A Role for Platelets and Endothelial Selectins in Tumor Necrosis Factor-α–Induced Leukocyte Recruitment in the Brain Microvasculature

Juliana Carvalho-Tavares, Michael J. Hickey, Jamie Hutchison, Jean Michaud, Ian T. Sutcliffe, Paul Kubes

Abstract—The mechanisms mediating leukocyte recruitment into the cerebral nervous system during inflammation are still poorly understood. The objective of this study was to investigate the leukocyte recruitment in the brain microcirculation by intravital microscopy. Superfusion of the brain with artificial cerebrospinal fluid did not induce leukocyte rolling or adhesion. However, intraperitoneal tumor necrosis factor-α (TNF-α) caused marked leukocyte rolling and adhesion in the brain microcirculation. Histology revealed that the recruitment was primarily of neutrophils. Both E- and P-selectin were required for TNF-α–induced leukocyte recruitment, as rolling was reduced after treatment with either anti–E- or anti–P-selectin antibody and eliminated in E- or P-selectin–deficient mice. A significant increase in brain P- and E-selectin expression was seen after TNF-α treatment, but both were an order of magnitude less than in any other tissue. We observed significant platelet paving of TNF-α–stimulated endothelium and found that anti-platelet antibody reduced leukocyte rolling and adhesion, as did acetylsalicylic acid (aspirin). However, depletion of platelets did not reduce cerebral P-selectin expression. Moreover, chimeric mice lacking P-selectin on endothelium but not platelets had significantly decreased P-selectin expression and reduced leukocyte recruitment in the brain. This suggests a role for endothelial P-selectin in cerebral leukocyte recruitment. In conclusion, TNF-α–induced neutrophil recruitment into the brain requires both endothelial E-selectin and P-selectin as well as platelets, but platelet P-selectin was not a major contributor to this process. (Circ Res. 2000;87:1141-1148.)

Key Words: selectin ■ tumor necrosis factor-α ■ neutrophil ■ inflammation ■ cerebral

Inappropriate leukocyte infiltration into tissues associated with stroke, multiple sclerosis, arthritis, and other inflammatory disorders have been postulated to cause tissue dysfunction. Visualization of leukocyte–endothelial cell interactions in vivo in inflamed vessels has revealed that leukocytes must first tether and roll along the venule before they can firmly adhere and emigrate out of the vasculature. Leukocyte tethering and rolling has been demonstrated to primarily be mediated by the selectin family of adhesion molecules. P-selectin is induced in minutes on endothelium stimulated by mediators such as histamine, H2O2, thrombin, C5a, and cysteinyl leukotrienes and supports early recruitment of leukocytes. E-selectin and P-selectin are also synthesized after stimulation with lipopolysaccharide, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), or antigen but require 2 to 4 hours for functional expression. In tissues studied to date, P-selectin or P-selectin and E-selectin pathways had to be inhibited to reduce lipopolysaccharide- or TNF-α–induced leukocyte recruitment. To our knowledge, an exclusive role for E-selectin (distinct from P-selectin) in leukocyte recruitment has not been demonstrated.

In addition to endothelium, platelets can also express P-selectin. This source of P-selectin can induce platelet-leukocyte aggregates after the induction of mesenteric ischemia/reperfusion. Moreover, Lehr and colleagues reported increased leukocyte-platelet aggregates and leukocyte rolling in response to oxidized lipoprotein infusion into the systemic circulation, and the platelet-leukocyte aggregates were dependent on P-selectin. Massberg et al reported that platelets did accumulate in the postischemic intestine, but whether platelets could function as a bridge to tether and adhere leukocytes in vivo was not addressed. In fact, a systematic assessment of the contribution of platelets and platelet P-selectin to leukocyte recruitment into inflammatory sites has not been done. Although a number of investigators have demonstrated in vitro that platelets immobilized to collagen can indeed support leukocyte rolling via platelet P-selectin, whether
platelets express P-selectin after binding injured or activated endothelium in vivo is not clear. Therefore, the endothelium-platelet-leukocyte pathway of leukocyte recruitment has been postulated but not demonstrated.

