Brain Pathology 10: 127-135(2000)

SYMPOSIUM: Role of Inflammation Following Stroke and Neurotrauma

Leukocyte Recruitment and the Acute Inflammatory Response

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Leukocyte recruitment is a hallmark feature of the inflammatory response. This review summarizes the generally accepted paradigm of leukocyte recruitment based on studies using intravital microscopy to visualize the microcirculation. The role of selectins and α_4-integrin in rolling as well as integrin-mediated adhesion is discussed. However, it is becoming increasingly clear that the recruitment cascade within organs differs and therefore the review also attempts to highlight what is and is not known regarding leukocyte recruitment into the brain microvasculature. In the second part of this review, we provide some discussion of mechanisms by which the inflammatory response may be terminated. Particular emphasis on nuclear factor NfκB and how IL10, IL13 and secreted leukocyte protease inhibitor (SLPI) may impact upon the NfκB-dependent inflammatory response is presented.

Introduction

It is becoming increasingly clear that the acute inflammatory response and specifically leukocyte recruitment, although essential to survival, can also severely harm the host. Most of the relevant information supporting this conclusion derives from studies that focus in organs other than the central nervous system (CNS). Many of the principles so far learned can be at least in part extrapolated to inflammatory responses in the CNS. Nevertheless, as will be seen some critical differences do indeed exist among organs. In this brief review, we will summarize some of the work pertaining to leukocyte recruitment and acute inflammation in various organs, but also comment on some of the information pertaining to acute inflammation in the brain. Areas that require further investigation will be highlighted.

Figure 1 demonstrates a schematic of the generally accepted cascade of events that lead to leukocyte recruitment. First, leukocytes moving at extremely high speed in the mainstream of blood make initial transient contact with endothelial cells lining the vessel wall and then roll along at a greatly reduced velocity relative to red blood cells. Only when leukocytes begin to roll can they firmly adhere and finally emigrate out of the vasculature. It should be noted that this is an interdependent series of events inasmuch as inhibiting leukocyte rolling prevents subsequent leukocyte adhesion and ultimately leukocyte emigration out of the vasculature (see reviews 2, 81). A number of cytokines and inflammatory mediators can rapidly mobilize the inflammatory response as well as induce various genes to further synthesize adhesive and inflammatory proteins. Not shown are potential anti-inflammatory systems which will be mentioned at the end of this review.

Leukocyte Recruitment

Leukocyte rolling is dependent upon the selectin family of adhesion molecules. P-selectin is induced in minutes in response to histamine (and other mediators) or following reperfusion of ischemic tissue and E-selectin (4-6 hrs for maximal induction) is expressed on LPS or TNFα-activated endothelium. L-selectin is found constitutively expressed on leukocytes. Each of these selectins contribute significantly to the rolling event at overlapping but also distinct times during inflammation (1, 9, 25, 35, 43 ,75). Although firm adhesion is mediated by the integrins including β_2-integrin (CD11/CD18) and β_1-integrin (see reviews 19, 8, 28), the β_1-integrin and more specifically, α_4-integrin can also mediate leukocyte rolling (3, 8) particularly in eosinophils and monocytes (54, 69). The identified lig-

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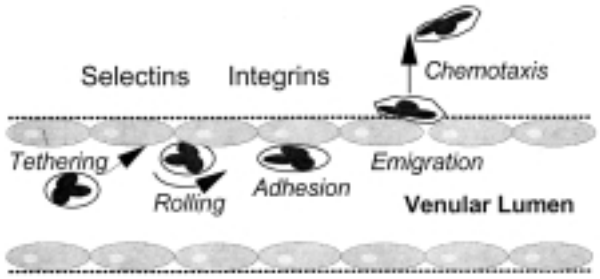

Figure 1.

ands for these adhesion molecules include ICAM-1 and VCAM-1 respectively. Finally, PECAM-1 is thought to be in part important in leukocyte emigration out of the vasculature (60).

