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Leukocyte Recruitment and Ischemic Brain Injury

Gokhan Yilmaz and **D. Neil Granger**
Department of Molecular & Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, LA 71130-3932, USA

D. Neil Granger: dgrang@lsuhsc.edu

Abstract

Leukocytes are recruited into the cerebral microcirculation following an ischemic insult. The leukocyte–endothelial cell adhesion manifested within a few hours after ischemia (followed by reperfusion, I/R) largely reflects an infiltration of neutrophils, while other leukocyte populations appear to dominate the adhesive interactions with the vessel wall at 24 h of reperfusion. The influx of rolling and adherent leukocytes is accompanied by the recruitment of adherent platelets, which likely enhances the cytotoxic potential of the leukocytes to which they are attached. The recruitment of leukocytes and platelets in the postischemic brain is mediated by specific adhesion glycoproteins expressed by the activated blood cells and on cerebral microvascular endothelial cells. This process is also modulated by different signaling pathways (e.g., CD40/CD40L, Notch) and cytokines (e.g., RANTES) that are activated/released following I/R. Some of the known risk factors for cardiovascular disease, including hypercholesterolemia and obesity appear to exacerbate the leukocyte and platelet recruitment elicited by brain I/R. Although lymphocyte–endothelial cell and –platelet interactions in the postischemic cerebral microcirculation have not been evaluated to date, recent evidence in experimental animals implicate both CD4+ and CD8+ T-lymphocytes in the cerebral microvascular dysfunction, inflammation, and tissue injury associated with brain I/R. Evidence implicating regulatory T-cells as cerebroprotective modulators of the inflammatory and tissue injury responses to brain I/R support a continued focus on leukocytes as a target for therapeutic intervention in ischemic stroke.

Keywords

Leukocyte–endothelial cell adhesion; Platelet adhesion; Ischemia-reperfusion; Stroke; Endothelial cell adhesion molecules; Blood–brain barrier

Introduction

Stroke and other cerebrovascular diseases remain a major cause of disability and death in industrialized countries; however, treatments that effectively limit the tissue injury and brain dysfunction in these conditions remain elusive. While fibrinolytic therapy has gained widespread acceptance as an effective approach for restoring blood perfusion to the brain during acute ischemic stroke, the consequent reperfusion of previously ischemic brain tissue can initiate a series of cellular responses that result in neuroinflammation. Since the recruitment and activation of inflammatory cells in postischemic tissue can accelerate and extend the brain infarction that is initiated by the ischemic insult, efforts have been undertaken to define the nature and underlying mechanisms of inflammatory cell recruitment in the brain, with the hope that anti-inflammatory agents may provide an effective therapeutic strategy for stroke. These

Correspondence to: D. Neil Granger, dgrang@lsuhsc.edu.

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studies have revealed that leukocyte–endothelial cell adhesion, a rate-determining step in inflammation, is largely absent in the normal healthy cerebral microcirculation, but becomes robust after the initiation of brain ischemia.

The accelerated recruitment of leukocytes and other blood cells (e.g., platelets) can be attributed to an increased expression of adhesion molecules on both cerebral endothelial cells and circulating blood cells. These adhesion molecules allow for the coordinated sequential recruitment of different populations of inflammatory cells (initially neutrophils, then mononuclear leukocytes), as well as platelets, into the cerebral microvasculature after ischemia-reperfusion. The blood cell recruitment further compromises cerebral microvascular perfusion and contributes to the evolution of the ischemic infarct. Much of the published work dealing with leukocyte involvement (recruitment and injury induction) in ischemic stroke has focused on the early injury response and has placed emphasis on the importance of neutrophils. However, more recent animal studies have revealed a role for lymphocytes in cerebral microvascular dysfunction and tissue injury that is manifested a day after the initiating ischemic insult.

In this report, we (a) briefly review the available literature that addresses leukocyte and platelet recruitment in cerebral microcirculation after ischemia-reperfusion, (b) summarize evidence that implicates leukocyte–endothelial cell adhesion as a critical determinant of the injury response to I/R, and (c) speculate on mechanisms that potentially underlie the deleterious effects of leukocyte/platelet recruitment in the postischemic cerebral microcirculation. Additional objectives of this review were to briefly summarize: (d) new evidence that implicates lymphocyte recruitment in the pathogenesis of ischemic stroke, and (e) potential mechanisms involved in the activation of lymphocytes after stroke.

Leukocyte Recruitment and Ischemic Stroke

It is now well-recognized that ischemia induces an inflammatory phenotype in brain tissue that is associated with the activation of different resident cell populations, including endothelial cells, microglia/macrophages, and astrocytes. This activation process leads to the synthesis, expression, and/or secretion of a variety of inflammatory proteins that attract and activate leukocytes, which bring their own repertoire of inflammatory mediators and cytotoxic factors that serve to amplify the inflammatory response. In the cerebral microcirculation, the inflammatory phenotype is characterized by an enhanced production of reactive oxygen species, activation of oxidant-sensitive transcription factors (e.g., NFκ B), diminished blood brain–barrier function, increased expression of endothelial cell adhesion molecules, the recruitment of inflammatory cells and platelets, and leukocyte capillary plugging. Whether or not (and how) these responses are linked remains unclear. However, there is a large and growing body of evidence that indicates that the inflammatory phenotype assumed by the cerebral microvasculature and the subsequent recruitment of leukocytes contributes to the initiation and evolution of brain injury after ischemic stroke (Yilmaz and Granger 2008; Gavins et al. 2007).

