Multiple sclerosis as a vascular disease

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Multiple sclerosis (MS) has traditionally been viewed and researched as an immune-mediated disease with principal emphasis on the role of activated inflammatory cells, oligodendrocytes and astrocytes in its pathogenesis. Abnormalities of cerebral endothelial cells (CECs) is an under explored facet of MS pathogenesis and vascular abnormalities play a crucial role in formation of the MS lesions and disease progress, at least in the initial stages of disease. This review will focus on MS as a central nervous system (CNS) disease with a strong vascular constituent and examines abnormalities within CECs in MS and their role in the loss of blood–brain barrier and transendothelial migration of activated leukocytes into the CNS. One goal of this paper is to persuade and promote research on the endothelial abnormalities in pathogenesis of MS and to exploit existing knowledge on endothelial injury. A deeper understanding of endothelial pathophysiology in MS may help develop effective treatments through stabilization of endothelial function, translating into delay or arrest of MS disease onset and disability in MS patients. [Neurol Res 2006; 28: 230–235]

Keywords: Multiple sclerosis; blood–brain barrier; cerebral endothelial cells; transendothelial migration

INTRODUCTION

Multiple sclerosis (MS), the most common cause of neurological disability in young individuals, remains enigmatic. Despite major advances in neuroimmunology and molecular biology, it is unclear why some patients develop clinically asymptomatic MS with a less aggressive course, while others experience massively devastating relapses, which leave them with permanent neurological deficits. In the 21st century, despite major achievements in exploring the pathogenic mechanisms of MS, we still ask: can we elucidate the molecular basis of relapses and remissions? Can we integrate the roles of CD4+ T lymphocytes, macrophages, cerebral endothelial cells (CECs), oligodendrocytes-myelin complex, cytokines and chemokines in pathogenesis of MS? It is a fact that MS is more than ‘an inflammatory demyelinating process affecting mainly subcortical white matter of the central nervous system (CNS)’. Injury in the context of MS extends beyond the oligodendrocyte/myelin complex affecting other cells such as CECs and neurons. However, our knowledge of the role of CECs in the inflammatory phase of MS has been only recently improved owing to major advances in neuroimmunology of MS and development of disease modifying drugs. With the introduction of monoclonal antibodies for treatment of MS, the role of CECs in pathogenesis of MS became more prominent and endothelial cells have been looked upon as potential therapeutic targets in MS.

Here we focus on MS as a vascular disease. The reason for such an explicit manuscript is to clearly show the role of CECs as the doorway for trafficking inflammatory cells to provoke the flood of cytokine and chemokines within the CNS. The concept of endothelial dysfunction in MS is not new but is certainly under-investigated. We hope to share our inclination that endothelial cells are key elements in MS pathogenesis and consequently to promote vascular targeting as the next frontier for the treatment of this incurable condition.

CECS: UNIQUE FEATURES

The blood-brain barrier (BBB) creates and separates the peripheral and the CNS circulations, establishing a normally impermeable barrier to proteins, cellular elements of periphery, peptides and majority of the circulating substances in blood. Three cellular elements of cerebral microvasculature create the BBB: CECs, astrocytes and pericytes. Of these, CECs are the most well studied. CECs possess structural features which set them fundamentally apart from other types of human endothelial cells. CECs lack fenestrations, have sparse pinocytic vesicular transport and possess a wide network of tight junctions (TJs) with high electrical resistance. TJs restrict the paracellular flux of hydrophilic molecules. However, lipophilic molecules (CO2 and O2) diffuse freely across the BBB along their concentration gradients. Electron microscopic examination of TJs has demonstrated them as a group of continuous, anastomosing intramembranous fibrils with apparent fusion of the...
outer leaflets of plasma membrane of adjacent CECs (Figure 1). Ultrastructurally, TJs contain three major classes of membrane proteins: claudins, occludin and the junction adhesion molecules, attached to groups of cytoplasmic linkage proteins such as zonula occludens (ZO)-1, ZO-2, ZO-3, cingulin, etc. Of the three structural proteins of TJs, occludin is probably the most well studied. Occludin is a 65 kDa phosphoprotein with four transmembrane domains, a long COOH-terminal cytoplasmic domain and a short NH2-terminal cytoplasmic domain. The paracellular barrier of the TJs is created by the two extracellular loops of occludin and claudin originating from opposed CECs. Because occludin is expressed at higher levels in human CECs compared with those outside of the CNS, it has been proposed that occludin is a major contributor to the maintenance of the BBB.

