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SYMPOSIUM: Role of Inflammation Following Stroke and Neurotrauma

Inflammatory Mediators of Cerebral Endothelium: A Role in Ischemic Brain Inflammation

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Brain inflammation has been implicated in the development of brain edema and secondary brain damage in ischemia and trauma. Adhesion molecules, cytokines and leukocyte chemoattractants released/presented at the site of blood-brain barrier (BBB) play an important role in mobilizing peripheral inflammatory cells into the brain. Cerebral endothelial cells (CEC) are actively engaged in processes of microvascular stasis and leukocyte infiltration by producing a plethora of pro-inflammatory mediators. When challenged by external stimuli including cytokines and hypoxia, CEC have been shown to release/express various products of arachidonic acid cascade with both vasoactive and pro-inflammatory properties, including prostaglandins, leukotrienes, and platelet-activating factor (PAF). These metabolites induce platelet and neutrophil activation and adhesion, changes in local cerebral blood flow and blood rheology, and increases in BBB permeability. Ischemic CEC have also been shown to express and release bioactive inflammatory cytokines and chemokines, including IL-1β, IL-8 and MCP-1. Many of these mediators and ischemia *in vitro* and *in vivo* have been shown to upregulate the expression of both selectin and Ig-families of adhesion molecules in CEC and to facilitate leukocyte adhesion and transmigration into the brain. Collectively, these studies demonstrate a pivotal role of CEC in initiating and regulating inflammatory responses in cerebral ischemia.

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Introduction

In the majority of acute stroke patients only a small area of the affected brain tissue, the ischemic core, is irreversibly damaged at the initial onset of stroke. A much larger volume of the brain tissue surrounding the ischemic core, known as penumbra, has the potential to recover most of its functions under favorable conditions provided by therapeutic intervention. Therefore, the final outcome of stroke is not determined solely by the volume of the ischemic core, but also by the extent of secondary brain damage inflicted to penumbral tissues by brain swelling, impaired microcirculation, and inflammation (87). Secondary brain damage typically develops after a delay of hours or days and has been observed after ischemia, trauma or subarachnoidal haemorrhage (60). Although the pathophysiological mechanisms of such damage are not clearly understood, recent studies implicate brain inflammation as an important component of this process (31, 32, 38, 39, 50).

Brain inflammation in cerebral ischemia is believed to develop as a consequence of two sequential, but closely linked processes: *i)* the activation of microglia and resident perivascular/parenchymal macrophages (47), and *ii)* the mobilization and infiltration of peripheral inflammatory cells into the brain (13, 38). The activated glia has been shown to produce a myriad of pro-inflammatory mediators (120) effecting molecular and phenotypic changes of cerebral endothelium that then orchestrate peripheral leukocyte recruitment into the brain (32).

The development of postischemic brain inflammation is co-ordinated by the activation, expression, and secretion of numerous pro-inflammatory genes/mediators from both brain parenchymal and vascular cells. Brain microvascular endothelium, a highly specialized endothelial tissue that performs the function of blood-brain barrier (BBB) (95), appears to be an important responsive and regulatory component of cerebral inflammation. Secretion of vasoactive/ inflammatory

Stimulus	Adhesion molecule	Model	Reference
IL-1β	ICAM-1	human CEC in culture	116, 118, 128 , 136
	VCAM-1		129
	E-selectin		130
TNFα	ICAM-1	human CEC in culture	79, 116, 118
	VCAM-1		79, 118
	E-selectin		79, 118
LPS	ICAM-1	human CEC in culture	80, 128
	VCAM-1		129
	E-selectin		80, 130
IFN-γ	ICAM-1, VCAM-1, E-selectin	human CEC in culture	80
ET-1, ET-2, ET-3	ICAM-1, VCAM-1, E-selectin	human CEC in culture	79, 80
Phorbol ester	ICAM-1	human CEC in culture	116
Hypoxia/Ischemia	ICAM-1	human CEC in culture	55, 58, 116, 118
		human brain tissue	70
	VCAM-1, E-selectin	human CEC in culture	118
Brain tumors	ICAM-1	human brain tissue	97

Table 1. The expression of adhesion molecules in human cerebral endothelial cells (CEC) in response to various stimuli.

mediators by cerebral endothelium during ischemia may lead to microvascular vasoparalysis, abnormal blood rheology, and additional clotting of microcirculation, thus exacerbating initial ischemic damage. This article reviews knowledge accumulated thus far on the ability of cerebral endothelial cells to respond to pro-inflammatory stimuli, to produce/express active mediators of inflammation under ischemic conditions, and to regulate infiltration of peripheral inflammatory cells into the brain.