Several approaches have been used to demonstrate the recruitment of leukocytes into brain tissue in human and experimental stroke. Histopathological examination of brain tissue has revealed the accumulation of neutrophils within and outside of cerebral venules after ischemia (Barone et al. 1992). The appearance of polymorphonuclear leukocytes (PMNs) within brain parenchyma is evident within several minutes to a few hours after the ischemic insult (Clark et al. 1994). Single photon emission computed tomography (SPECT) imaging of radiolabeled PMNs has confirmed the early recruitment of PMNs in postischemic human brain (Akopov et al. 1996; Price et al. 2004). While leukocyte recruitment persists for days to weeks following

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the ischemic insult, the population of recruited cells shifts from PMNs to mononuclear leukocytes (Barone et al. 1995).

Cell–Cell Interactions

Intravital microscopic analysis of leukocyte recruitment into cerebral venules after brain ischemia-reperfusion has revealed an early accumulation of adherent leukocytes that is evident within a few minutes after reperfusion. The number of adherent leukocytes has been shown to rise progressively up to 48 h after reperfusion (Park et al. 1999; Ishikawa et al. 2005), with the value detected at 4 h after reperfusion reflecting approximately 50% of the 24 h value (Fig. 1a). Based on a comparison of the leukocyte adhesion responses to brain I/R in normal and neutropenic mice (Yilmaz et al. 2006), it is predicted that neutrophils account for the majority (~85%) of the total adherent leukocytes detected at 4 h following reperfusion, while neutrophils account for only about 20% of total adherent leukocytes detected at 24 h after reperfusion. While the identity of the non-PMN leukocyte population that accounts for 80% of the adherent cells in postischemic cerebral venules is not known, mononuclear cells such as monocytes and T-lymphocytes are likely candidates.

The leukocyte adhesion that is elicited by cerebral I/R is also accompanied by the recruitment of rolling and adherent platelets (Fig. 1b). Platelet recruitment in postischemic cerebral venules generally lags behind leukocyte recruitment (i.e., adherent leukocytes but not platelets are detected at 30 min after reperfusion) and a large proportion of the platelet accumulation in these microvessels can be attributed to the binding of platelets to already adherent leukocytes. Neutrophil-dependent platelet accumulation dominates at 4 h after reperfusion, but neutrophil-independent mechanisms underlie the continued recruitment of platelets into cerebral microvessels at 24 h after reperfusion (Yilmaz et al. 2006). The later response likely reflects a combination of platelet binding to the non-PMN leukocytes as well as the direct adhesion of platelets to endothelial cells. A potential consequence of the attachment of platelets to leukocytes is enhanced cell activation. It has been shown that the attachment of activated platelets to neutrophils enables the latter to produce larger quantities of superoxide (Suzuki et al. 2001) and platelet activating factor (Herd and Page 1994) than either cell is capable of producing alone. Another possible consequence of platelet–leukocyte interactions within cerebral venules is the formation of platelet–leukocyte aggregates (PLA) that may be dislodged by shear forces and gain access to the general circulation. Ischemic stroke in both human (Htun et al. 2006) and experimental animals (Ritter et al. 2005) is associated with a significant increase in the number of PLA in systemic blood. PLA may represent an important circulating source of inflammatory mediators that can help sustain and/or amplify an inflammatory response (Li et al. 2000).

Adhesion Molecules

There are several published reports that address the identity of the adhesion molecules that mediate the recruitment of rolling and firmly adherent leukocytes in the postischemic cerebral microvasculature. Immunostaining has revealed an increased expression of different adhesion molecules on endothelial cells of cerebral microvessels, including the selectins, ICAM-1 and VCAM-1 (Stanimirovic et al. 1997). Increased P-selectin expression is observed as early as 15 min following an ischemic insult and persists for at least 24 h. The later prolonged upregulation of P-selectin may reflect both increased expression on cerebral endothelial cells and the binding of P-selectin positive platelets to the vessel wall (Ishikawa et al. 2003). The transcription-dependent expression of E-selectin and ICAM-1 is noted as early 1–2 h of ischemia and remains elevated for several hours (Zhang et al. 1998; Okada et al. 1994). Soluble circulating levels of E-selectin, ICAM-1 and VCAM-1 are elevated in plasma of patients suffering an acute ischemic stroke (Frijns et al. 1997). Since these soluble adhesion molecules

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are shed from the surface of activated endothelial cells, they provide a useful surrogate marker of CAM expression in acute and chronic inflammatory states.

Direct evidence for the participation of different adhesion molecules in the leukocyte–endothelial cell adhesion elicited by brain I/R is provided in animal studies employing blocking antibodies and adhesion molecule-deficient mice (Ishikawa et al. 2003, 2004). These studies indicate that P-selectin-mediated adhesive interactions account for the large influx of rolling leukocytes in post-ischemic cerebral venules, while interactions between beta-2 integrins (CD11a/CD18 and CD11b/CD18) on leukocytes and ICAM-1 on brain endothelial cells account for the recruitment of firmly adherent leukocytes (Fig. 2). The relative contributions of these adhesion mechanisms to I/R-induced leukocyte recruitment differ however between the early phase that is dominated by neutrophils and the later phase involving non-PMN leukocyte populations (Arumugam et al. 2004).

Less attention has been devoted to defining the adhesion molecules that mediate the recruitment of platelets into postischemic cerebral microvessels. Blocking the platelet adhesion glycoprotein GP/IIb/IIIa (which can mediate both platelet adhesion and aggregation) has little or effect on I/R-induced platelet adhesion in cerebral venules. However, blocking adhesion molecules that mediate leukocyte adhesion, such as P-selectin, CD18, and ICAM-1, can also reduce the number of adherent platelets in postischemic cerebral venules (Ishikawa et al. 2003, 2004). This observation, coupled to reports showing that platelet accumulation lags behind leukocyte accumulation (Tailor 2005), is consistent with a mechanism of platelet recruitment that involves the binding of platelet-associated P-selectin to its ligand PSGL-1 on leukocytes, which in turn utilize endothelial cell P-selectin and ICAM-1 to attach to venular endothelial cells (Fig. 2). Such a mechanism is consistent with data demonstrating that much of the platelet accumulation in the postischemic cerebral microvasculature is linked to leukocyte recruitment (Ishikawa et al. 2003, 2004).

