[52–54] Berger *et al.* have shown that the CCR3 and CXCR4 chemokines are abundantly expressed in CEC, indicating that these may be key elements in controlling leukocyte migration across the BBB.[55] Therefore, chemokines, cytokines and adhesion molecules cooperate in the control of leukocyte-endothelial adhesion and motility and determine the cellular composition of inflammatory infiltrate in MS[21,56] and BBB properties.[57]

BBB junction remodeling in MS

The tight junctions of the CEC contain occludin,[58–60] claudin-1 and claudin-5,[61–64] junctional adhesion mole-

cules 1–3,[65–67] 7H6, and cingulin.[10] In patients with MS, disturbances in the BBB may reflect 1) diminished expression and/or 2) increased destruction of tight and/or adherens elements, 3) changes in junctional binding, 4) internalization/repositioning of junctional elements, and 5) increased cytoskeletal tension.

Changes in junctional protein expression in MS

Proinflammatory cytokines like IFN-γ and TNF-α are elevated in MS, and can affect barrier integrity through several mechanisms, particularly inhibition of junctional proteins expression. *In vitro*, we reported that, IFN-γ decreased endothelial expression of occludin.[43] IFN-γ decreased endothelial occludin expression and affected barrier function over 48 hours, without concomitant reductions in VE-cadherin.[42,43] Similarly, TNF-α and IFN-γ reduced expression of occludin and barrier in epithelia[68] and might be expected to do the same in CEC. However, Wachtel *et al.* reported in CEC that TNF-α does *not* affect occludin expression; on the other hand, astrocytes (which express occludin, claudin-1, ZO-1, and ZO-2) show decreased occludin after TNF-α, with no effect on ZO-1. This is presumed to be an NF-kB dependent phenomenon.[69]

We observed that occludin structure and content in endothelial cells were diminished by IFN-γ,[70] an effect that was blocked by IFN-β1b or IFN-β1a.[71] Interestingly, we also observed that, alone, both IFN-β1a and IFN-β1b could *enhance* occludin expression in cultured EC.[71] This phenomenon is currently under study in our laboratory and could in part explain some of the therapeutic effects of IFN-β in MS therapy.

Possible alterations of junctional binding in MS

It is generally accepted that microfilament organization parallels junctional bond strength (and thus barrier); this has only recently been demonstrated.[72] The mechanisms through which inflammatory mediators (cytokines, autacoids, oxidants) fragment the cytoskeleton and might lead to barrier failure remain unknown.

Oxidant stress and cytokines decrease endothelial barrier[73] and increase the tyrosine phosphorylation of occludin, zonula occludens (ZO-1), E-cadherin, and beta-catenin, which dissociates these complexes.[74,75] Similarly, we have shown in EC that oxidants decrease EC barrier by disintegrating occludin and ZO-1 binding, events that are MAPK dependent.[76] Cytokines like TNF-α can induce the phosphorylation of VE-cadherin,[75] which controls its integration into cell junctions and barrier function.

Sequestration of classical cadherins and VE-cadherin[77,78] may provide an additional mechanism for regulating junction assembly and barrier and has been reported following endothelial exposure to oxidants and other proinflammatory mediators.

Many groups support tyrosine phosphorylation as the critical trigger for junctional disorganization in response to several inflammatory mediators, which include oxidants and growth factors. Conversely, re-establishment of the barrier and junctional restitution also requires activity of tyrosine kinases as well.[79]

cAMP has been shown to play a significant role in organizing elements in the BBB, like microfilaments, and may act through vasodilator stimulated phosphoprotein (VASP), which is an important regulator of actin-tight junction interactions.[80]

Endothelial growth factors in MS: possible role in reorganization of the junctions

In addition to cytokines, endothelial growth factors may also modulate junctional molecular structure and function in MS. Vascular endothelial growth factor (VEGF) mediates various aspects of endothelial cell physiology, including regulation of growth, permeability, and inflammation. Kevil *et al.*[81] demonstrated that VEGF increases albumin permeability across endothelial monolayers *in vitro* and suggested that permeability increases through rearrangement of two major endothelial junctional proteins, occludin and VE-cadherin. Increased expression of VEGF is seen both in active and chronic MS lesions.[82] Thus, it is possible that, particularly during acute relapses of MS, excessive expression of VEGF contributes to disruption of the BBB and alters the endothelial barrier function by the lowering the expression of key junctional proteins.

Role of matrix metalloproteinases in MS pathogenesis

Transendothelial trafficking of leukocytes from vascular compartment into the brain parenchyma involves matrix metalloproteinases (MMPs), which remodel extracellular matrix (ECM) and junctional components. MMPs consist of a group of >20 Zn^{2+}-dependent endopeptidases, which are implicated in the pathogenesis of MS. Expression of MMPs is regulated at transcriptional, translational, and post-translational levels with tissue inhibitor of metalloproteinase (TIMP). There is growing evidence that MMPs are involved in various steps of pathogenesis of MS, e.g., focal BBB damage, perivascular lymphocyte infiltration, destruction of myelin, formation of demyelinated plaques, and loss of axons.[83,84] Nearly all cells in the BBB (astrocytes, microglia, CEC, T cells, macrophages) can form MMPs, which can disrupt the BBB.[85,86] Immunochemical studies of brain tissue from MS patients have shown elevated production of MMPs-1, -2, -3, -7, -9 by macrophages in active lesions.[87,88] MMP-9 levels in MS serum are elevated compared with controls;[89–91] the serum levels of MMP-9 rise rapidly during relapses of MS and are correlated with gadolinium-enhancing lesions on MRI.[91]