Cerebral endothelial cell adhesion molecules

The process of leukocyte recruitment into injured tissues is believed to begin with the 'activation' of endothelial cells by inflammatory mediators resulting in *i)* rapid secretion of P-selectin from Wiebel-Palade bodies followed by *ii)* sequential transcriptional induction of E-selectin and immunoglobulin family of cellular adhesion molecules (CAMs) (for review see ref. 22 and 63). In most vascular beds, selectins have been shown to mediate initial attachment and 'rolling' of leukocytes along vessel walls, whereas a firm adhesion is produced by the interaction of leukocyte integrins with CAMs expressed on endothelial cells (22).

Similarly, adhesion molecules that mediate cell-cell and cell-matrix interactions in the cerebrovasculature have emerged as key players in leukocyte-endothelial cell interactions induced by ischemia (29, 50, 63). However, some ostensible differences have been observed in cerebral endothelial cell responses. For example, while P-selectin is rapidly externalized by thrombin and histamine from storage granules called Weibel-Palade bodies in peripheral microvascular beds, it appears that constitutive P-selectin expression is largely absent in cultured murine brain microvascular endothelial cells (12) but can be transcriptionally up-regulated by cytokines IL-1β and TNFα (12). Also, leukocyte recruitment across the BBB in experimental autoimmune encephalomyelitis appear not to involve either E- or P-selectin (36). Nevertheless, both selectin- (88, 126) and CAM-family adhesion molecules are up-regulated in microvessel walls in ischemic brain (88, 126, 127).

Regulation of adhesion molecule expression has been extensively studied in cultured cerebral endothelial cells (CEC) from various species, including human. Cultured human CEC have been shown to up-regulate adhesion molecules from both selectin- and CAM- families in response to cytokines, bacterial lipopolysaccharide, phorbol ester, and neuropeptides (summarized in Table 1). We have recently shown that hypoxia and simulated *in vitro* ischemia (oxygen-glucose deprivation; OGD) trigger a 'pro-inflammatory' activation of human CEC resulting, among other changes, in the up-regulation of adhesion molecules ICAM-1 (116), VCAM-1 and E-selectin (118) along with the ICAM-1/CD18 dependent adhesion of allogenic neutrophils to endothelial monolayers (116). Moreover, the exposure of either peripheral (44) or brain (35) endothelial monolayers to cytokines

has been shown to facilitate neutrophil transmigration across endothelial barrier *in vitro*.

Hypoxia *in vitro* has been shown to cause a significant imbalance in cerebral endothelial cell redox status (i.e, GSSG/GSH ratio) (86). Parenthetically, pharmacologically induced redox imbalance in endothelial cells has been shown to trigger a transcription-independent and transcription-dependent surface expression of different endothelial cell adhesion molecules, including E- and P-selectins and ICAM-1, followed by a two-phased neutrophil-endothelial adhesion response (64). Recent studies suggest that leukocyte adhesion to cerebral endothelial cells induces ICAM-1 cross linking and the activation of Rho-linked signaling pathway(s) resulting in phosphorylation of endothelial cytoskeletal proteins and induction of transcription factors (37). This signaling cascade likely leads to transcriptional regulation of target genes and endothelial cell shape changes necessary for leukocyte migration through the BBB (37).

Experiments in animal stroke models have demonstrated ICAM-1 up-regulation in microvessel walls and have positively correlated neutrophil brain infiltration with the levels of ICAM-1 expression (13, 77, 107, 126, 127). Recently, ICAM-1 up-regulation has also been demonstrated in tissue sections of human brains from stroke patients (70). In addition to ICAM-1, increased expression of VCAM-1 (126), E-selectin (126), and P-selectin (88) has been observed in brain capillaries and microvessels in stroke models.

The significance of adhesion molecule-mediated leukocyte infiltration into ischemic brain tissue for the development of brain damage has been corroborated in experiments showing that: *i)* depleting circulating neutrophils (i.e., neutropenia) reduces infarct volume and edema in various experimental models of cerebral ischemia (23, 77, 107); *ii)* the administration of an anti-ICAM-1 antibody (19), as well as antibodies against leukocyte-expressed adhesion molecules (24, 26, 77) to experimental animals before and/or after ischemia limits leukocyte infiltration into the brain and decreases infarct size and brain swelling, and *iii)* transgenic ICAM-1-deficient mice develop smaller infarcts than their wild-type counterparts (109).

However, despite significant evidence accumulated from both *in vitro* and *in vivo* studies suggesting a pathogenic role of neutrophil infiltration in stroke damage, the potential efficacy of strategies designed to reduce the expression of adhesion molecules on CEC to or block CEC/leukocyte interactions in clinical treatment of stroke remains to be validated.

Inflammatory mediators expressed/released by cerebral endothelial cells

Cerebral endothelial cells are an important source of vasoactive, permeabilizing and pro-inflammatory mediators that are key local regulators of microcirculation, blood cell activation, and blood rheology under both physiological and stress-conditions. These mediators target local environment via both paracrine and autocrine pathways and are believed to play a key role in the development of microcirculatory stasis and the BBB breakdown following stroke. The participation of some of these mediators in ischemia-induced brain inflammation is reviewed below.