A role for platelet P-selectin in platelet–leukocyte adhesion is supported by animal studies demonstrating that the non-selective selectin inhibitor fucoidin effectively prevents the appearance of circulating platelet–leukocyte aggregates in peripheral blood after ischemic stroke and reperfusion (Ritter et al. 2005). While platelet GPIIb/IIIa does not appear to contribute to the binding of platelets to adherent leukocytes (Ishikawa et al. 2004), a GPIIb/IIIa antagonist was shown to be effective in preventing the formation of circulating platelet–leukocyte aggregates that appear in peripheral blood after ischemic stroke and reperfusion (Ritter et al. 2005).

Mediators

The molecular mechanisms that underlie the cell activation and subsequent upregulation of adhesion molecules following cerebral ischemia-reperfusion remain poorly understood. Cytokines, reactive oxygen metabolites, lipid mediators are among the large number of substances released into postischemic brain tissue and plasma that may activate the transcription-dependent and -independent pathways that result in adhesion molecule upregulation. However, little attention has been devoted to defining the contributions of these potential mediators to the recruitment of adherent leukocytes and platelets in the postischemic cerebral microcirculation. There are reports describing a role for the CD40/CD40L (Ishikawa et al. 2005) and Notch (Arumugam et al. 2006) signaling pathways in the regulation of I/R-induced leukocyte (and platelet) adhesion in cerebral venules, with mice genetically deficient in either CD40 or CD40L exhibiting a blunted leukocyte recruitment response to I/R. Gamma secretase (an enzyme that cleaves the Notch receptor and allows the translocation of Notch intracellular domain to the nucleus) inhibition has been shown to reduce I/R-induced ICAM-1 expression and the recruitment of adherent leukocytes and platelets in mouse brain.

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Few attempts have been made to directly assess the role of specific cytokines in the blood cell–vessel wall interactions elicited by brain I/R. RANTES (regulated on activation normal T-cell expressed and secreted), a C–C motif 5 chemokine produced by T-lymphocytes, platelets, endothelial cells, smooth muscle cells, and glial cells, has been recently implicated in the blood cell recruitment associated with cerebral I/R (Terao et al. 2008b). This chemokine, which is known to bind avidly to the surface of endothelial cells where it can promote leukocyte chemotaxis and transendothelial cell migration, exhibits profoundly elevated concentrations in brain tissue after I/R. RANTES-deficient ($RANTES^{-/-}$) mice show a markedly attenuated leukocyte and platelet adhesion, with corresponding reductions in infarct volume and blood–brain barrier (BBB) permeability after middle cerebral artery occlusion and reperfusion (MCAo/R). Since wild-type mice transplanted with bone marrow from $RANTES^{-/-}$ mice (bone marrow chimeras) exhibited similar protection against blood cell recruitment and tissue injury, it has been proposed that the RANTES mediating these effects was likely derived from blood cells, probably platelets (Terao et al. 2008b).

Leukocyte–Perivascular Cell Interactions

Once leukocytes emigrate from the postischemic cerebral microvasculature, they encounter and can interact with perivascular cells such as astrocytes and microglia. Astrocytes and microglia (the resident macrophages of the brain) are believed to exert both beneficial and harmful effects in the postischemic brain. For example, microglia, which proliferate in response to brain ischemia, can either afford protection through phagocytosis of infiltrating neutrophils or mediate injury by releasing reactive oxygen and nitrogen species. Both cell populations are activated by ischemia (Stoll et al. 1998; Danton and Dietrich 2003), which leads to the production and liberation of proinflammatory cytokines that can mediate the recruitment of leukocytes. Activated astrocytes express adhesion molecules (ICAM-1, VCAM-1) that can bind infiltrating leukocytes. Leukocyte-derived cytokines (e.g., IL-1) are known stimulants of adhesion molecule expression by astrocytes (Moynagh 2005). Furthermore, adhesion-dependent interactions between leukocytes and astrocytes are associated with an accelerated production of cytokines such as IL-1α, TNFα, and MCP-1 (Andjelkovic et al. 2000), which would be expected to amplify that inflammatory condition elicited by I/R. Less is known about adhesion-dependent interactions between leukocytes and microglia.

Cardiovascular Risk Factors

Much of the work on experimental ischemic stroke has been undertaken using animals that are otherwise healthy, despite the fact that the incidence of stroke in the human population is linked to well-established risk factors, including hypertension, obesity, hypercholesterolemia, and diabetes. Recent studies have addressed the influence of hypercholesterolemia and obesity on the inflammatory and tissue injury responses to MCAo/R. Diet-induced hypercholesterolemia has been shown to exaggerate the oxidative stress and recruitment of rolling and adherent leukocytes and platelets in postischemic cerebral venules (Ishikawa et al. 2004). Since hypercholesterolemic recipients of platelets derived from mice placed on either a normal or cholesterol-enriched diet exhibit a robust platelet adhesion response, while normocholesterolemic recipients of platelets from hypercholesterolemic donors exhibit only a modest platelet adhesion response, it was concluded that the blood vessel wall, rather than platelets per se, must be exposed to the hypercholesterolemic milieu in order to realize the platelet adhesion response to this risk factor. The study also revealed that the hypercholesterolemia-induced enhancement of I/R-induced blood cell adhesion is largely absent in mice that are genetically deficient in the gp91phox protein subunit of NADPH oxidase, suggesting that oxidants derived from this enzyme are largely responsible for the blood cell recruitment. A similar analysis of the responses to brain I/R in obese mice has shown a comparable exaggeration of leukocyte and platelet adhesion in cerebral venules as well as larger infarcts and increased BBB permeability (Terao et al. 2008a). Leptin-deficient ob/ob