Much of this work is derived from intravital microscopy which is a technique that permits visualization of very small post-capillary venules (20-40 μm diameters). To date the mesentery and cremaster muscle have been the primary tissues used because of their translucent properties and using these tissues, the universal concept of leukocyte recruitment of selectin-dependent rolling followed by integrin-dependent firm adhesion was proposed. However, it is becoming increasingly clear that this concept may not apply to all tissues and in fact each tissue studied may have its own profile of leukocyte recruitment. For example there is a growing body of evidence that neither the liver nor the lung always require selectins to recruit leukocytes (58, 89). Intravital microscopy of the liver revealed that much of the leukocyte recruitment occurs in the sinusoids rather than post-sinusoidal venules (89). The space constraints of these vessels do not allow leukocytes to roll and instead leukocytes squeeze through the capillaries. Not surprisingly, much of the adhesion occurs independent of selectins and rolling (89). Similarly, in the lung, in response to certain infectious agents, the three selectins do not contribute to neutrophil recruitment (58). This work underscores the importance of systematically examining leukocyte recruitment in the brain microvasculature. It is surprising how relatively little information is available regarding the adhesion molecule profiles and associated leukocyte recruitment in the brain microvasculature.

P-selectin

P-selectin is a very likely mediator of the early phase of leukocyte rolling and therefore leukocyte recruitment in acute inflammation. Lawrence and Springer (48) demonstrated that leukocytes exposed to shear forces, rolled on artificial lipid bilayers containing purified P-selectin but not with other adhesion molecules (ICAM-1). This selectin is stored in Weibel-Palade bodies of endothelial cells and is expressed within minutes on the cell surface following activation by a variety of inflammatory mediators including histamine, thrombin, LTC4 and oxidants (25). Histamine and thrombin could induce P-selectin-dependent leukocyte rolling on human umbilical vein endothelial cells (HUVECs) under shear conditions *in vitro* (35, 68) and similar results have been reported in rat mesentery *in vivo* for histamine (35, 46), thrombin (52, 93), LTC_4 (41) and oxidants (70). However, recent work has demonstrated that in the hepatic microvasculature, histamine even at 1000 μM did not induce leukocyte rolling whereas LTC_4 induced adhesion (no rolling) that was entirely P-selectin independent (12). I raise this fact to simply highlight the heterogeneity of responses in two different tissues. This raises the question as to what happens in the brain? The data however, are not very clear. Barkalow *et al*., (6) have cultured murine brain endothelium and have reported that there is no pre-stored pool of P-selectin. Moreover, the endothelium could not express P-selectin in response to histamine or a far more potent activator Ca2+ ionophore. However, we have reported that growing endothelium in culture dramatically reduces basal levels of P-selectin suggesting that this may be an issue of cell culture (44). Nevertheless, Barkalow and colleagues (6) also had difficulty demonstrating P-selectin in the murine brain microvasculature *in vivo*, similar to our own inability to induce rolling with histamine in mice (13).

In vivo, results by Yong *et al*., published last year, suggested that histamine superfusion on the pial microvasculature could induce P-selectin-dependent leukocyte recruitment in SJL/J but not BALB/c mice (91). As the former are a strain particularly susceptible to autoimmune disease, it is conceivable that P-selectin simply is not pre-synthesized in the brain microvasculature of commonly used mouse strains. However, Weber *et al*., (87) have reported in rat brain microvasculature that the early rolling in pial vessels in response to bacterial meningitis is dependent upon histamine and therefore a role for P-selectin may be inferred, suggesting additional variables in addition to strain differences. Other issues that need to be considered include 1) testing of numerous other P-selectin inducers and 2) potential problems associated with intravascular versus extravascular delivery of mediators across the blood-brain barrier. Finally, and most importantly it will be important to establish whether the P-selectin-dependent leukocyte recruitment leads to firm adhesion and if so which mediators is responsible.