Autocoids. *Prostaglandins.* Eicosanoids are bioactive metabolites of arachidonic acid and affirmed mediators of inflammation and circulatory disorders (99). The arachidonic acid cascade is initiated by the activation of phospholipases A_2 (PLA_2) and C (PLC), brought about by various receptor-agonist interactions, increases in free cytosolic calcium, and/or ischemia (43, 48, 99). Arachidonic acid released from brain phospholipids during ischemia/reperfusion is a major source of free radicals and a putative mediator of the BBB disruption and brain edema (1, 48). Once produced, arachidonic acid is converted by cyclooxygenase (COX) to prostaglandin H_2 (PGH_2) which is further metabolized to a variety of prostaglandins, prostacyclin, and thromboxane A_2 (43). COX possesses both cyclooxygenase and hydroperoxidase (i.e., radical-generating) activities, and is inhibited by aspirin (ASA) and nonsteroid anti-inflammatory drugs (43). Two COX isoforms were recognized at the molecular level - COX-1 which is constitutively expressed in many cell types, including endothelial cells, and is responsible for the production of prostaglandins under physiological conditions, and COX-2 which is rapidly inducible by proinflammatory stimuli, including mitogens, cytokines, and lipopolysaccharide, in cells *in vitro* and at inflamed sites *in vivo* (43, 74).

While prostaglandins occur naturally in the brain, their concentrations are maintained at very low levels under normal conditions. However, both prostaglandin synthesis and levels have been shown to dramatically increase concentration in the brain during cerebral ischemia/reperfusion (30). In addition to various other functions in the brain, members of the eicosanoid family have been shown to exert potent and often opposing vasomotor effects (eg., vasoconstriction by TxA_2, $PGF_{2\alpha}$, and vasodilation by PGI_2, PGE_2, and PGD_2), to increase microvascular and BBB permeability (17, 30), and to act

as chemoattractants, primers, or activators of neutrophils (99).

It has become apparent that endothelial cells derived from various microvascular beds express different regulatory enzymes of the arachidonic acid cascade, and thus secrete different eicosanoid profiles. Cerebral endothelial cells were found to express and up-regulate COX-2 mRNA in response to inflammatory stimuli, including cytokine IL-1β and lipopolysaccharide (21). In peripheral endothelial cells, both COX-2 expression (105) and prostaglandin synthesis (83) are up-regulated by hypoxia. Unlike peripheral microvascular endothelium that secretes high levels of PGI_2, the predominant vasoactive eicosanoid released from cultured primary and immortalized rat brain endothelial cells was shown to be PGE_2 (62), whereas human cerebral capillary endothelial cells were found to secrete high levels of PGD_2 and to express the metabolic machinery for its conversion into vasoconstrictive 9α, 11β-prostaglandin F_2 (112, 115). The vasoconstrictive peptides, endothelin-1 (ET-1), arginine-vasopressin (AVP), and angiotensin II (Ang-II) have been shown to induce the release of arachidonic acid and the secretion of various prostaglandins from human cerebromicrovascular endothelial cells in culture (110, 111, 115).

Leukotrienes. Leukotrienes are bioactive compounds generated by the 5-lipoxygenation of AA to 5(S)-hydroperoxy-eicoateraenoic acid (5-HPETE), which is subsequently dehydrated by the same enzyme to an unstable epoxide, leukotriene A_4 (LTA_4) (99). LTA_4 is either enzymatically hydrolyzed to the potent neutrophil chemoattractant, LTB_4, or is conjugated with glutathione (GSH) by the action of glutathione-S-transferase (GST) to yield pro-inflammatory LTC_4 (99).

Leukotrienes play multiple roles as mediators of inflammation and allergy in various tissues (99). In peripheral microvascular beds, the peptidoleukotrienes exert two simultaneous effects: they constrict the microvessels, but also cause plasma leakage in capillaries and associated postcapillary venules (99). Peptidoleukotrienes have also been shown to induce endothelium-dependent contraction of cerebral arteries and arterioles (78, 96). However, the putative role of these compounds in mediating BBB permeability and brain edema is still controversial.

In most studies, intracarotid injection, brain superfusion, or intraparenhimal injection of higher than physiological (i.e., 10^{-9}-10^{-8} M) concentrations of peptidoleukotrienes did not result in significant BBB disruption (6, 7, 17). However, a correlation between LTC_4 content and BBB breakdown has been shown in human brain tumors (7, 17, 18) and the peritumoral edema was attenuated by lipoxygenase inhibitors (7). Moreover, intracarotid infusion of low doses of LTC_4 opened the BBB in peritumoral areas (7) and increased BBB permeability when administered 72 hours after permanent middle-cerebral artery occlusion (MCA-O) in rat (6).