On the basis of findings that anti-platelet drugs such as acetylsalicylic acid (ASA) reduce brain inflammation, it is our hypothesis that platelets may contribute to leukocyte recruitment into the brain microvasculature. In fact, the brain microvasculature may be strikingly different from all other vascular beds. First, the brain is considered an immune, privileged organ because of the existence of the blood-brain barrier and therefore is thought not to induce very significant leukocyte recruitment. Anderson et al.20 reported a minimal amount of leukocyte recruitment into the brain parenchyma after proinflammatory cytokine (TNF-α, IL-1, or formyl-methionyl-leucyl-phenylalanine [fMLP]) injection into the brain, and Engelhardt et al.21 reported that selectins were not important in leukocyte recruitment into an experimental autoimmune encephalomyelitis model of brain inflammation. This group hypothesized that reduced expression of the selectins in the brain may be responsible for the lack of function. However, adhesion molecule upregulation (including E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and platelet endothelial cell adhesion molecule-1) has been described on endothelium in neural diseases including multiple sclerosis and stroke,22,23 and leukocyte recruitment is a common feature of these disorders.

Therefore in this study, we systematically assessed the mechanism(s) involved in leukocyte recruitment into the brain microcirculation by visualizing these events online using intravital microscopy and intervening in the recruitment process by immunoneutralization and gene-targeting approaches. The results reveal a novel essential role for E-selectin in cytokine-induced leukocyte recruitment into brain. Moreover, by visualizing the brain microvasculature, we reveal an as-yet-undescribed, critical role for platelets in TNF-α-induced leukocyte-endothelium interactions in inflamed brain microvessels.

Materials and Methods

Animals

All animals used in this study were C57BL/6 mice weighing between 20 and 30 g. Mice deficient in P-selectin and E-selectin were generated by gene targeting in embryonic stem cells as described previously and bred onto a C57BL/6 background.23,24 All of the animals were maintained on a purified laboratory diet in specific pathogen-free facilities. Animal protocols were approved by the University of Calgary Animal Care Committee and met the Canadian Council on Animal Care standards. All procedures were performed in accordance with the University of Calgary Animal Care Committee’s Policies and Procedures and the guidelines of the Canadian Council on Animal Care.

Intravital Microscopy in Mouse Brain

Animals were anesthetized by intraperitoneal injection of a mixture of 10 mg/kg xylazine (MTC Pharmaceuticals) and 200 mg/kg ketamine hydrochloride (Rogar/STB). The tail vein was cannulated to administer rhodamine 6G, an additional anesthetic, and experimental reagents as required. Rectal temperature was continuously monitored and maintained at 36–37°C using a heating pad (Fine Science Tools Inc). A craniotomy was performed with a high-speed drill (Fine Science Tools Inc) in the right parietal bone. Stripping the dura from the site exposed the brain pial vessels. This did not disrupt the vascular barrier of the pial microvasculature, as fluorescently labeled proteins remained within the vasculature. The surface of the brain was continuously superfused with artificial cerebrospinal fluid (CSF) (ionic composition in mmol/L: NaCl 132, KCl 2.95, CaCl₂ 1.71, MgCl₂ 0.64, NaHCO₃ 24.6, dextrose 3.71, and urea 6.7) at 37°C, pH 7.35. The superfuse was bubbled continuously with 10% O₂, 6% CO₂, and 84% N₂, which maintains a gas tension and pH comparable to the cerebral vessels in response to TNF-α-induced leukocyte-endothelium interactions in inflamed brain microvessels. The surface of the brain was continuously superfused with artificial cerebrospinal fluid (CSF) (ionic composition in mmol/L: NaCl 132, KCl 2.95, CaCl₂ 1.71, MgCl₂ 0.64, NaHCO₃ 24.6, dextrose 3.71, and urea 6.7) at 37°C, pH 7.35. The superfuse was bubbled continuously with 10% O₂, 6% CO₂, and 84% N₂, which maintains a gas tension and pH comparable to those of normal CSF.