Barrier modulation by leukocyte emigration: possible role in MS

CEC activation is the initial step in enhanced trafficking of leukocytes, particularly CD4+ T cells into the CNS of MS patients. Transendothelial migration of activated leukocytes during the inflammatory cascade of MS is perhaps the most significant consequence of the BBB disruption. While some forms of leukocyte migration do not alter barrier or junctional integrity.[3] In other cases, leukocyte migration itself may be enhanced by reduced junctional integrity in MS, and may further contribute to structural modification of endothelial junctions during chronic inflammation like that in MS.[92,93] This remodeling process may involve NF-kB-dependent signaling,[93,94] secretion of proteases, e.g., elastases,[94,95] and may target components in both tight and adherens junctions. Thus, while modest levels of leukocyte migration might not damage the BBB, the large numbers of cells migrating during active episode of MS, or the level of their activation could potentially remodel junctions with destructive consequences.

Endothelial microparticles in multiple sclerosis

Another interesting feature of the CEC activation by the cytokine and chemokine storm of MS is shedding of endothelial microparticles (EMP) into plasma. Upon activation by cytokines, IFN-γ and TNF-α, endothelial cells shed small membrane vesicles, which bear adhesion molecules from the activated parent endothelial cells. EMP were first studied *in vitro*, but recently have been studied *in vivo* as markers of endothelial stress.[96,97] Some of the markers carried by EMP include PECAM-1 (CD31), CD51, endoglin (CD105), E-selectin, and VCAM-1. High plasma levels of EMP carrying CD31 has shown a positive association with the presence of contrast enhancing lesions by brain MRI in MS patients and may represent a new marker of disease activity.[42]

Role of oxidative stress and NO in BBB disruption in MS

Oxidative stress may, in the form of $O_2^{\bullet}$, H_2O_2, $OH^{\bullet}$, peroxyl radicals and hydroperoxides, contribute to development of endothelial injury in MS.[98,99] While reactive oxygen species (ROS) act as tertiary messengers, excess oxidants reduce barrier function, and involve reorganization of tight junction architecture involving ZO-1 and occludin pairing.[73] ROS also activate kinase pathways and transcription factors, such as NF-kB[100,101], poly-ADP ribose polymerase,[102] to induce inflammatory genes, including ECAMs, MMPs, and iNOS.

Levels of $NO^{\bullet}$ and its metabolites are elevated in blood, urine, and CSF of MS patients,[103] but it remains controversial whether $NO^{\bullet}$ plays a protective, an injurious, or both roles in pathogenesis of MS.[103] In the BBB, CEC form $NO^{\bullet}$ via endothelial $NO^{\bullet}$ synthase (eNOS, NOSIII) and may decrease or increase BBB. CEC sense $NO^{\bullet}$ by a cGMP-pathway that may phosphorylate junction associated proteins (like VASP),[104] which strengthen paraendothelial barrier and limits endothelial permeability. Although by scavenging oxidants NO may also serve as an important antioxidant defense in CEC,[105] several reactions between oxidants and $NO^{\bullet}$ can lead to formation of highly reactive species, like peroxynitrite, which disintegrate the BBB.[106] The induction of iNOS by cytokines in several cells within the BBB could release sufficient $NO^{\bullet}$ to form toxic levels of $NO^{\bullet}$ metabolites that disrupt the BBB.[107]

Therapeutic implications

The current treatments for MS, glatiramer acetate, IFN-β, affect the cytokine milieu through different mechanisms and result in a shift in cytokine pattern from proinflammatory to anti-inflammatory. IFN-β may also have beneficial effects on the BBB and on the trafficking of the activated leukocytes into the CNS. However, such effects have not been fully investigated yet. New experimental therapies for MS have been proposed, which restore the normal cytokine balance, block transendothelial migration

Table 1 Current available and experimental treatments for MS with effects on the blood brain barrier

Treatment strategy	*Target in the BBB*
Adhesion molecules	Antiadhesion molecule(s) monoclonal antibodies (Antegren: anti-VLA-4 monoclonal antibody)
	Interferons (β1a and β1b)[110]
	Corticosteroids[111]
Matrix metalloproteinase(s)	Matrix metalloproteinase inhibitors[112,113]
	Tissue inhibitor of matrix metalloproteinase (TIMP)[114]
	Interferons[115]
Chemokines/cytokines	Corticosteroids[116]
Nitric oxide synthase	Nitric oxide synthase inhibitor(s)[117]

of activated leukocyte, and/or endothelial adhesive determinants, all of which apparently help to restore or stabilize the BBB (Table 1). Monoclonal antibodies are a new promising class of drugs that can potentially alter the future of MS treatment. These monoclonal antibodies against adhesion molecules block binding interactions of these molecules to their ligands. Natalizumab (Antegren), a humanized α4 integrin antibody, has been used in clinical trials for treatment of patients with RRMS.[108,109] Natalizumab binds α4 integrin on the surface of activated lymphocytes and monocytes effectively and blocks their adhesion to VCAM-1 and MAdCAM-1 on activated endothelium. This effect potentially impairs leukocyte trafficking across the failing endothelial barrier in MS patients. Our expanding knowledge of the biochemical and cellular events which drive MS (and other chronic inflammatory states) should help improve existing therapies and aid in the design of safer and more effective treatments for MS.

Acknowledgements

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