The rapid breakdown of LTC_4 to LTD_4 in brain capillaries occurs via γ-glutamyl transpeptidase (GGTP) (18, 41). GGTP is concentrated in the walls of brain capillaries and is notably absent in regions lacking BBB properties (41). It has been suggested that in normal brain capillaries, high levels of GGTP act as an enzymatic barrier protecting the BBB against permeabilizing effects of LTC_4 (17, 18). Indeed, in both tumor and ischemic tissue, in which leukotrienes act as BBB permeabilizers (6, 7), the affected cerebral capillaries were found to be depleted of GGTP activity (17, 18). We have recently shown that *in vitro* hypoxia/ischemia reduces GGTP activity in cultured human CEC and this renders the cells more vulnerable to permeabilizing actions of leukotrienes (86). Simultaneously, LTB_4 generated in this process is a potent neutrophil chemoattractant that facilitates neutrophil transmigration across the BBB.

The production/lmetabolism of leukotrienes has been demonstrated in the brain and the cerebral microvessels (68, 69) and the release of leukotrienes has been shown to increase during cerebral ischemia/reperfusion in animal models (85). However, it appears that cerebral endothelial cells, similar to peripheral endothelium (25), lack 5-lipoxygenase to generate leukotrienes from arachidonic acid (unpublished observation). In peripheral endothelium, peptidoleukotriene A_4 (LTA_4) is delivered to the endothelial cells transcellularly by transiently activated neutrophils (25, 73) and is then processed further to inflammatory LTC_4 by endothelial enzymatic machinery. It is reasonable to suggest that a similar process likely occurs in cerebral endothelial cells stimulated by cytokines or ischemia to express adhesion molecules and interact with neutrophils. Therefore, the ischemic damage to the enzymatic barrier against leukotrienes and the production of pro-inflammatory and chemoattracting leukotrienes at the site of BBB may contribute to the development of the ischemic BBB breakdown and increased leukocyte infiltration into the brain.

Platelet-Activating Factor (PAF). Vascular endothelial cells have been shown to produce another potent lipid mediator of inflammatory reactions and thrombosis, platelet-activating factor (PAF). The molecular

structure of PAF is defined as 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine. PAF is produced by the hydrolysis of ether phospholipids *via* PLA_2 and is associated with the liberation of arachidonic acid that is further metabolized to prostanoids and leukotrienes as described in previous sections (81, 82). Interestingly, leukotrienes LTC_4 and LTD_4 have been shown to stimulate human endothelial cells to produce PAF and bind neutrophils (81), closing a loop likely involved in augmenting inflammatory actions of various arachidonic acid metabolites.

Satoh and colleagues have recently found that cultured porcine cerebral endothelial cells produce PAF in response to the stimulation with calcium ionophore A23187 and thrombin and bradykinin agonists (101, 102; Table 2), compounds that have previously been shown to stimulate PAF production in umbilical vein endothelial cells (82). Therefore, there is no apparent functional specificity of brain microvascular endothelial cells, as compared to the cells from large vessels, in regard to the production of PAF (102). Quantitatively, however, there is a large difference between brain microvascular endothelial cells and aortic or umbilical vein endothelial cells: the amount of PAF produced in porcine brain microvascular endothelial cells is roughly one fourth of that in aortic endothelial cells obtained from the same animal (102). Coincidentally, cerebral endothelium also produces a low amount of prostacyclin (102), an autocoid that exhibits opposing activities to PAF.

PAF is incorporated into membrane phospholipid bilayer and exerts its activities at the cell surface (138) in both peripheral and brain endothelial cells (93). PAF is vasoactive and vascular smooth muscle cells are important target for these actions. This led to the hypothesis that PAF is released from the basal surface of endothelial cells towards the medial smooth muscle layer. However, brain endothelial cells cultured on a collagen-based cell culture insert did not release PAF into either upper or lower compartment separated by the insert (101).

Zimmerman and associates (72, 92, 138) have demonstrated that endothelial PAF production is coupled with the cell surface expression of P-selectin. Agonists known to stimulate PAF production in endothelial cells also stimulate fusion of Weibel-Palade bodies with the plasma membrane leading to the externalization of P-selectin. PAF expressed on the endothelial cell surface activates leukocytes tethered by the action of P-selectin (56). The initial leukocyte activation by PAF is essential for subsequent firm adhesion of leukocytes mediated by ICAM-1 (4). We have demonstrated that the adherence of polymorphonuclear neutrophils to bradykinin-stimulated brain endothelial cells is, at least partly, mediated by PAF (102). Again the adherence was much lower in brain endothelial cells as compared to aortic endothelial cells obtained from the same animal (102). Hypoxia has been shown to induce neutrophil adhesion to peripheral endothelial cells in a PAF-dependent manner (4). PAF then primes adherent neutrophils for enhanced production of free radicals and increased release of arachidonic acid (56). Both these compounds have been shown to increase permeability of brain endothelial cell monolayers (117, 121) and cause breakdown of the BBB (17).