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mice were shown to exhibit a more profound recruitment of adherent leukocytes and platelets in response to cerebral I/R than their lean counterparts. Ob/ob mice with reconstituted plasma leptin levels did not show a blunted blood cell recruitment response, despite evidence in other vascular beds invoking a role for leptin in inflammation (Singer and Granger 2007), suggesting that leptin deficiency per se does not account for the exaggerated inflammatory and injury responses in the obese mice. Additional work is needed to define the mechanisms that underlie the exaggerated inflammatory and prothrombogenic responses to ischemic stroke in the presence of cardiovascular risk factors.

Adherent Leukocytes and Ischemic Tissue Injury

The contention that leukocytes contribute to the tissue injury associated with brain ischemia and reperfusion is supported by studies demonstrating that animals rendered neutropenic (with antineutrophil serum) exhibit smaller infarcts and improved neurological outcome (Matsuo et al. 1994; Hudome et al. 1997). Additional support is provided by studies of ischemic stroke that employed either antagonists or blocking antibodies against specific leukocyte or endothelial cell adhesion molecules (Mori et al. 1992; Matsuo et al. 1994; Zhang et al. 1996). For example, targeting selectin-mediated leukocyte rolling using either non-selective antagonists or ligands of the selectins (e.g., fucoidin), E- and P- (but not L-) selectin specific antibodies, or P-selectin deficient mice has generally yielded protection against I/R-induced brain injury (Connolly et al. 1997; Huang et al. 2000; Yenari et al. 2001; Ruehl et al. 2002). Similar protection has been reported when the adhesion molecules mediating the firm adhesion and transendothelial migration of leukocytes (ICAM-1, CD11a/CD18, CD11b/CD18) were targeted (Connolly et al. 1996; Zhang et al. 2003).

While the outcome of many animal studies supports the therapeutic potential of leukocyte adhesion-directed therapy in ischemic stroke, there are several reports that contradict this view. The absence of protection against I/R injury in animals treated with neutrophil-blocking antibodies and in some adhesion molecule-deficient mice suggests that leukocyte recruitment is not a critical determinant of the injury response and that leukocyte–endothelial cell adhesion is more likely a consequence of the injury process. However, some of the inconsistent findings may be the result of differences in experimental models/techniques, the duration and magnitude of the ischemic insult and reperfusion periods, and/or the time of administration of the anti-adhesion therapy. Indeed, anti-adhesion molecule strategies have generally proven to be more effective in models of transient, but not permanent, ischemia. This observation suggests that adhesion-directed therapeutic strategies are more likely to offer some benefit in patients receiving tissue plasminogen activator (tPA) to achieve reperfusion following ischemic stroke. This possibility is supported by reports of an improved outcome and extended therapeutic window of tPA following an ischemic stroke in animals receiving a combination of anti-b2-integrin (CD11/CD18) and tPA, and an extension of tPA's therapeutic window (Zhang et al. 2003). Post hoc analysis of data from a Phase 2 clinical study of 900 patients with acute ischemic stroke who were treated with a CD11b-specific antagonist (UK-279,276) revealed a slight improvement in those patients who received a combination of tPA and the CD11b antagonist (Krams et al. 2003). However, other clinical trials employing either a humanized CD11b antibody (Becker 2002) or an anti-murine ICAM-1 monoclonal antibody have not shown any clinical improvement in stroke patients (Enlimomab Acute Stroke Trial Investigators 2001).

Lymphocytes and Ischemic Stroke

Recent reports have implicated lymphocytes in the recruitment of adherent leukocytes and/or tissue injury that is manifested within 24 h after an ischemic insult (followed by reperfusion) in mouse brain (Yilmaz et al. 2006; Hurn et al. 2007). While lymphocytes have been implicated in the pathogenesis of stroke in both humans and experimental animals, previous efforts suggest

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that lymphocytes mediate a later stage of postischemic injury. This assertion is largely based on histopathological and flow cytometric data that places the appearance of lymphocytes in brain tissue 18 h to 7 days after the ischemic stroke (Schroeter et al. 1994; Jander et al. 1995; Campanella et al. 2002; Stevens et al. 2002; Gelderblom et al. 2009). The new findings in animal models suggest that lymphocytes may exert an influence on the evolution of tissue inflammation and injury prior to their appearance in the extravascular brain compartment.