**MS is an immune-mediated disease**

Pathologically, MS is characterized by demyelination, the presence of peri-venular activated leukocytes, astrogliosis, loss of the oligodendrocyte/myelin complex and neurodegeneration. The prevailing hypothesis of MS pathogenesis suggests that following exposure to certain, still as yet unknown, environmental antigen(s), in genetically susceptible individuals, CD4+ T cells become activated towards myelin basic protein, myelin oligodendrocyte glycoprotein or proteolipid protein complexes, triggering a massive inflammatory cascade which eventually leads to transendothelial migration of activated leukocytes and macrophages from the vascular space into the brain and intermittently, a continuous, vicious cycle of tissue destruction within the CNS. These antigen(s) (the strongest candidate are members of myelin basic protein family) are presented to the CD4+ T cell receptor in the context of human lymphocyte antigen (HLA) class II molecules expressed by antigen presenting cells. It has been shown that under inflammatory conditions such as MS, CECs can also act as antigen-presenting cells (APCs) and present antigens to activated lymphocytes. Activated CD4+ T cells and

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*Figure 1: Upon activation by myelin basic protein and related antigen(s) Th1-CD4 lymphocytes release pro-inflammatory cytokines such as interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha. CECs, exposed to these inflammatory mediators, undergo certain biological changes such as release of endothelial microparticles carrying various adhesion molecules from their ‘parent’ cells, loss of solute barrier and disintegration of tight junction owing to a loss of occludin and cadherins. Together these events promote transendothelial migration of activated leukocytes from the periphery to the CNS.*
macrophages (the major cellular players in pathogenesis of MS) along with B cells migrate through the disrupted BBB and enter the CNS. There they are exposed to more members of myelin basic protein family as potential antigens, which in turn promotes and enlarges inflammatory response (epitope spread). The entry of activated leukocytes through a disrupted BBB into the CNS occurs through a number of stages: capture, rolling, arrest/adhesion, opening of CECs junctions, degradation of extracellular matrix and transendothelial migration of activated leukocytes. The focus of this review is the role of CECs in each of these steps in the pathogenesis of MS. The initial capture of tethering of leukocyte represents the first step in leukocyte-endothelium interaction and requires close approach of leukocytes to the underlying endothelium. Leukocytes then roll along the underlying endothelial layer once the selectin-type molecular bonds are formed. Rolling is not a metabolically active process and can continue even during endothelial metabolic inhibition. Based on the studies of the leukocyte-endothelial cell interactions (mostly using neutrophils) capture and rolling are necessary for firm adhesion of leukocyte to the underlying endothelium. In peripheral endothelial beds, selectins are involved in rolling of activated leukocyte over the underlying endothelium and are also expressed by naive T and B cells and macrophages. Selectins are expressed by both leukocytes (L-selectin) and endothelial cells (P-selectin and E-selectin). Interestingly, the role of selectins in the pathogenesis of leukocyte-CECs interactions in MS remains controversial. Indeed, involvement of P-selectin glycoprotein ligand 1 (PSGL-1) and its ligand on CECs, P-selectin, in the process of transendothelial migration of activated leukocytes in pathogenesis of MS, has been challenged. Engelhardt et al.\(^5\) studied transendothelial migration of encephalogenic T cells in SJL and C57BL/6 mice with experimental autoimmune encephalomyelitis (EAE). The investigators reported that expression of PSGL-1 by activated encephalogenic T cells was not involved in T cell migration and neither antibodies against PSGL-1 nor the lack of PSGL-1 (in PSGL-1-deficient mice) blocked the recruitment of inflammatory cells across the BBB, therefore, PSGL-1 may not be required for transendothelial migration of activated leukocytes in EAE or MS.

Firm adhesion of activated leukocyte to the activated CECs involves expression of integrins, a process which is promoted by chemokines. Of the integrins involved in pathogenesis of MS, \(\alpha_4\beta_1\)-integrin is perhaps the most significant one. \(\alpha_4\beta_1\)-integrin which is expressed on the surface of activated lymphocytes binds to its ligand on the endothelial cells (vascular cell adhesion molecule-1/VCAM-1). Platelet endothelial cell adhesion molecule-1/PECAM-1, a highly N-glycosylated Ig-family membrane protein in endothelial cells and platelets, is involved in regulation of extravasation of activated leukocytes. Elevated serum levels of both soluble and insoluble PECAM-1 in MS patients\(^6\) have been reported, but whether this reflects active extravasation is not clear.