Inhibition studies with anti-P-selectin therapy have been used successfully to reduce leukocyte rolling in a number of models of inflammation. For example, a role for P-selectin in ischemia/reperfusion (I/R)-induced leukocyte recruitment has been documented in many tissues (18, 24, 45, 88) and recent work suggests that P-selectin-deficient mice have no rolling in response to ischemia/reperfusion in striated muscle for at least 3 hrs (42). P-selectin expression has been shown to increase in the brain microcirculation following an ischemic episode (65) but only one study to date has examined a functional role for P-selectin. Connolly *et al*., reported that isolated, purified, radiolabeled neutrophils accumulated in postischemic brain and that there were fewer neutrophils accumulating in P-selectin-deficient mice (16). Although there was a subtle increase even at 30 min of reperfusion whether this was due to P-selectin-dependent rolling and subsequent adhesion, whether this was due to neutrophil plugging in capillaries or whether it was a result of trapped platelet-neutrophil aggregates was not determined. The issue of platelets is not trivial in light of the fact that platelets express P-selectin and may tether leukocytes to injured or activated endothelium (67, 86).

Initial work suggested that thrombin, histamine and LTC_4 all induce P-selectin expression for only a brief 30-60 min period (25, 46). However, functional evidence that P-selectin recruits leukocytes for many hours in response to antigen, TNFα, IL-4 and oncostatin M both in mouse and human now exists (33, 40, 53). P-selectin is thought to be dependent upon protein synthesis and is significantly increased at 8 hrs in the brain microcirculation of mice and remains elevated for at least 24 hrs in response to LPS (21). However, the role for P-selectin appears to diminish in more chronic inflammatory models. Although, leukocyte recruitment is important in 4-24 hr cytokine-induced meningitis it does not contribute to experimental autoimmune encephalomyelitis (20).

E-selectin

Minimal or no E-selectin is expressed on endothelium in non-inflamed tissue. De novo synthesis of E-selectin expression on the surface of endothelium occurs at 2-4 hrs following exposure to LPS, IL-1, or TNFα and lasts for at least 4-8 hours (1, 9). E-selectin expressed on transfected L-cells (fibroblasts), cytokine-treated endothelial cells, or substrates bearing purified E-selectin all support neutrophil rolling (1, 49). The role of E-selectin dependent leukocyte-endothelial cell interactions *in vivo* is somewhat controversial in part due to the profound, overlapping role of P-selectin in some tissues. Olofsson *et al*. (66), reported that prolonged IL-1 treatment of rabbit mesenteries supported leukocyte rolling via an E-selectin-dependent mechanism and TNFα causes E-selectin-dependent leukocyte rolling in mouse cremaster (47). By contrast, TNFα and LPS did not induce E-selectin dependent rolling in the cat mesentery (31) nor the rat mesentery (38) respectively potentially due to contribution from other selectins. Recently, a role for E-selectin in skin but not muscle was demonstrated for leukocyte recruitment in response to antigen challenge (30). Using a radiolabeled antibody system in this study it was demonstrated that E-selectin was only expressed in skin but not muscle. These data clearly demonstrate that different stimuli in the same tissue and the same stimuli in different tissues will evoke very different adhesion molecule profiles.

Only a limited number of studies have examined a role for E-selectin in cerebrovascular inflammatory models. E-selectin contributed to the recruitment of leukocytes in cytokine-induced meningitis (83) but not bacterial infection (59) or experimental autoimmune encephalomyelitis (20).