Autocoid	Stimulus	Model	Reference
Arachidonic acid	Angiotensin II	Human CEC	110
	Arginine vasopressin		110
	Hypoxia		111
PAF	Bradykinin	Porcine CEC	101, 102
	Calcium ionophore		
6-keto-$PGF_{1\alpha}$	LPS	Rat CEC	33
	IL-1β		
	IL-6		
	Bradykinin	Porcine CEC	101, 102
	Calcium ionophore		
	ET-1	Human CEC	115
	Phorbol ester		115
	Angiotensin II		110
	Arginin-vasopressin		110
TxB_2	ET-1	Human CEC	115
	Phorbol ester		115
	PGD_2		112
	Angiotensin II		110
	Arginin-vasopressin		110
$PGF_{2\alpha}$	ET-1	Human CEC	115, 119
	Phorbol ester		115
	PGD_2		111, 112
	Angiotensin II		111, 112, 119
	Arginin-vasopressin		110, 111
PGE_2	LPS	Rat CEC	33
	IL-1β		
	IL-6		
	ET-1	Human CEC	115
	Phorbol ester		115
	Angiotensin II		110
	Arginin-vasopressin		110
PGD_2	ET-1	Human CEC	112, 115
	Phorbol ester		115
	Angiotensin II		110, 111
	Arginin-vasopressin		110, 111

Table 2. Autocoids release/expression in cultured cerebral endothelial cells.

The production/release of various autocoids from cultured cerebral endothelial cells in response to different stimuli is summarized in Table 2.

PAF acetylhydrolase is a plasma lipoprotein-associated enzyme that specifically inactivates PAF (113). Plasma PAF acetylhydrolase activity is reduced in diseases such as septic shock and acute myocardial infarc-

Stimulus	IL-1β		ICE
	RT-PCR (% control)	ELISA (pg/ml)	RT-PCR (% control)
Control	100	2.5 ± 0.4	100
100 u/ml TNFα			
4 h	568 ± 22*	n.d.	354 ± 42*
24 h	360 ± 32*	8.4 ± 0.6*	260 ± 35*
40 nM TPA			
4 h	672 ± 49*	n.d.	125 ± 18
24 h	432 ± 28*	n.d.	202 ± 13*
OGD (4 h)	252 ± 12*	4.2 ± 0.6	161 ± 12*
Recovery			
4 h	354 ± 45*	10.4 ± 1.8*	212 ± 41*
16 h	307 ± 84*	21.5 ± 4.2*	204 ± 32*
24 h	321 ± 64*	54.6 ± 13.4*	170 ± 36*

Values are means ± S.D. of 4 RT-PCR gels and 6 replicates in ELISA experiments. n.d. - not determined
Asterisks indicate a significant difference (ANOVA, P<0.01) from control values. Semi-quantitative RT-PCR was performed using β-actin as internal standard and the intensity of bands was estimated by densitometry.

Table 3. The expression of IL-1β and ICE in cultured human cerebral endothelial cells in response to cytokine TNFα, phorbol ester (TPA) and oxygen-glucose deprivation (OGD).

tion (59).

Plasma lipoproteins attenuate the adherence of polymorphonuclear leukocytes (PMNs) to endothelial cells and this activity is abolished by the pretreatment of lipoproteins with an inhibitor of PAF acetylhydrolase (92). Plasma PAF acetylhydrolase activity is increased in stroke patients as compared to healthy control population (103). Experimental findings suggested that the higher PAF acetylhydrolase activity seen in stroke patients might be due to the enhanced PAF generation in stroke (100). Inherited form of plasma PAF acetylhydrolase deficiency is caused by a missense mutation in the gene encoding the enzyme (Val279Phe) (114). A higher prevalence of this mutation was found in stroke patients than in healthy controls (57). The prevalence was almost equal in patients with ischemic and hemorrhagic strokes (132), but no linkage to hypertension could be established (132).

In summary, it appears that the endothelial PAF production is an important factor determining susceptibility to stroke and is involved in the pathogenesis of stroke and inflammation. Hence, PAF should be considered as a potential therapeutic target for attenuating consequences of stroke (65).

Cytokines. Ischemic brain has been shown to generate inflammatory cytokines (38, 39) and increased levels of both pro-inflammatory and anti-inflammatory cytokines have been detected in the cerebrospinal fluid of stroke patients (122). Cytokine actions in the brain have most often been considered a part of the damaging response (98). For example, both interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) have been implicated as mediators of ischemic brain injury (38, 39). However, inflammation has also been suggested to contribute to brain healing mainly through processes associated with the removal of damaged tissue (54, 98). In addition to directly affecting neurons and parenchymal brain cell, cytokines exert multiple actions on cerebral microvasculature, including cerebral endothelial cells.