Lymphocytes and Stroke Patients

Studies of lymphocyte function in stroke patients have revealed the presence of activated T- (but not B-) lymphocytes and increased antigen-specific T-cell responses within 60 days after stroke onset (Tarkowski et al. 1991; Tarkowski et al. 1995). Circulating CD4+ and CD8+ T cells that recognize myelin protein-derived peptides in stroke patients is consistent with antigen-dependent activation of lymphocytes in this population (Pette et al. 1990; Tsuchida et al. 1994). It has also been noted that the lymphocytes of individuals with ischemic brain damage exhibit a reduced proliferation capacity and cytolytic activity in response to IL-2 and mitogens (Rogers et al. 1988). A change from Th1 to Th2 responses (Chamorro et al. 2006; Theodorou et al. 2008) with reductions in TNF-α and IFN-γ production (Haeusler et al. 2008) as well as a reduction in the number of circulating CD4+ lymphocytes (Vogelgesang et al. 2008) occurs after stroke. Generally, CD8+ T-lymphocytes are also reduced while regulatory T-cells (Tregs) are increased after acute stroke. The initial severity of stroke has been shown to be correlated with the low number of T lymphocytes (Urra et al. 2009). Some specific subpopulations of T-cells have received particular attention in stroke patients, including CD4+ CD25+ Foxp3 regulatory cells (Yan et al. 2009) and CD4+ CD28− T-cells (with a high tissue-damaging potential), which both increase. The latter cell population appears to be predictive of poor outcome and increased risk of death (Nadareishvili et al. 2004). CD4+ CD28− cells carry Killer-cell immunoglobulin-like receptors (KIRs) that can be activated by MHC-I molecules (Zal et al. 2008). Reports in mice of increased Treg cells that are accompanied with splenic atrophy after ischemic stroke suggests a balancing effort by the regulatory immune system against the massive proinflammatory responses triggered by brain ischemia (Offner et al. 2006a; b). Overall, the lymphocyte responses to ischemic stroke (altered T-cell function, lower number of circulating cells, and appearance of aberrant T-cell populations) result in immune suppression. This apparent effort to prevent an excess autoimmune reaction to self-antigens also leads to an increased susceptibility to infections (Chamorro 2007).

Lymphocytes and Experimental Stroke

Lymphocyte-deficient Rag-$1^{-/-}$ (Yilmaz et al. 2006) and SCID (Hurn et al. 2007) mice have recently been used to evaluate the role of lymphocytes in the inflammatory and tissue injury responses to ischemic stroke. These studies reveal a major role for lymphocytes in the infarct progression and neurological deficits following cerebral I/R. Since mice that are genetically deficient in either CD4+ and/or CD8+ T-lymphocytes also exhibit protection against brain inflammation and injury following-MCAo/R (Yilmaz et al. 2006), it has been proposed that both T-cell subsets contribute to the deleterious responses by acting through a common pathway. A direct assessment of the contribution of B-lymphocytes in experimental stroke (using B-cell deficient mice) revealed no role for this immune cell population in promoting either the blood cell–endothelial cell interactions or tissue injury following cerebral I/R (Yilmaz et al. 2006).

Other studies in experimental animals are also consistent with a role for T-lymphocytes in ischemic stroke. For example, mice deficient in key lymphocyte signaling molecules such as lymphocyte function-associated antigen-1 (LFA-1) or CD40/CD40L exhibit reduced leukocyte and platelet adhesion into cerebral venules and better outcome following focal cerebral ischemia-reperfusion (Arumugam et al. 2004, Ishikawa et al. 2005). E-selectin-specific

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regulatory T cells (Tregs) primed by repetitive intranasal administration of recombinant E-selectin has been shown to protect spontaneously hypertensive rats (SHR) against the brain injury and functional deficits elicited by permanent MCAo (Ishibashi et al. 2009). The E-selectin-tolerized SHR also exhibited increased Tregs in the ischemic brain. It remains unclear whether this novel approach for targeting Tregs to injured regions of the brain dampens the recruitment of adherent leukocytes and platelets in cerebral venules. The cerebroprotective effects of regulatory T-cells have also been demonstrated in mice depleted of CD4+ CD25+ Foxp3+ regulatory T lymphocytes, which exhibited an augmented postischemic activation of resident and invading inflammatory cells, an exaggeration of delayed brain damage and worse functional outcome following MCAo (Liesz et al. 2009). TNF-α and IFN-γ were shown to mediate the early and delayed development of the cerebral infarct in the Treg cell-depleted mice. The transfer of Treg cells derived from IL-10 deficient mice abolishes these protective effects of regulatory T cells, underscoring the critical role of IL-10 in Treg-mediated immunomodulation.

While animals studies provide convincing evidence for the participation of T-cells in the inflammation and injury responses observed within 24 h following ischemic stroke, there is no direct evidence that T cells engage in direct adhesive interactions with the cerebral microvasculature. The data presented in Fig. 1a shows a progressive recruitment of non-neutrophilic leukocytes beginning after 4 h of reperfusion of the ischemic mouse brain, which may represent the adhesion of lymphocytes. However, it is also conceivable that T-cells can modulate the brain inflammation after I/R in the absence of direct adhesive interactions with blood vessels via the secretion of inflammatory cytokines. This possibility is supported by studies demonstrating that IFN-γ knockout mice exhibit an attenuated recruitment of adherent leukocytes and platelets in cerebral venules after MCAo/R, and that the protection against recruitment of the adherent leukocytes that afforded by lymphocyte deficiency in Rag-1$^{-/-}$ mice can be abolished to some extent by the administration of splenocytes derived from IFN-γ knockout mice (Yilmaz et al. 2006).

Platelet–Lymphocyte Interactions

The extent to which lymphocytes contribute to the formation of platelet–leukocyte interactions detected in blood of patients and experimental animals suffering from an ischemic stroke remains unclear. Lymphocytes appear to have a much lower potential to form heterotypic aggregates with platelets than either granulocytes or monocytes (Rinder et al. 1991). Nonetheless, platelets will bind to all lymphocyte subpopulations (T- and B-cells) to form platelet–lymphocyte aggregates, with larger and more activated T-lymphocytes more readily forming heterotypic aggregates (Li et al. 2006). While platelets express a variety of adhesion molecules that enable them to bind to lymphocytes, P-selectin ligation appears to be essential, although GPIIb/IIIa, CD40L and ICAM have also been implicated in this heterotypic conjugation (Li et al. 2006). Activated platelets can also modulate T-cell responses by releasing mediators such as platelet-released platelet factor 4 (PF4) or through cell surface receptor (e.g., CD40L) signaling (Li 2008). The contribution of platelet–lymphocyte interactions to ischemic brain injury remains unknown. However, the ability of platelets to modify lymphocyte function may allow anti-platelet therapies (anti-aggregants, thrombolytics) to influence the contribution of lymphocytes to the pathogenesis of ischemic stroke.