Leukocyte Parameters

Animals were anesthetized by intraperitoneal injection of a mixture of 0.3 mg/kg ketamine hydrochloride (Rogar/STB). The tail vein was cannulated with a 20-gauge needle. Rectal temperature was continuously monitored using an infrared thermometer (Infra Red). A craniotomy was performed with a high-speed drill (Fine Science Tools Inc) in the right parietal bone. Stripping the dura from the site exposed the brain pial vessels. This did not disrupt the vascular barrier of the pial microvasculature, as fluorescently labeled proteins remained within the vasculature. The surface of the brain was continuously superfused with artificial cerebrospinal fluid (CSF) (ionic composition in mmol/L: NaCl 132, KCl 2.95, CaCl₂ 1.71, MgCl₂ 0.64, NaHCO₃ 24.6, dextrose 3.71, and urea 6.7) at 37°C, pH 7.35. The superfuse was bubbled continuously with 10% O₂, 6% CO₂, and 84% N₂, which maintains a gas tension and pH comparable to those of normal CSF.

Leukocyte Parameters

Animals received rhodamine 6G intravenously (0.3 mg/kg body weight) to label leukocytes. Rhodamine 6G–associated fluorescence was visualized by epi-illumination at 510 to 560 nm, using a 590-nm emission filter. A microscope (Optiphot-2; Nikon Inc) with ×20 lens (Nikon) was used to observe the microcirculatory events in the cerebral vessels. A silicon-intensified camera (model C-2400-08; Hamamatsu Photonics) mounted on the microscope projected the image onto a monitor, and the images were recorded for playback analysis using a videocassette recorder. The number of rolling and adherent leukocytes was determined offline during video playback analysis. Leukocytes were considered adherent to the venular endothelium if they remain stationary for a minimum of 30 seconds. Rolling leukocytes were defined as white cells moving at a velocity less than that of erythrocytes within a given vessel. Pial vessels with diameters ranging from 50 to 120 μm were used, as most adhesion occurred in vessels of these sizes. Because of the greater variability in size of these vessels (compared with that of other tissues studied using intravital microscopy), we expressed leukocyte adhesion as number of cells/mm². A significant amount of platelet deposition was also noted on endothelium. This was quantified as the percentage area of vessel covered by fluorescently labeled platelets.

Experimental Protocol

TNF-α (0.5 mg/animal IP) was administered 4 hours before surgical exposure of the brain microvasculature and initial recording of leukocyte rolling and adhesion. To determine whether TNF-α–induced leukocyte recruitment is mediated by selectins, animals were treated with an anti–P-selectin antibody (Ab) (RB 40.34; Pharmigen; 20 μg/animal) or with an anti–E-selectin Ab (9A9; 100 μg/animal), generously provided by Dr Barry Wolitzky (Hoffmann-La Roche Pharmaceuticals, Nutley, NJ). In addition, TNF-α–induced leukocyte recruitment was studied in P-selectin– and E-selectin–deficient animals. As very significant platelet recruitment was noted in the cerebral vessels in response to TNF-α, the animals were pretreated with rabbit anti-mouse thrombocyte serum (0.5 mL/kg; Accurate Chemical and Scientific Corp) 1 hour before TNF-α to determine whether platelets contributed to leukocyte recruitment. In an additional series of experiments, ASA (aspirin) (1 mg/10 g body weight IP; Sigma Chemical Co) was injected 60 minutes before TNF-α administration.