ENDOTHELIAL JUNCTIONAL ABNORMALITIES IN MS PATHOGENESIS

Serum pro-inflammatory cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\), which are elevated before clinical exacerbations of MS and can affect CECs and alter CNS endothelium barrier function through a number of mechanisms. A decrease or inhibition of expression of endothelial junctional proteins may play a significant role in this abnormal situation. Using an in vitro model, Minagar et al.\(^7\) considered the effects of serum from MS patients with active disease on the expression of the junctional proteins, occludin and vascular endothelial (VE)-cadherin in cultured endothelial cells. Sera from MS patients in exacerbation, remission, and normal controls were incubated with cultured endothelial cells and the expression of occludin and VE-cadherin were measured by immunoblotting. The study found that sera from MS patients in exacerbation reduced the expression of occludin and VE-cadherin by brain endothelial cells and this effect was more pronounced for occludin expression. The study concluded that elevated serum levels of pro-inflammatory cytokines in MS patients during exacerbation provoked down-regulation of VE-cadherin and particularly occluding. This was later demonstrated using IFN-\(\gamma\).\(^8\)

In another study using single and double immunofluorescence, Kirk et al.\(^9\) quantified the uneven distribution of tight junction pathology and its association with BBB disruption in MS. The investigators studied frozen sections from plaques and normal-appearing white matter in 14 MS patients versus six patients with other neurological disorders and five normal controls. The tight junction-associated protein ZO-1 was assessed across tissues from these three groups in relation to fibrin leakage. The investigators reported significant differences in the incidence of tight junction abnormalities which were detected between different lesion types in MS and control white matter. These abnormalities of TJs were more prominent in active MS lesions and also concluded that TJs disintegrate in MS, promoting BBB leakage.

Another abnormality within CECs during MS involves the endothelial basement proteins, which can play a role in the flux and transport of leukocytes. van Horssen et al.\(^10\) characterized the molecular composition of the endothelial and astroglial basal membranes in chronic active and active MS lesions. That group observed differential expression of specific laminin chains in endothelial and astroglial basement membranes and detected the presence of fibrous-like depositions of extracellular matrix inside inflammatory cuffs. These fiber-like depositions showed immunoreactivity for a number of laminin isoforms, fibronectin, collagen IV and heparin sulfate proteoglycans. Based on this description, the authors concluded that presence of basement membrane molecules in the inflammatory cuffs (and in close proximity to activated leukocytes such as myelin-laden macrophages) may act as conduit or tracks for myelin-containing macrophages supporting their movement into the CNS from the peripheral circulation.
Expression of HLA molecules (mainly class II and HLA-G, a non-classical major histocompatibility complex class I antigen) is another significant pathologic feature within CECs during active pathogenesis of MS. Under normal circumstances CECs do not express class II HLA molecules and minimally express HLA-G. However, during pathogenesis of MS these constitutive features of CECs are radically altered. van der Maesen\(^1\) and his colleagues examined brain biopsy specimens obtained from MS patients during acute demyelinating attacks for the expression of class II HLA molecules by CECs. Immunostaining and electron microscopic examination of biopsied brain tissues specimens revealed presence of HLA class II molecules which were localized to the cytoplasm of CECs. The investigators concluded that during acute demyelinating events of MS, CECs can express the HLA class II molecules acting as antigen presenting cells. Wiendl \(^{\text{et al.}}\)\(^{14}\) investigated the expression of HLA-G by cerebral tissue of MS patients. The investigators observed that in MS brains, HLA-G expression was significantly increased in acute and chronic active MS plaques as well as in perilesional areas and areas of normal appearing white matter. The investigators detected that CECs associated with microglial cells and macrophages were the primary source of expression of HLA-G. The investigators also found that Ig-like transcript 2 (ILT2) (a receptor for HLA-G) present in MS brain specimens supporting an inhibitory function for HLA-G, which down-regulates the deleterious effects of T cell infiltration to the CNS in pathogenesis of MS.