L-selectin

L-selectin is expressed constitutively on most leukocytes, and is involved in lymphocyte recirculation (22) and leukocyte-endothelial cell interactions at peripheral sites of inflammation (71, 80, 85). For example, leukocyte infiltration into the inflamed peritoneum is significantly reduced by intravenous (i.v.) administration of a mAb or soluble recombinant L-selectin (71, 85). In addition, L-selectin blockade by antibodies provides partial protection from acute inflammation in the heart, lung and other organs (55, 62, 73). L-selectin-deficient mice show a significant impairment in migration to the inflamed peritoneum, to non-specific skin irritants, as well as resistance to lipopolysaccharide induced septic shock (5, 14, 84). Additionally, these mice have an impairment in contact hypersensitivity responses to reactive haptens (84), an observation confirmed by others (14, 90). Whether the observed reduction in hapten-induced inflammation is due to early events in antigen sensitization or more delayed effector mechanisms remains unclear. One complicating factor in the study of this molecule is the lack of identity of the L-selectin ligand in the periphery. Bovine brain endothelium did appear to express an L-selectin ligand and lymphocyte-endothelium interactions were reduced with an L-selectin antibody in that study (39). However, to date, only one paper has attempted to examine a role for L-

selectin in a stroke model. In acute thromboembolic stroke an L-selectin antibody in the presence of tPA reduced some of the brain injury. No mention of leukocyte recruitment was made (7). Therefore, a more systematic role for L-selectin is warranted under acute conditions, following ischemia/reperfusion as well as in delayed leukocyte recruitment associated with cytokines. A caution here is that L-selectin likely has significant overlapping functions with the other selectins requiring assessment of the role of multiple selectins simultaneously (36, 43).

α-integrin/VCAM-1 interactions

Although it is generally accepted that leukocyte rolling and tethering are mediated by the selectin family of adhesion molecules and their carbohydrate ligands, there is at least one other pathway which is capable of supporting leukocyte rolling. Recently, a number of reports have suggested that the α_4-integrin ($\alpha_4\beta_1$, VLA-4) known to be involved in leukocyte adhesion, is also capable of mediating leukocyte rolling (3, 37, 82). Alon *et al*. (3) illustrated that the α_4-integrin on lymphocytes was capable of mediating tethering, rolling and firm adhesion on its ligand VCAM-1. It should be noted, however, that the entire sequence of events in this study occurred at a shear stress of less than 1 dyn/cm^2. At higher shear stresses where selectins can clearly tether leukocytes and support rolling, the α_4-integrin may not support leukocyte tethering to VCAM-1. On the other hand, α_4-integrin-dependent tethering and rolling may occur *in vivo* in the presence of red blood cells, which can facilitate leukocyte-endothelial cell interactions (76).

Indeed VCAM-1 was able to recruit monocytes, eosinophils and even some neutrophils from whole blood at relatively high shear forces *in vitro* (74). Another critical difference between *in vitro* and *in vivo* systems may be the site density and distribution of VCAM-1 for the α_4-integrin. The use of various *in vitro* substrata instead of microvascular endothelium may greatly underestimate the site density of ligands (eg., vascular cell adhesion molecule-1 [VCAM-1]) for α_4-integrins. Therefore, it is conceivable that under inflammatory conditions, α_4-integrin ligands may be expressed in sufficient numbers to gain the capacity to mediate leukocyte tethering, rolling and adhesion independent of selectins. In the rat mesentery it was shown that α_4-integrin could indeed support rolling, but the initial catching or tethering to the endothelium did not appear to be dependent on this pathway (37). Using mice deficient in both E- and P-selectin confirmed that either P-selectin or E-selectin were required for the initial tether after which α_4-integrin can mediate subsequent rolling in response to antigen (40). Recently, it was reported that IL-4 induced recruitment could be mediated via α_4-integrin in the absence of the other 3 selectins in cremaster muscle (29). Although the role of α_4-integrin has not been assessed in acute brain inflammation, in no organ has α_4-integrin therapy been more successful than in the brain raising the possibility that in this microvasculature the α_4-integrin pathway may dominate. Indeed, in the EAE model, neither E-selectin nor P-selectin were important in blocking leukocyte recruitment (20) and yet α_4-integrin dramatically reduced leukocyte recruitment (57) suggesting that at least endothelial selectins may not be required for α_4- integrin function in the brain. Nevertheless, a potential role for L-selectin cannot be dismissed. These data raise sufficient questions to investigate the importance of the α_4-integrin/VCAM-1 pathway in the brain in the different cytokine models and in ischemia/reperfusion. Indeed, since this is an important pathway for monocyte recruitment and monocytes have been shown to infiltrate postischemic brain this is a reasonable hypothesis to test.