Generation of P-Selectin Chimeric Mice

Bone marrow was isolated from donor mice euthanized by spinal cord displacement. Recipient mice were irradiated with 2 doses of 500 rad (Gammacell 40 β-irradiation source), with an interval of 3 hours between the first and second irradiations. This protocol has previously been shown to destroy 99% of existing bone marrow cells. Cells (8 × 10⁶) of the donor bone marrow were injected into the tail vein of recipient, irradiated mice. In the following 8 weeks, the mice were kept in clean, germ-free microisolate cages to allow full humoral reconstitution. Preliminary work confirmed that ~99% of cells were derived from donor bone marrow as assessed using Thy.1.1 and Thy.1.2 congenic mice. To test for a selective role for platelet P-selectin, 8 weeks after bone marrow transplant, mice expressing P-selectin on platelets but not on endothelium were used to assess leukocyte recruitment by intravital microscopy.
P- and E-Selectin Expression in the Brain and Other Tissues

Expression of P-selectin and E-selectin was quantified in the brain, as well as in other organs including lung, heart, cremaster muscle, small intestine, and skin in untreated mice and at 4 hours after TNF-α injection. We used a quantitative dual-radiolabeled Ab technique as previously described. Briefly, animals were injected intravenously with a mixture of either 10 µg 125I-labeled anti-P-selectin (RB40.34) or 10 µg 125I-labeled anti-E-selectin (10E6) Ab, and a dose of 131I-labeled nonbinding Ab (P-23) was calculated to achieve a total injected 131I activity of 400 000 to 600 000 cpm (total volume 200 µL). The Abs were allowed to circulate for 5 minutes, and then a blood sample was obtained from a carotid artery. The mice were exsanguinated by blood withdrawal through the carotid artery catheter and simultaneous intravenous infusion of bicarbonate-buffered saline. Whole organs were harvested and weighed. Both 111I and 125I activities were measured in plasma and tissue samples. Both P-selectin and E-selectin expression were calculated per gram of tissue by subtracting the accumulated activity of the nonbinding Ab (131I-labeled P-23) from the accumulated activity of the binding Ab (125I-labeled RB40.34 or 125I-labeled 10E6). Data for E-selectin and P-selectin expression were represented as the percentage of the injected dose of Ab per gram of tissue. The radioactivity can be displaced with cold Ab. As there are 2 sources of P-selectin (endothelial and platelet), we also report herein that this technique detects primarily endothelial P-selectin, as chimeric mice lacking P-selectin on endothelium (not platelets) had almost no detectable radioactivity in tissues. Finally, the P-selectin and E-selectin Ab bind primarily in postcapillary venules as assessed by immunohistochemistry. 28 This approach can detect small but significant amounts of constitutive P-selectin in wild-type mice relative to no P-selectin in P-selectin–deficient mice. 27

P-Selectin Detection on Platelets

In additional experiments, mice were anesthetized as previously described, and blood was drawn. Platelets were collected from blood treated with acid-citrate-dextrose by density centrifugation and analyzed for P-selectin using an anti-P–selectin Ab (RB40.34) labeled with FITC (Pharmigen). Samples were subjected to flow cytometry analysis using a fluorescence-activated cell sorter (FACScan; Becton Dickinson). Ab IgG2Ak was used as an isotype control.

Histology

Brains were removed quickly after intravital microscopy and placed in 10% formalin. The brain was divided into 4 coronal blocks, embedded in paraffin, sectioned (4 µm), and stained with hematoxylin-phloxin-saffron. Brain sections were examined in a blinded fashion by a neuropathologist and microscopist.

Statistical Analysis

Data are presented as mean±SE. A one-way ANOVA and Student t test with Bonferroni correction were used for multiple comparisons. Statistical significance was set at P<0.05.

Results

TNF-α–Induced Leukocyte Recruitment Is Dependent on Both Endothelial Selectins

Exposure of the murine pial microvasculature to CSF revealed essentially no baseline rolling in any of the vessels over the first 30 minutes with a slight increase in rolling at 60 minutes (Figure 1A). No adhesion occurred over this time (Figure 1B), consistent with the view that this approach did not cause significant preparation-induced inflammation. Figures 1A and 1B demonstrate that 4 hours of TNF-α by contrast caused a significant increase in both leukocyte rolling and leukocyte adhesion, the latter increasing essentially from no adherent leukocytes to >500 cells/cm². Histology revealed that most of the recruited cells in the brain sections examined were neutrophils, although some monocytes were also noted (Figure 2), suggesting a consistent response to TNF-α throughout this tissue.