Mechanisms of Lymphocyte Activation

Both antigen-dependent and independent mechanisms may contribute to the lymphocyte activation associated with ischemic stroke. While less attention has been devoted to antigen-independent mechanisms, studies of lymphocyte involvement in the pathogenesis of angiotensin-induced hypertension may be relevant to ischemic stroke. There are several lines of evidence that implicate angiotensin II (AngII) in ischemic brain injury, including the

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decreased brain injury reported in wild-type mice treated with angiotensin receptor blockers and in mutant mice lacking the angiotensin gene (Maeda et al. 1999; Walther et al. 2002). Transgenic mice carrying the human renin and angiotensinogen genes, which yield higher quantities of AngII in brain tissue and in plasma, exhibit an exaggerated brain injury response to focal cerebral ischemia (Inaba et al. 2009). AngII appears to mediate its deleterious effects on vascular function and blood pressure via an AT-1 receptor-mediated mechanism that activates T-lymphocytes to produce and secrete the cytokine TNF-alpha (Guzik et al. 2007). Lymphocyte-deficient Rag-1$^{-/-}$ mice do not develop the vascular dysfunction and hypertension in response to chronic AngII administration and this is reversed following adoptive transfer of T lymphocytes to the Rag-1$^{-/-}$ mice (Guzik et al. 2007). These findings, coupled to the evidence implicating AngII in ischemic stroke, raise the possibility that AngII serves as a signaling molecule that may activate T cells following stroke in the absence of an antigenic stimulus.

An antigen-dependent mechanism has also been proposed to explain the lymphocyte activation associated with ischemic stroke (Fig. 2). This mechanism requires the exposure of CNS antigens, such as neurofilaments, NMDA receptors, and S100B (a calcium-binding protein that is predominantly expressed and secreted by astrocytes in vertebrate brain), to blood following disruption of the BBB, which then leads to the development of autoimmune reactive T cells (Wang et al. 1992;Bornstein et al. 2001;Dambinova et al. 2003). In a recent study, using a neuro-antigen-specific immunomodulatory agent that inhibits autoreactive T cells administered after ischemia was shown to reduce ischemic brain damage in experimental stroke (Subramanian et al. 2009). The findings of this study support the hypothesis that T lymphocytes are being activated by neuro-antigens following ischemia and autoreactive T cells contribute to ischemic brain injury in a specific antigen-dependent manner. Depending on the status of the immune system at the onset of stroke, tolerance, or sensitization to the CNS antigens may develop (Becker et al. 2005). The T lymphocytes are primed with antigens that are in close contact with antigen presenting cells (APCs) to form immunological synapses, which require an interaction of MHC-I or -II with CD8+ T or CD4+ lymphocytes receptors, respectively, as well as the binding of LFA-1 on lymphocytes to ICAM-1 expressed on APCs (Kupfer and Kupfer 2003;Dustin 2008;Yewdell et al. 2003). Focal ischemia induces the expression of MHC class I antigens in brain tissue, which contribute to the activation of CD8+ T-cells (Kato et al. 1996). IFN-γ, which increases in brain tissue after ischemia (Li et al. 2001), can also induce MHC class II molecule expression in rat brain endothelial cells and astrocytes, which may contribute to the priming of CD4+ T cells. (Male et al. 1987;Etienne et al. 1999). In other tissues (liver and kidney), ischemia is known to elicit the endothelial expression of proteins that act as co-stimulatory molecules that optimize the activation of T-cells (Satoh et al. 2002;Kojima et al. 2001). Although the ischemic brain is reported to exhibit the expression of B7.1 co-stimulatory proteins (Becker et al. 2005), the contribution of such co-stimulatory or accessory proteins to the inflammatory and tissue responses in ischemic and postischemic brain remains undefined.

If MHC-1 presentation achieves a level that can activate CD8+ T cells, cytotoxic molecules such as perforin and Fas ligand/TNFSF6 are released into brain tissue where they can destroy target cells by inducing membrane damage and apoptosis (Shresta et al. 1998; Russell and Ley 2002; Stinchcombe and Griffiths 2003) (Fig. 2). After activation, naïve CD4+ T cells differentiate into effector (Th1, Th2, Th17), regulatory T (Treg) and memory T cells which orchestrate an adaptive immune response that involves the release of the pro-inflammatory cytokines IL-2, IL-12, IFN-γ, TNF-α from Th1 cells (Zhu and Paul 2008), while Th2 cells release the neuroprotective cytokines IL-4, IL-5, IL-6, IL-10, and IL-13 (Hendrix and Nitsch 2007). Hence, the Th2 cells appear to counterbalance and limit the Th1-mediated brain inflammation and injury following ischemic stroke (Fig. 2) (Chamorro et al. 2006; Theodorou et al. 2008). Th17 cells, a potentially harmful subtype of CD4+ cells that can cross and disrupt

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the BBB by releasing IL-17 and IL-22 and subsequently promote CNS inflammation through CD4+ recruitment, have also been implicated in human and experimental stroke (Kebir et al. 2007; Li et al. 2001, 2005). However, the contribution of Th17 cells to stroke pathogenesis remains unclear.