Other adhesive pathways

Probably the best documented pathway is the ICAM-1/β_2-integrin pathway for leukocyte recruitment. In fact this pathway has been shown to be important in neutrophil recruitment in the brain microvasculature. ICAM-1 has been shown to increase in ischemia/reperfusion of the brain and antibodies directed against the β_2-integrin pathway or ICAM-1 have revealed significant inhibition as early as 1-2 hrs as well as in delayed reperfusion models (10, 56, 92). Moreover, the ICAM-1 and CD18 deficient mice also protected from cerebral ischemia/reperfusion (17, 72, 79).

A final pathway of leukocyte recruitment that has received limited attention *in vivo* is the leukocyte-endothelium interaction where platelets function as a bridging mechanism. The platelet attaches to endothelium via vW factor and numerous other adhesion molecules (see reviews 11). The platelets may then express P-selectin and tether leukocytes to their surface. In addition the platelets express numerous pro-inflammatory molecules including PAF which then cause neutrophils to tether and firmly adhere (67). *In vivo* work has implicated platelets as a potential "other" source of P-selectin-dependent leukocyte recruitment, however a systematic assessment of the importance of this pathway remains unknown. This pathway may be quite important in light of the fact that 1) platelets have been reported to

accumulate in significant numbers in cerebral ischemia (23) and 2) there is a significant amount of platelet adhesion in the inflamed brain circulation (4, 23). Clearly, investigating this pathway in thrombin and ischemia/reperfusion-induced leukocyte recruitment will be extremely important.

Finally, to date almost all leukocyte recruitment has been observed in small postcapillary venules. At this stage, the exception is the liver and lung which recruit leukocytes in capillaries/sinusoids (32). Whether physical trapping contributes to recruitment of leukocytes in capillaries and small venules of the brain remains entirely unknown.

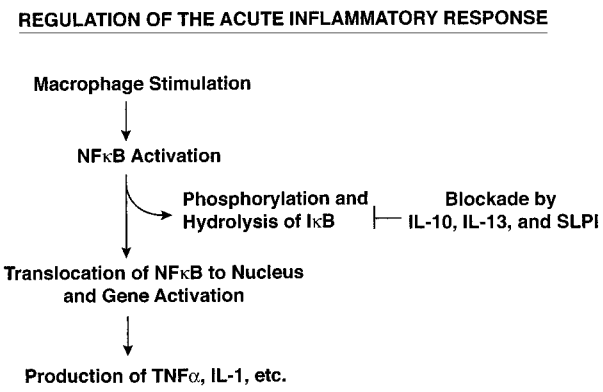

Figure 2.

Turning off the inflammatory response

Although much attention has been given to the mechanisms that activate their acute inflammatory reaction and the adhesion molecule profiles, the genes activated to reduce inflammation have not been studied in detail. We will limit this discussion to a single organ and use the lung as an example. When the acute inflammatory response is triggered in lung following airway deposition of IgG immune complexes (36), intensive gene activation occurs in a manner that is NFκB-dependent (51). The first wave of mediators generated includes the powerful pro- inflammatory cytokines, TNFα and IL-1. The chief function of these cytokines is to bring about upregulation of vascular adhesion molecules such as E-selectin and intercellular adhesion molecule-1 (ICAM-1) (15, 63), both of which react with "counter-receptors" on neutrophils to bring about both intermittent and sustained adhesive interactions between blood neutrophils and the activated endothelium as already summarized. Transmigration of neutrophils subsequently occurs, leading to release of toxic oxygen products and proteases from the infiltrating neutrophils (and from activated tissue macrophages), causing damage to cells and to extracellular matrix components.