In P-selectin–deficient mice, the number of rolling and adherent cells was decreased to near control values (Figures 3A and 3B), suggesting a very important role for this adhesion molecule in TNF-α–induced leukocyte recruitment into the brain microvasculature. Although E-selectin alone has not been shown to play a notable role in leukocyte recruitment, particularly in models in which P-selectin plays the dominant role, 13,14,29 for completeness we examined the role of E-selectin. Surprisingly, the E-selectin–deficient mice also revealed very little rolling and a significant reduction in adhesion in the brain microvasculature (Figures 3C and 3D). To confirm this observation, we also tested the effects of a P-selectin and an E-selectin Ab in the TNF-α–induced leukocyte recruitment (Table). There was a reduction in leukocyte rolling and adhesion in animals treated either with P-selectin or E-selectin Ab, albeit that the phenotype was not as profound as that noted in the selectin-deficient mice. A simple explanation may be the more complete inhibition of
the selectins in the gene-deficient animals than in the Ab-treated mice.

Consistent with the rolling data is quantitative expression of selectins within the brain microvasculature (Figures 4A and 4B). There is almost no basal P-selectin expression and no E-selectin expression in the noninflamed brain microvasculature, consistent with no basal rolling in this tissue. With TNF-α, a 6-fold increase in P-selectin and a significant increase in E-selectin were noted. For comparison, Figures 4C and 4D reveal that the lung per unit weight expresses 20 times more E-selectin and 50 times more P-selectin than that of the brain. Other tissues, such as cremaster muscle, which has been used to visualize leukocyte responses to TNF-α, also reveal at least a 10- to 20-fold greater expression of endothelial selectins per unit weight relative to brain.

Platelets Also Contribute to Leukocyte Recruitment in the Brain Microvasculature

Visualization of leukocyte recruitment using intravital microscopy revealed that in addition to leukocytes, platelets were a dominant cell recruited to the surface of brain microvascular endothelium, an event not noted in, for example, the liver or cremaster muscle in response to TNF-α. Platelets covered 13.5 ± 4.0% of the venules under study in the TNF-α–treated brain microvasculature. A platelet-depleting Ab significantly decreased the platelet-endothelium interactions (2.6 ± 0.6%) and significantly reduced leukocyte rolling and adhesion in response to TNF-α administration (Figures 5A and 5B). The anti-platelet Ab depleted platelets

### Effects of P- and E-Selectin Antibodies in the TNF-α–Induced Leukocyte Recruitment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Rolling, No. Cells/Field</th>
<th>Adhesion, No. Cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (0.5 μg/animal)</td>
<td>14.33 ± 6.59</td>
<td>431.12 ± 81.59</td>
</tr>
<tr>
<td>TNF-α + P-selectin Ab (RB40)</td>
<td>0 ± 0</td>
<td>168.85 ± 64.20</td>
</tr>
<tr>
<td>TNF-α + E-selectin Ab (9A9)</td>
<td>5.61 ± 3.38</td>
<td>292.62 ± 95.78</td>
</tr>
</tbody>
</table>

Figure 3. The role of P- and E-selectin in TNF-α–induced leukocyte recruitment. P- and E-selectin–deficient (KO) animals were treated with TNF-α, and both leukocyte rolling (A and C) and adhesion (B and D) were assessed. *P < 0.05 relative to same time point in control group.
by 96.5%, whereas circulating leukocyte counts were not affected, suggesting that the anti-platelet serum depleted platelets specifically and that the platelets played an important role in the leukocyte recruitment in the cerebral vasculature. To further verify this potentially important role of platelets, we also treated animals with the therapeutic agent ASA (the therapeutic of choice for prevention of strokes) at concentrations known to impair platelet function in rodent systems. ASA inhibited platelet adhesion to endothelium (1.5 ± 0.3) and in part reduced the leukocyte recruitment (Figures 5C and 5D).

Figure 5. The effect of anti-platelet Ab and aspirin in TNF-α–induced leukocyte recruitment. Shown are numbers of rolling and adherent leukocytes in TNF-α–treated animals with and without the anti-platelet Ab (A and B) or aspirin (C and D) administered at the start of TNF-α treatment. *P<0.05 relative to TNF-α alone at the same time point.