In another study exploring molecular mechanisms of human cerebral endothelial cells (HCECs) abnormalities under inflammatory stimulation, Franzen \(^{\text{et al.}}\)\(^{15}\) performed gene expression profiling of HCECs activated by TNF-\(\alpha\). The investigators applied Affymetrix gene arrays and proteomics (2D-gel electrophoresis and mass spectrometry) to assess the early changes in the HCECs stimulated by TNF-\(\alpha\). The experiments were duplicated with human umbilical vein endothelial cells (HUVECs) as a reference system. The investigators observed that HCECs and HUVECs responded similarly in regard to expression of adhesion molecules, chemotaxis, apoptosis and oxidative stress molecules. However, certain proteins such as nuclear factors NF-kB1 and NF-kB2, plasminogen activator inhibitor-1 and cofilin were expressed exclusively by the HCECs. The authors suggested that the genes activated by TNF-\(\alpha\) in HCEC activation (and perhaps in MS), involve the urokinase plasminogen activator system and the cytoskeletal rearrangement.

**MATRIX METALLOPROTEINASES IN PATHOGENESIS OF MS**

Matrix metalloproteinases (MMPs), a family of Zn\(^{\text{2+}}\)-dependent endopeptidases with >20 members, are involved in MS pathogenesis by disintegrating the BBB extracellular matrix, facilitating transendothelial migration of the activated leukocytes, an effect mediated by the increased release of pro-inflammatory cytokines such as TNF-\(\alpha\), and is involved in the breakdown of the myelin sheath\(^{16}\). Interestingly, these enzymes may also be involved in repair and regeneration within CNS\(^{17}\). It has been shown that in the pathogenesis of MS, MMPs are generated and secreted by both endothelial cells (constitutive expression of MMP)\(^{18}\) and activated leukocytes\(^{19}\). In MS patients with acute relapses, serum and CSF levels of MMP-9 are elevated and correlate with the degree of BBB disruption (documented by presence of contrast-enhancing lesions on brain MRI\(^{20,21}\)). By attacking the basal lamina of the CECs, secreted MMPs proteolytically disrupt the BBB. This production of MMP-9 by activated leukocytes is decreased by IFN-\(\beta\), while the production of TIMP-1 is increased\(^{22}\) suggesting MMPs as essential targets in interferon therapy.

**ENDOTHELIAL MICROPARTICLES AND THEIR SIGNIFICANCE IN PATHOGENESIS OF MS**

Diseased or stressed endothelial cells release small vesicles from their cell membrane known as endothelial microparticles (EMP). Because of their significance in inflammatory endothelial injury and in MS, in particular, assays for EMP as markers of inflammation have gained interest in basic and clinical science arenas. EMP are sub-microscopic membranous vesicles with a size \(<\)1.5 \(\mu\)m, released by endothelial cells under inflammatory conditions in response to activation by pro-inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\). Released EMP carry the parent endothelial cell adhesion molecules such as VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and PECAM-1 which can be measured by flow cytometry.

Minagar \(^{\text{et al.}}\)\(^{23}\) reported a significant elevation of EMP carrying PECAM-1/CD31+ (CD31 + EMP) during relapses of MS in contrast to remission. Jy \(^{\text{et al.}}\)\(^{24}\) further explored the role of EMP in pathogenesis of MS and assessed functional interactions between EMP and leukocytes. The investigators observed that EMP preferentially bound to monocytes and activated them. The number of EMP-monocyte complexes was significantly raised in MS patients in relapse compared with remission status and such elevated values demonstrated a strong correlation with presence of contrast enhancing lesions on brain magnetic resonance imaging (MRI). Using an in vitro model for transendothelial migration of monocytes through monolayers of CECs, Jy \(^{\text{et al.}}\)\(^{25}\) investigated the role of EMP in transendothelial migration of monocytes. The investigators used this model to study transendothelial migration of monocyte U937 cells through a monolayer of CECs in the presence of plasma from MS patients versus normal control subjects with and without EMP. The authors reported that MS plasma enhanced transendothelial migration of monocytes and pre-treatment of monocytes with EMP increased their migration through CECs monolayers. They also reported that EMP-monocyte complexes showed a higher rate of transendothelial migration versus monocytes alone, therefore not only are EMPs

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*Neurological Research, 2006, Volume 28, April*
markers of disease, but may be an important element driving MS activity.