Epilogue

Much progress has been made in understanding the nature, magnitude, and underlying mechanisms of the leukocyte recruitment elicited by brain ischemia. While time-dependent contributions of neutrophils and mononuclear leukocytes to the brain inflammation and tissue injury after brain I/R are supported by the existing data, it is increasingly apparent that T-lymphocytes exert an earlier influence on these processes than previously considered. The nature of the influence of T-cells and their inflammatory products on the early activation of vascular endothelium, neutrophils, and platelets after I/R is poorly understood and warrants more attention. Similarly, more effort should be directed toward defining the consequences of platelet–leukocyte aggregate formation within the postischemic cerebral vasculature and their appearance in systemic blood of stroke patients. It remains unclear whether these heterotypic cell interactions further impair perfusion of the cerebral microvasculature by physically plugging microvessels or promoting microthrombus formation, and whether strategic targeting of the adhesion molecules that mediate PLA formation as well as leukocyte–endothelial cell adhesion has therapeutic potential for improving brain function after stroke.

Current knowledge of the cell–cell interactions that fuel the brain inflammation elicited by ischemia and reperfusion is based on imaging technologies that allow for observation and quantification of cell adhesion to the walls of intact cerebral microvessels. Important limitations of these approaches include the need to examine microvessels on or near the brain surface and labeling the total leukocyte population. With improvements in imaging technology and labeling methods, future studies can focus on microvessels deeper within brain tissue and on the trafficking of distinct leukocyte subsets. This would allow for a direct assessment of the early and late adhesive interactions of neutrophils, lymphocytes, and monocytes with cerebral microvascular endothelium and with each other. Imaging methods of the future are also likely to provide the opportunity to visualize and quantify the activation state of these cells following heterotypic adhesion or aggregation within microvessels. The application of such imaging technologies and approaches to the cerebral microcirculation should help to address some important unresolved issues about how leukocytes and platelets contribute to brain injury following ischemic stroke.

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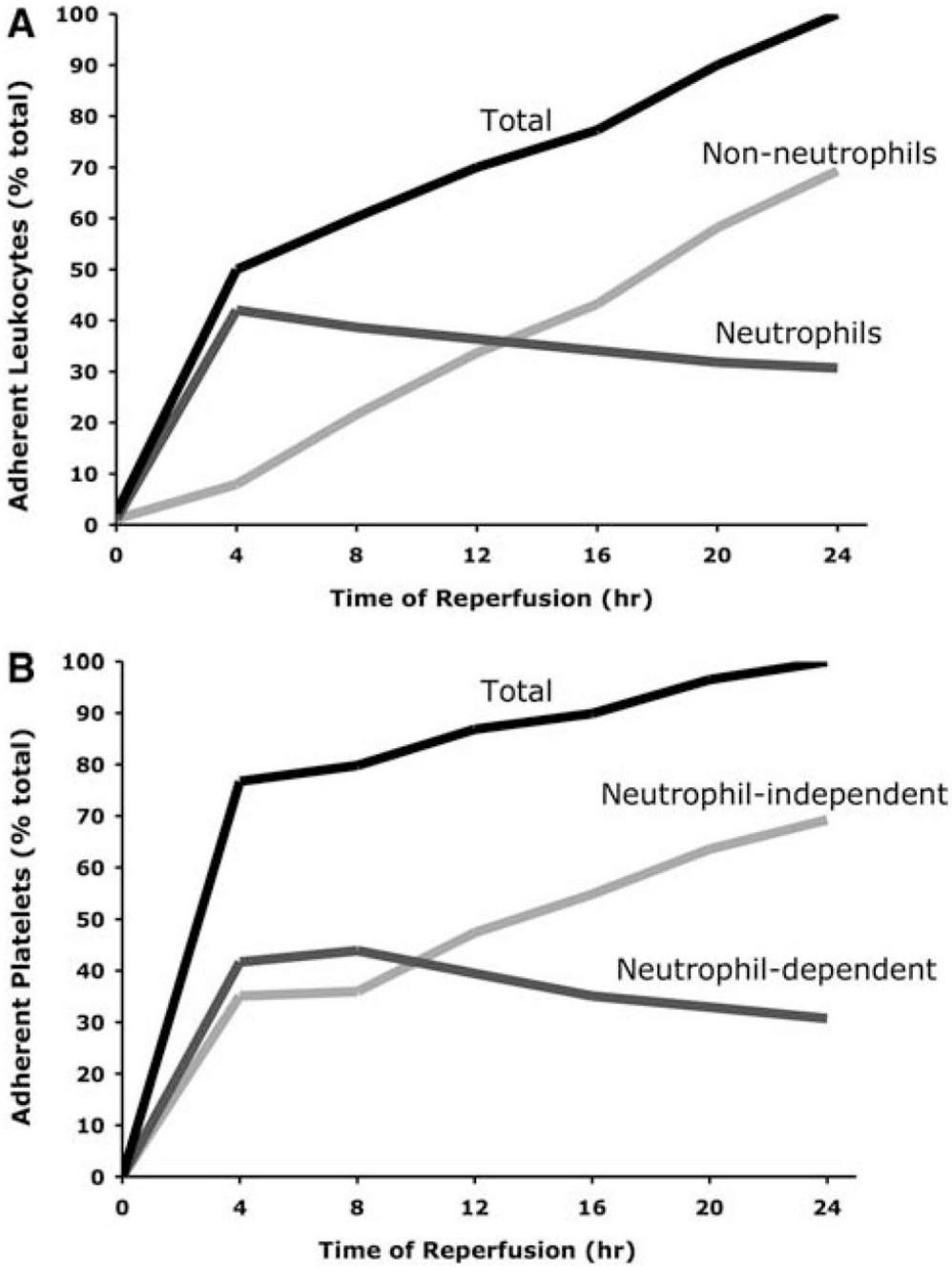

Fig. 1.