Platelet P-Selectin Does Not Contribute to TNF-α–Induced P-Selectin Expression or Leukocyte Recruitment

Chimeric mice with P-selectin only on platelets or on endothelium were made. No P-selectin was expressed by platelets 8 weeks after transfer of P-selectin−/− marrow into wild-type mice (Figure 6). By contrast, P-selectin−/− mice receiving wild-type marrow had P-selectin expression detected on the surface of the majority of platelets (Figure 6). Chimeric mice lacking P-selectin on endothelium had a very significant reduction in rolling (Figure 7A) and 50% reduction in adhesion (Figure 7B) on brain endothelium, suggesting that

Figure 4. Quantitative expression of P- and E-selectin; data using the dual-radiolabeled technique showing P- and E-selectin expression in the brain with and without TNF-α (A and B). For comparison, numerous other tissues (C and D) were also examined for P-selectin and E-selectin in untreated animals and in TNF-α–treated animals. *P<0.05 relative to baseline group (A and B) and relative to brain values (C and D). % I.D./g indicates percentage of the injected dose of Ab per gram of tissue.
platelet P-selectin could not recruit leukocytes effectively in the absence of endothelial P-selectin.

Figure 7C shows that depletion of platelets did not reduce the expression of P-selectin within the inflamed brain microvasculature, whereas chimeric animals with no endothelial P-selectin had almost no P-selectin expression in the inflamed brain microvasculature. Clearly, endothelium was the dominant source of P-selectin expression within the brain microvasculature and was necessary for leukocyte recruitment (Figures 7A and 7B). Adhering platelets may release various proinflammatory products that could affect adhesion molecule expression. Because depletion of platelets did not reduce P-selectin (Figure 7C) or E-selectin (data not shown) on the surface of brain endothelium of TNF-α-treated mice, our data suggest that secretory products from adhering platelets could not account for the adhesion molecules expressed on endothelium.

**Discussion**

In this study, we have examined the mechanisms of action underlying cytokine–induced recruitment of leukocytes in the brain microvasculature. The results suggest that both P-selectin and E-selectin are expressed and absolutely required for the delivery of neutrophils to the inflamed brain microvasculature. These data are unlike any other reported with respect to P-selectin and E-selectin. In murine tissue examined to date, an exclusive role for E-selectin has not been observed. This includes cremaster, skin, mesentery, and liver. The use of intravital microscopy also permitted us to document a role for platelets as tethers between the endothelium and leukocytes. However, the adherent platelets were not responsible for the P-selectin expression on brain endothelium, and endothelial rather than platelet P-selectin was involved in the neutrophil recruitment.

Direct examination of leukocyte adhesion to cerebral endothelium in response to cytokines has been examined in vitro. Barkalow et al observed that 4 to 24 hours of IL-1β and TNF-α yielded an induction of P-selectin. An adhesion assay performed under static conditions revealed a very significant increase in neutrophil adhesion to IL-1 or IL-1/TNF-α–treated brain endothelium in both wild-type murine microvascular endothelium and in P-selectin–deficient murine microvascular endothelium. Only a 36% decrease in
adhesion was noted on cultured brain endothelium from P-selectin–deficient versus wild-type mice. On the basis of our data, it might be expected that no adhesion would be seen on P-selectin–deficient endothelium. However, the key difference in these studies is that the in vitro work was performed under static conditions rather than under shear conditions. Static conditions diminish the importance of selectins, as tethering and rolling can be bypassed such that most of the adhesion in the static assay is CD18 dependent.