The acute inflammatory response also triggers gene activation that results in production by tissue macrophages of IL-10 and IL-13. Both of these Ig's are *powerful anti-inflammatory products* which, when produced in adequate amounts, very efficiently down regulate the inflammatory response (64). IL-10 and IL-13 are known to have the ability *in vitro* to reduce macrophage production of pro-inflammatory mediators such as TNFα and IL-1. In the context of acute inflammatory responses *in vivo*, the endogenous release of IL-10 and IL-13 acts as a "brake" on the inflammatory responses, causing a rapid turn-off of *in vivo* production of TNFα and IL-1. This leads to a curtailment of the inflammatory response, including a rapid drop in tissue levels of TNFα, cessation in any additional recruitment of neutrophils, no further increase in expression of vascular adhesion molecules, and abrogation of any additional vascular damage (as reflected by extravascular leak of ^{125}I-albumin) (77). If blocking antibodies to IL-10 or IL-13 are employed *in vivo*, the inflammatory response becomes more intense, higher tissue levels of TNFα occur, more neutrophils are recruited, and tissue injury is magnified, supporting the concept that IL-10 and IL-13 function as endogenous regulators of the acute inflammatory response.

There is now information that explains how IL-10 and IL-13 function *in vivo* to suppress the inflammatory response. This can be understood by the information in Figure 2. When macrophages are activated *in vitro* or *in vivo*, this results in activation of NFκB, this process requiring phosphorylation of a protein, IκBα, which form a complex with NFκB and prevents the ability of NFκB to translocate into the nucleus where it binds with promoter regions of DNA (26). Phosphorylation of IκB sets the stage for its intracellular hydrolysis by a cytoplasmic 26S proteasome enzyme. Once accomplished, NFκB then translocates to its binding sites on DNA. This process of degradation is blocked by the presence of either IL-10 or IL-13, resulting in a sustained NFκB·IκB complex that prohibits translocation of NFκB into the nucleus (51). Thus, IL-10 and IL- 13 interfere with gene activation that is critical for initialization the acute inflammatory response.

A second class of factors is produced during the acute inflammatory response and has anti-inflammatory effects. These products include the *protease inhibitors*, tissue inhibitor of metalloprotease-2 (TIMP-2) and secreted leukocyte protease inhibitor (SLPI) (27, 61). As indicated above, proteases are incriminated as factors

that are released from phagocytic cells and cause tissue damage during the acute inflammatory response. TIMP-2 has its targets metalloproteases (MMP), 2 and 9, which are abundant in neutrophils and macrophages. Introduction into lung of blocking antibody to TIMP-2 significantly enhances the intensity of lung injury in a manner associated with increased recruitment of neutrophils (27). Precisely how the enzyme targets of TIMP-2 are involved in fitting into events of the acute inflammatory response is not clear.

The naturally occurring protease inhibitor, which inhibits serine proteases, is SLPI, which is also upregulated during the acute inflammatory responses (27, 34). Exogenous administration of SLPI depresses neutrophil accumulation tissues, caused reduced levels of TNFa and diminishes the extent of ensuing tissue injury (50). *In vivo* blockade of endogenous SLPI intensifies the intensity of the inflammatory response in lung and the degree of tissue injury. The key to understanding how SLPI regulates the acute inflammatory response is the observation that, like IL-10 and IL-13, SLPI impairs NFκB activation. Unlike IL-10 and IL-13, SLPI prevents in some unknown manner breakdown of IκBβ, another in the family of proteins that bind to NFκB and prevent its translocation to DNA binding site in the nucleus (50). Thus, IL-10, IL-13 and SLPI function as intrinsic regulators of the acute inflammatory response. Their involvement in regulation of acute inflammatory events in the CNS awaits definition.

The hope remains that once a full understanding is gained of what we term inappropriate inflammation, therapeutic intervention may become a real possibility. Although the adhesion molecules per se may become a therapeutic target, another very reasonable approach may be to suppress the genes that induce adhesive interactions. The latter may be possible using the body's own anti-inflammatory defense mechanisms.

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