Interleukin-1. The IL-1 family consists of IL-1α, IL-1β, two IL-1 receptors, and IL-1 receptor antagonist (IL-1Ra) (3, 28, 34). IL-1β, a 153 amino-acid protein of 17.5 kDa, is cleaved from the inactive, 269 amino-acid precursor, pro-IL-1β, by IL-1 converting enzyme (ICE) (28). ICE is a cysteine protease belonging to a family of proteolytic enzymes, caspases, implicated in apoptotic cell death (3, 42, 84). Increased levels of IL-1β and induced expression of ICE as well as other members of Ice gene family (5, 51) have been demonstrated in the animal brain after focal cerebral ischemia. Moreover, the inhibition of Ice family proteases (51), as well as ICE gene knockout in mice (104) have been shown to reduce excitotoxic and ischemic brain injury. IL-1β binds two types of receptors, a signaling type I (IL-1RI) receptor, and type II (IL-1RII) receptor, a decoy that inhibits IL-1 activity (3, 28, 34). Blockade of IL-1 receptor function by intracerebroventricular infusion of IL-1Ra has been shown to reduce infarct lesion, neuronal death and neurological deficit in experimental models of stroke (34, 45).

The presence of IL-1 receptors has recently been demonstrated in both rat (125) and human CEC (123, 137). Consistent with this, IL-1β has been found to potently stimulate pro-inflammatory activation of cerebral endothelial cells. As shown in Tables 1 and 2, IL-1β increases the expression of adhesion molecules and neutrophil adhesion to human CEC, and has also been found to induce COX-2 expression and the release of prostaglandins from cerebral vasculature (21). Recently, we have shown that IL-1β up-regulates the expression and the release of bioactive neutrophil chemoattractant, IL-8, and monocyte chemoattractant, MCP-1, in human CEC (136, Table 4). The pro-inflammatory activation of human CEC by IL-1β is shown to be receptor-mediated since it is prevented in the presence of IL-Ra (137). Therefore, the neuroprotection seen with IL-1Ra in *in vivo* models of cerebral ischemia (45) is likely in part mediated by IL-1Ra ability to reduce inflammatory activation of CEC.

In addition to being a target cell for IL-1β produced

Stimulus	IL-8		MCP-1		Chemotaxis
	RT-PCR (% control)	ELISA (pg/ml)	RT-PCR (% control)	ELISA (pg/ml)	(number of neutrophils)
Control	100	89 ± 6	100	192 ± 12	465 ± 24
100 u/ml IL-1β					
4 h	251 ± 32*	1980 ± 130*	168 ± 11*	2980 ± 110*	n.d.
24 h	360 ± 35*	31400 ± 2450*	227 ± 29*	9760 ± 434*	4240 ± 278*
OGD (4 h)	148 ± 24	75 ± 9	168 ± 40	222 ± 17	489 ± 38
Recovery					
4 h	170 ± 27*	225 ± 18*	154 ± 8*	710 ± 37*	1520 ± 72*
16 h	148 ± 15*	628 ± 54*	192 ± 15*	1265 ± 76*	n.d.
24 h	128 ± 34	697 ± 62*	198 ± 41*	1130 ± 55*	1480 ± 56*

Values are means ± S.D. of 4 RT-PCR gels, 6 replicates in ELISA experiments, and 4 replicates in chemotaxis experiments. Asterisks indicate a significant difference (ANOVA, P<0.01) from control values. Semi-quantitative RT-PCR was performed using β-actin as internal standard and the intensity of bands was estimated by densitometry. Chemotaxis of neutrophils was induced by media of cells treated as described using a ChemoTx #101-5 assay (Neuro Probe, Gaithersburg, MD). n.d..- not determined.

Table 4. Chemokine expression in human cerebral endothelial cells (HCEC) and neutrophil chemotaxis induced by chemoattractants released by HCEC in response to cytokines and oxygen-glucose deprivation (OGD).

by brain parenchymal or blood cells, CEC are a potentially important source of IL-1β. Rat brain vasculature has been shown to constitutively express genes encoding for interleukin-1 system (131) and cerebral vessels up-regulate IL-1β expression after focal brain ischemia (133). TNF-α, phorbol ester, and oxygen-glucose deprivation *in vitro* were found to up-regulate both ICE and IL-1β expression in human CEC (134; Table 3). Ice-like proteases have recently been implicated in hypoxia/reperfusion injury and death of peripheral endothelial cells (52). Increased release of IL-1β by hypoxic human CEC (Table 3) indicates a possibility of autocrine brain endothelial activation. In peripheral systems, hypoxia-induced endothelial cell synthesis and release of IL-1 results in an autocrine enhancement of adhesion molecule expression (108)

Tumor-necrosis factor α. TNF-α is synthesized as a 26-kDa polypeptide of 233 amino-acid residues and is predominantly present as a biologically active membrane-bound form (16). The membrane-bound form of TNF-α has been shown to be the prime ligand in activating the 80 kDa TNF receptor in various cell systems (16) including endothelium (89), thus acting as a local, site-restricted regulator of inflammatory response (16). The levels of TNF-α were found to increase in the brain of animals subjected to focal cerebral ischemia (39). In addition to glial cells (101), TNF-α can be released by the infiltrating myeloid cells (39, 98). TNF-α is found to stimulate the expression of adhesion molecules in peripheral endothelium (75) and adhesion molecules (Table 1) and chemokines in cultured cerebral endothelial cells (134, 136).