Although a role for P-selectin in leukocyte recruitment has been reported in a number of models of brain inflammation, including (1) a TNF-α and IL-1β mixed-cytokine infusion, (2) traumatic brain injury, and (3) permanent middle cerebral artery occlusion, a role for E-selectin per se has not been noted. The only study to our knowledge is that of Tang et al., who reported that P-selectin/E-selectin–double-deficient mice had greater inhibition of leukocyte recruitment into the brain microvasculature than did P-selectin–deficient mice. Data to date from the E-selectin–deficient mice have not revealed impairments in the development of inflammatory responses. However, E-selectin has been purported to contribute to the leukocyte rolling velocity, as a lack of slow rolling in E-selectin–deficient mice has been identified in the cremaster muscle. Our data for the first time demonstrate that leukocyte recruitment is impaired in E-selectin–deficient mice within the brain microvasculature, and this impairment is due to a reduction in rolling leukocytes leading to few adhering cells. These data suggest that E-selectin was directly contributing to rolling and subsequent adhesion in the brain microvasculature.

A likely reason for the need for both endothelial selectins is perhaps the low expression level of either selectin in the brain. Indeed, because rolling in the cerebral microvasculature was reduced in each of the mutant mice in response to TNF-α, the data would suggest that in the absence of P-selectin, E-selectin expression was not sufficient to recruit leukocytes, and similarly in the absence of E-selectin, insufficient P-selectin expression must exist. Because other organs do not display an essential need for both selectins, we hypothesized that in other organs there is sufficient expression of either endothelial selectin to compensate for a lack of the other selectin. Our data reveal that the brain expressed less P-selectin and E-selectin than any other organ examined. In fact, the lung, mesentery, and muscle expressed 20 to 50 times more endothelial selectins than the brain. The vascular density of the brain is certainly at least as large as muscle or mesentery, so lower vascularity per weight tissue in brain cannot explain the lower adhesion molecule expression. Clearly, the brain microvasculature responds with a much less robust P-selectin and E-selectin expression than other tissues, and this may explain the absolute dependence on both selectins for leukocyte recruitment.

Our data strongly suggest an important role for platelets in the leukocyte recruitment into the inflamed brain microvessels. This contention is based on the significant reduction in leukocyte recruitment into inflamed cerebrovascular in mice depleted of platelets. The fact that platelet depletion did not alter P-selectin expression in the brain, and chimeric mice lacking P-selectin on endothelium lacked significant levels of P-selectin in inflamed brain microvessels, argues against the adherent platelets contributing significantly to the P-selectin expression observed in our model of cerebrovascular inflammation. In vitro work has clearly demonstrated that platelets adherent to extracellular proteins express copious amounts of P-selectin for recruitment of leukocytes. By contrast, activated brain endothelium must not provide the same stimulating signals to platelets as extracellular matrix, as endothelium-adhering platelets did not express P-selectin in significant quantities in this study. Alternative platelet molecules, including GPⅡbⅢa or GPⅠb, may contribute to leukocyte recruitment in this model.

These data may have some very real therapeutic implications. At present, ASA is used for stroke prevention as well as perioperative stroke management. Although the mechanism of action is certainly inhibition of platelet function, our data would extend this to suggest that the reduction in platelet adhesivity directly impacts on leukocyte recruitment into the brain microvasculature. Interestingly, our data suggest more favorable reductions of leukocyte recruitment with anti-selectin therapy than with ASA (Figure 3 versus Figure 5), raising the possibility that using anti–E-selectin therapy prophylactically to prevent perioperative strokes may be useful. In fact, E-selectin therapy may be useful to enhance the anti-inflammatory efficacy of ASA. E-selectin rather than general selectin inhibition is also appealing, as this approach to date appears to have no antiadhesive and hence immunosuppressive activity in other organs.

In summary, the data in this study provide evidence that anti-selectin therapy as well as anti-platelet therapy could conceivably reduce the leukocyte recruitment into brain microvasculature after stroke, in sepsis, and perhaps in trauma-associated brain pathologies. The importance of platelets in leukocyte recruitment in brain microvasculature is underscored by the benefit provided by ASA in stroke patients and the lower incidence of leukocyte recruitment in our study with ASA. The observation that E-selectin or P-selectin are sufficient to reduce leukocyte recruitment in the brain but not in other tissues may also be useful to target the brain without necessarily impacting on the ability of the immune system to counter the development of ongoing systemic infections.

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