**IFN-BETA AND THE BLOOD–BRAIN BARRIER**

The introduction of IFN-β (Betaseron, Rebif and Avonex) as treatments for relapsing-remitting MS has changed the natural course of disease and provided clinicians with new therapeutic tools. Application of IFN-β in the MS population has been associated with a decrease in the annual relapse rate and a reduction of development of new lesions on brain MRI scans in individuals. Adhesion of IFN-β to cell surface receptors initiates a number of signaling pathways with generation and secretion of a number of antiviral, anti-proliferative and immunoproliferative mediators. Here we will only focus on the interactions of IFN-β with CECs. Mechanisms of therapy involving members of the IFN-β family and CECs include: (1) stabilization of CECs; (2) decrease in release of EMP by CECs; (3) block of transendothelial migration of monocyte-EMP complexes and (4) maintained expression of junctional proteins. Kraus et al.²⁹, in an *in vitro* model, studied the stabilizing effects of IFN-β on barrier characteristics of brain endothelial cells. The authors investigated the possible direct effects of IFN-β on the BBB integrity using an *in vitro* BBB model in which brain endothelial cells in co-culture with astrocytes formed a tight permeability barrier for ⁴H-inulin and ¹⁴C-sucrose. The authors observed that removal of astrocytes from the coculture or alternately addition of histamine resulted in an increased paracellular permeability of small tracers across the brain endothelial monolayer. When IFN-β was present in the culture medium, the leakiness of the brain endothelial monolayer under both conditions was reduced.

Minagar et al.¹⁰ in another set of experiments observed that occludin structure and content in endothelial cells were decreased upon exposure of these cells to IFN-γ and that this process was blocked by members of IFN-β family. In addition, the investigators observed that members of IFN-β family (IFN-β₁b and -β₁a) alone increased expression of occludin by cultured endothelial cells. Using a murine CECs line, Harzheim et al.²⁶ studied the expression of N-cadherin and vinculin by immunofluorescence staining and Western blot. The investigators first incubated the CECs with IFN-γ and observed decreased expression of these intercellular adhesion molecules. Then they treated CECs with IFN-β₁a and observed increased expression of both molecules. Lastly, they observed that combined treatment of murine CECs with both interferons did not affect the expression of N-cadherin and vinculin. They concluded that up-regulation of expression of junctional N-cadherin and vinculin in CECs by IFN-β₁a may contribute to the beneficial effects of IFN-β in treatment of MS.

In another set of experiments, Jimenez et al.²⁷ explored the effects of IFN-β₁b on the release of EMP and the transendothelial migration of monocytes and monocyte:EMP complexes. The investigators pre-incubated CEC cultures of IFN-β₁b before addition of plasma obtained from MS patients. The MS patients’ plasma was added initially to stimulate generation of EMP by CEC cultures. Three EMP phenotypes, CD₅₄, CD₆₂E and CD₃₁ were assayed. Plasma specimens from 20 patients with relapsing remitting MS (11 in exacerbation, MS-E, and nine in remission, ME-R) and ten healthy controls were applied. Incubation of CEC cultures with plasma from MS patients during exacerbation generated elevated levels of EMPCD₅₄, EMP₆₂E and EMP₃₁ relative to plasma from MS patients in remission and those of normal controls. Only plasma from MS patients who were in exacerbation augmented transendothelial migration of monocytes. Monocyte:EMP complexes further increased transendothelial migration of monocytes, but only in the presence of plasma from MS patients in exacerbation. The presence of IFN-β₁b inhibited transendothelial migration of monocytes and monocyte:EMP complexes by 20 and 30%, respectively. IFN-β₁b inhibited both release of EMP and transendothelial migration of monocytes, suggesting a role of EMP and a novel therapeutic mechanism for IFN-β₁b in MS.

**OTHER POSSIBLE MECHANISMS OF CECs ABNORMALITIES IN MS**

Anatomic studies have demonstrated that microfilaments of the endothelial cells strengthen junctional bond strength. Pro-inflammatory cytokines reorganize the cytoskeleton of endothelial cells, often with deleterious effects on endothelial barrier function. Oxidative and nitrosative stress also affect endothelial cells adversely and contribute to barrier failure in the context of inflammatory conditions, but oxidant mediated BBB dysfunction, while intriguing, has not yet been investigated in depth in MS.²⁸

**CONCLUDING REMARKS**

In conclusion, MS definitely shows many of the characteristics associated with vascular inflammatory phenomena, underscoring significant roles of the cerebral vascular system, cerebral vascular inflammation and defects with the blood–brain barrier in active MS. The well documented immune components of MS must interact with the vascular system in introducing and maintaining the cells which drive the inflammatory response and involve the expression of adhesive determinants (under the control of unbalanced cytokines), their penetration and destruction of the BBB and the development of the MS lesion, all of which may be future targets with immunomodulating drugs and regulatory cytokines.

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