a Kinetics of leukocyte adhesion in cerebral venules during the first 24 h following exposure to ischemia and reperfusion. The accumulation of adherent neutrophils reach a peak at 4 h following reperfusion. Thereafter, non-neutrophilic leukocytes are predominantly recruited into the postischemic microvessels. **b** Recruitment of adherent platelets in postischemic cerebral microvessels. Neutrophil-dependent and -independent mechanisms of platelet recruitment are evident in the initial 4 h of reperfusion. Thereafter, neutrophil-dependent platelet recruitment wanes and non-neutrophilic recruitment increases

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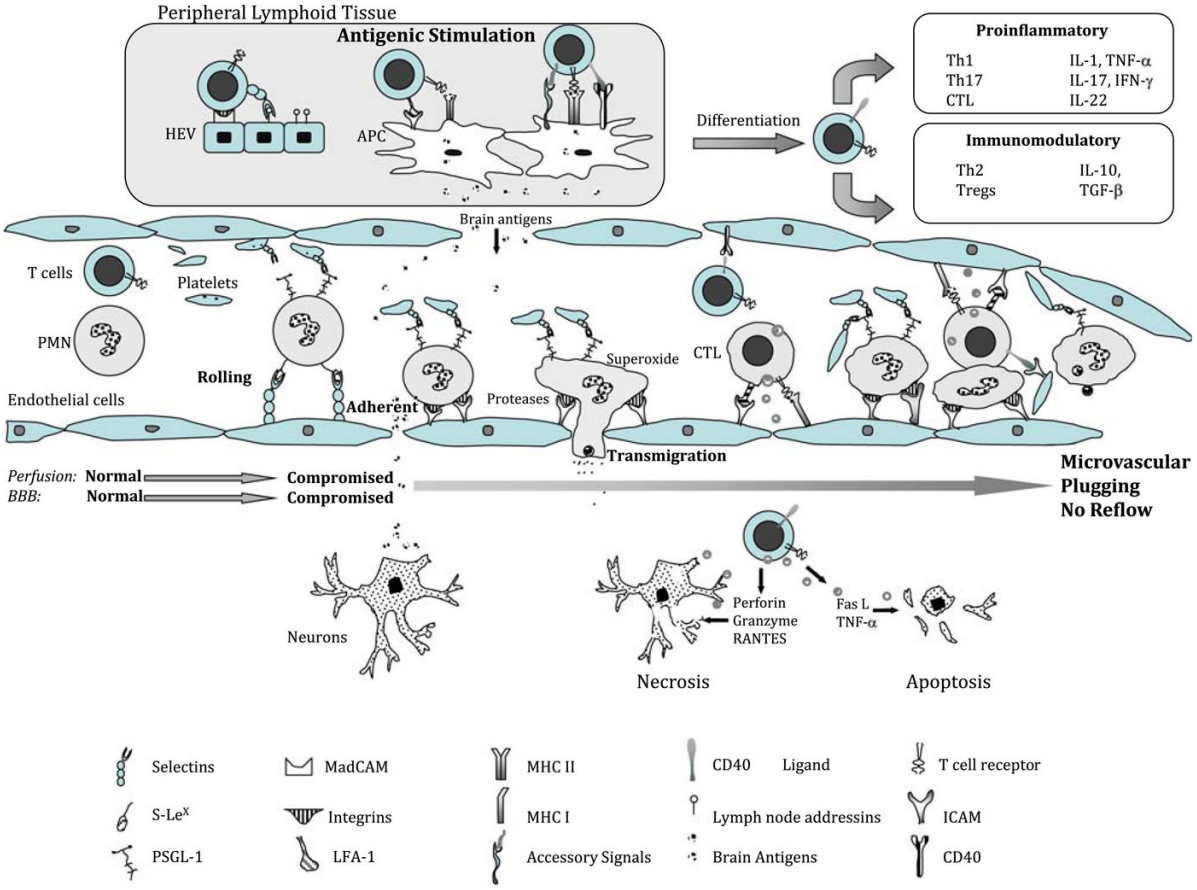

Fig. 2.

Mechanisms underlying the leukocyte and platelet adhesion in postischemic cerebral microvessels. With normal perfusion, few cell–cell interactions occur within the microvessels and blood–brain barrier permeability (BBB) is normal. Once perfusion is compromised (ischemia), adhesion molecules on endothelium and circulating cells are upregulated, resulting in the recruitment of rolling and adherent polymorphonuclear neutrophils (PMN) and platelets. The activated PMN releases proteases and superoxide to cause BBB disruption and exposes brain antigens to blood. In lymphoid tissue, brain antigens are processed by APCs and presented to T lymphocytes via major histocompatibility complex class II (MHC II) and accessory molecules. The recruitment and activation of T lymphocytes into lymphatic organs require expression of cell surface adhesion molecules such as MadCAMs (Mucosal addressin cell adhesion molecule) and lymph node addressins on high endothelial venules (HEV), lymphocytes and APCs. Upon activation by APCs, T cells differentiate into proinflammatory Th1, Th17 or cytotoxic T cells (CTL) and immunomodulatory Th2 and T regulatory (Tregs) cells. These cells exert their distant effects on neuronal tissue via the release of pro-and/or anti-inflammatory cytokines. Adhesion of T lymphocytes in cerebral microvessels may contribute to the injury process via CTLs that release membrane attacking and pro-apoptotic substances once activated by MHC I presenting APCs. The coordinated activation of T cells is time-dependent, contributing to the late phases of reperfusion injury. The neuronal cell apoptosis and necrosis that results from leukocyte activation products promotes further inflammation, which is accompanied by an accelerated decline in vascular perfusion due to plugging by leukocytes and platelet–leukocyte aggregates