Chemokines. The events subsequent to neutrophil-CEC adhesion that lead to the transient opening of the BBB and neutrophil transmigration into the brain involve several putative mediators released by activated neutrophils, including free radicals, metalloproteases, perchloric acid, and eicosanoids. Various neutrophil chemoattractants released at the site of injury are thought to then provide the driving force for neutrophil movement across the barrier (9, 53, 94).

Chemokines, a family of 8-12kD peptides, have been shown to entice selective leukocyte recruitment at peripheral inflammation sites (9, 10). Chemokine selectivity to subpopulation of leukocytes is determined by the distribution of four cysteines in a highly conserved N-terminal domain, such as that α (CXC; prototypic member IL-8) chemokines primarily attract neutrophils, β (CC; prototypic member MCP-1) chemokines attract both monocytes and lymphocytes, γ (C; lymphotactin) chemokines draw lymphocytes, and δ (CX3C; neurotactin) both neutrophils and monocytes (9, 10). Recent studies have shown that chemokines are expressed in a variety of cells, including human peripheral (20, 61) and brain endothelium (134) and glial cells (134, 136). Recently, chemokine binding sites/receptors have also been visualized on human brain microvessels (2).

Chemokines have been suggested to play a role in the pathophysiology of brain injury accompanying autoimmune (94), post-traumatic (53, 94), and, more recently, post-ischemic brain inflammation (94). Increased levels

of chemokines (76, 94) have been detected in the ischemic rat brain, and systemic administration of anti-IL-8 antibody has been shown to reduce cerebral edema, BBB permeability, and infarct size in experimental models of stroke (76). We have recently shown that human CEC and human astrocytes express and release increased amounts of bioactive IL-8 and MCP-1 and likely other neutrophil chemoattractants when exposed to ischemia *in vitro* (136; Table 4).

The endothelial cell surface presentation of chemokines (9, 10) and the proximity of presented chemokines to target cells (10) may be a key trigger of molecular changes in target cells necessary for chemotaxis to occur, such as the expression of integrins and cytoskeletal rearrangements. Elaboration of chemokines, such as IL-8 and MCP-1, by sentinel cells may also be responsible for inducing/sustaining strong adhesive interactions between rolling leukocytes and endothelium under flow conditions (10, 46). Therefore, it is plausible to suggest that the expression/presentation of chemokines including IL-8 and MCP-1 and the release of chemoattractants, such as LTB_4, by cerebral endothelium is a decisive event in initiating transmigration of adhering leukocytes across the BBB.

Regulation/modulation of inflammatory gene expression in CEC

Paracrine regulation. Inflammatory activation of CEC in cerebral ischemia likely commences via signals activated by hypoxia/ischemia in CEC themselves and is then sustained/augmented by paracrine mediators released by neighboring parenchymal cells. Astrocyte end-feet envelope brain capillaries and close interactions between the two cell types have been implicated in the regulation of phenotypic properties of CEC (14). Astrocyte/endothelial interactions may also be important in regulating inflammatory responses at the site of BBB. Activated glia has been shown to produce numerous pro-inflammatory mediators (46, 120), including IL-1β (134, 137) and chemokines (134, 136), that are capable of inducing 'pro-inflammatory' activation of cerebral endothelium.

We have recently demonstrated that human astrocytes exposed to hypoxia *in vitro* express and release bioactive IL-1β (137). IL-1β appears to be a key paracrine mediator effecting pro-inflammatory activation of human brain endothelium (137). Human astrocytes subjected to oxygen-glucose deprivation *in vitro* also secrete chemokines IL-8 and MCP-1, as well as other neutrophil chemoattractants (134-136). Similarly, signals produced by smooth muscle cells have been shown to up-regulate the expression of COX-2 in cerebral endothelial cells (90).

These studies suggest that CEC and other cells of the microvascular bed situated in close proximity likely cooperate in attracting, marginalizing and mobilizing neutrophils and monocytes into the brain parenchyma during cerebral ischemia.

Pharmacological agents. Pharmacological manipulation of the expression/release of inflammatory mediators in the brain is being considered as a potential therapeutic approach to reduce secondary ischemic brain damage. Two major approaches to attenuate/control postischemic inflammation in the brain have been developed: a) targeting immune and inflammation mediators (i.e., adhesion molecules, cytokines, etc.) and b) immunomodulation (31, 54). The difficulty in the first approach is associated with a notorious redundancy of inflammatory mediators that function as a multi-level cascade. Consequently, targeting any singular player in this cascade can easily be circumvented by an alternate pathway and is likely to be ineffective in the clinical setting. The second approach aims to reduce overall immune response including that of resident brain microglia, thus targeting the cascade as a whole. Among various drugs attempted, glucocorticoids had no apparent clinical benefit (60), whereas some non-steroid anti-inflammatory drugs have been shown to be neuroprotective in experimental models of stroke (31, 60). Immunophilin-binding agents such as cyclosporin A and tacrolimus (FK506), have been found to ameliorate brain damage after global and focal cerebral ischemia (31, 124) in the rat. However, the neuroprotective actions of these drugs have been commonly explained by their ability to suppress mitochondrial calcium transition (124) rather than inflammatory responses.

In order to clarify whether neuroprotective properties of anti-inflammatory drugs and immunosuppressants originate from direct neuronal actions or from their ability to modulate inflammatory responses, we have recently shown that drugs from both classes discussed above are capable of suppressing various aspects of pro-inflammatory activation of human CEC and human astrocytes subjected to hypoxic conditions *in vitro* (116, 135). For example, non-steroid anti-inflammatory drug, indomethacin, was found to inhibit both hypoxia-induced expression of ICAM-1 and the expression/secretion of chemokines IL-8 and MCP-1 in human CEC (116, 135). Dexamethasone, on the other hand, was found to selectively suppress hypoxia-stimulated expression/release of IL-1β from both human CEC

and human astrocytes (137). Immunosuppressants cyclosporin A and rapamycin reduced cytokine-induced expression of ICAM-1 in human CEC, whereas cyclosporin A decreased hypoxia-induced release of IL-8 and MCP-1 from human CEC (135).

However, regardless of the ability of some of these drugs to attenuate inflammatory responses *in vitro*, immunomodulation strategies remain highly non-selective and need for more targeted approaches has become an imperative. The novel approaches to simultaneously interfere with multiple inflammatory genes/mediators may arise from a better understanding of transcriptional regulation of these genes.

Transcription factors. Inflammatory genes are often transcribed as clusters and many genes are regulated through the same transcription pathway. NF-kB is a key transcription factor required for the expression of many proinflammatory genes (11, 27). NF-kB is a member of Rel family of proteins and is typically a heterodimer composed of p50 and p65 subunits (11). In quiescent cells, NF-kB resides in the cytosol in latent form bound to an inhibitory protein kB (IkB), which masks the nuclear localization sequences of p50/p65 (11). Stimulation of cells with cytokines, LPS, viruses or oxidants triggers a series of signaling events that ultimately lead to the phosphorylation and proteolytic degradation of IkB and activation of NF-kB which translocates into the nucleus and stimulates transcription by binding to cognate kB sites in the promoter regions of target genes including cytokines, chemokines and cell adhesion molecules (11, 15, 27). Regulated proteolysis of IkB is processed via the ubiquitin-proteasome pathway of protein degradation (49). Therefore, inhibition of an array of proinflammatory molecules can be achieved by suppressing NF-kB activation (8, 66, 71, 123).

Studies in animal models of cerebral ischemia have shown time-dependent changes in transcription factor binding activities, including NF-kB (106). Global ischemia has been shown to activate NF-kB in the forebrain and sensitive hippocampal neuronal layers (106) and NF-kB activation in focal cerebral ischemia has been implicated in the cell death-promoting pathways (106). Recently, NF-kB activation has been causally linked to ICAM-1 up-regulation in human endothelial cells subjected to cytokines (71) and hypoxia/reoxygenation (58). Similarly, hypoxic stimulation of COX-2 in human vascular endothelium has been associated with NF-kB activation (105). A transcription factor decoy for NF-kB (123), as well as a novel pharmacological inhibitor of the ubiquitin-proteasome pathway responsible for the degradation of IkB (66), have been shown to simultaneously suppress TNFα-induced NF-kB activation and the expression of IL-6 and cell surface adhesion molecules in peripheral human endothelium. Therefore, targeting (i.e., inhibiting) key transcription elements involved in regulating the expression of inflammatory gene clusters may provide both necessary selectivity as well as simultaneous targeting of multiple players in the inflammatory cascade.

Summary

Inflammatory genes/mediators are involved in molecular/biochemical cascades determining stroke outcome. Cerebral endothelial cells, targeted by inflammatory mediators produced in ischemic brain, undergo pro-inflammatory activation by expressing/secreting various inflammatory mediators, thus becoming a source of inflammation themselves. A unique position of the cerebral endothelial cells at the interface between blood and brain establishes their role as principal regulators of peripheral inflammatory cell recruitment into the brain during stroke. Cerebral endothelial cells also exhibit ability to regulate vasomotor responses of brain microcirculation and the permeability of the BBB, both important components of inflammation. Therefore, approaches to attenuate consequences of stroke must take into consideration cerebral endothelial cells as an easily accessible target to interfere with the ischemic brain inflammation.

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