Postischemic Cerebrovascular E-Selectin Expression Mediates Tissue Injury in Murine Stroke

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Background and Purpose—Although the deleterious role of several proinflammatory mediators, including P-selectin, in reperfused stroke is well established, the role of E-selectin has not been fully characterized.

Methods—E-selectin mRNA expression was studied at 4, 10, and 24 hours after reperfusion with reverse transcription and polymerase chain reaction in mice (n=18) subjected to transient intraluminal middle cerebral artery occlusion (MCAO). Mice received intravenous injection with anti–E-selectin monoclonal antibody (10, 35, or 50 μg), nonimmune IgG, or vehicle immediately before MCAO and 90 minutes later (n=85). Others received anti–E-selectin antibody 3 or 6 hours after MCAO (n=32). Myeloperoxidase activity was measured in sham-operated mice and after 10 hours of reperfusion in saline-, nonimmune IgG-, or anti–E-selectin IgG–treated cohorts (n=17). Serial cerebral blood flow was measured with laser-Doppler flowmetry, and outcomes were assessed by neurological deficits and infarct volumes with the use of planimetric analysis of triphenyltetrazolium chloride–stained sections.

Results—Upregulated E-selectin expression occurred in the ischemic cerebral vasculature within 4 hours of reperfusion and persisted for 24 hours. Anti–E-selectin antibody increased ischemic cortical cerebral blood flow up to 2.6-fold (P<0.05). In addition to dose-dependent reductions in neurological deficits (P<0.05), mortality, and infarct volumes (P<0.01 for 35 and 50 μg), anti–E-selectin treatment reduced cerebral neutrophil accumulation (P<0.05) and was neuroprotective even if delayed until 3 hours after ischemia (P<0.05).

Conclusions—These findings establish a functional role for E-selectin in the pathogenesis of tissue injury after cerebral ischemia and reperfusion and suggest that E-selectin blockade may be clinically useful in the treatment of reperfused stroke.

Key Words: cerebral ischemia, focal ■ cerebral ischemia, transient ■ E-selectin ■ gene expression ■ mice

Current therapeutic strategies to reduce or prevent acute ischemic stroke damage are limited. A 3-hour window of administration limits even the best-approved therapy, recombinant tissue plasminogen activator. Furthermore, even if reperfusion can be achieved with recombinant tissue plasminogen activator or other thrombolytic agents, the reperfusion itself can precipitate further cerebral injury. A number of studies have established the role of leukocyte adhesion receptors in cerebral reperfusion injury. In a murine model of stroke, neutrophil-depleted wild-type, homoyzous null intercellular adhesion molecule–1 (ICAM-1)–deficient, and homoyzous P-selectin null animals have smaller strokes, reduced microvascular failure, and improved functional outcomes. The contribution of leukocytes to ischemic cerebral tissue injury appears to be most pronounced in the setting of reperfusion, which reintroduces polymorphonuclear neutrophils (PMNs) into an adhesion receptor–rich neurovascular milieu. Despite evidence that absence of the ICAM-1 or P-selectin genes leads to improved regional cerebral blood flow, some degree of no-reflow still exists, suggesting that other mediators may be operational. Others have shown that E-selectin is upregulated after focal cerebral ischemia in a variety of animal models; nonetheless, the functional significance of this upregulation remains incompletely understood.

To explore the pathophysiological role of E-selectin in reperfused stroke, we used a murine model of transient focal middle cerebral artery occlusion (MCAO). Using this model, we tested the hypothesis that E-selectin expression is increased and contributes to leukocyte recruitment, postischemic hypoperfusion, and tissue injury in stroke.

Materials and Methods

Mice

Experiments were performed with C57BL/6 wild-type mice (Jackson Labs, Bar Harbor, Maine). Animals were aged 7 to 9 weeks and
weighed between 22 and 26 g. They were housed in a certified animal care facility and maintained on a standard laboratory diet. All experiments were conducted in a humane manner and approved by the Columbia University Institutional Animal Care and Use Committee.

**Murine Transient Cerebral Ischemia Model**

The details of the murine model of focal cerebral ischemia using an intraluminal suture have been described previously. In brief, mice were anesthetized with 0.3 mL of intraperitoneal ketamine (10 mg/mL) and xylazine (0.5 mg/mL) and positioned supine on a rectal temperature–controlled operating surface (Yellow Springs Instruments, Inc). Animal core temperature was maintained at 37 ± 2°C during surgery and for 90 minutes after surgery. A midline neck incision was created to expose the right carotid sheath under the operating microscope (6 to 40 zoom, Leica). The common incision was created to expose the right carotid sheath under the microscope during surgery and for 90 minutes after surgery. A midline neck incision was created to expose the right carotid sheath under the operating microscope (×6 to ×40 zoom, Leica). The common carotid artery was isolated with 4-0 silk, and the occipital, pterygopalatine, and external carotid arteries were each isolated, cauterized, and divided. MCAO was accomplished by advancing a 13-mm heat-blunted 6-0 nylon suture via an arteriotomy made in the external carotid stump. After placement of the occluding suture, the external carotid artery was cauterized to prevent bleeding through the arteriotomy, and arterial flow was established. After 45 minutes the occluding suture was removed, and electrocautery was used to close the arteriotomy. The wound was closed with surgical staples. Sham-operated animals underwent carotid artery exposure and ligation of the occipital, pterygopalatine, and external carotid arteries without placement of the suture.

Serial measurements of blood pressure and heart rate were obtained for mice in each of the treatment cohorts (Columbus Instruments). For hematological and arterial blood gas analyses, 0.6 to 0.9 mL of blood was withdrawn from the left ventricle for each mouse.

**Administration of Anti–E-Selectin Antibody**

A cohort of mice was given either saline vehicle (100 μL; n = 21), nonimmune IgG (35 μg in 100 μL normal saline; n = 27), or rat anti-mouse monoclonal antibody (Pharmergen; clone: 10E9.6; iso-type: Lewis rat IgG2a; 10 μg [n = 8], 35 μg [n = 21], or 50 μg [n = 8] in 100 μL normal saline) immediately before and 90 minutes after ischemia. Separately, mice were given nonimmune IgG (50 μg in 100 μL normal saline; n = 16) or anti–E-selectin IgG (50 μg in 100 μL normal saline) at 3 hours (n = 8) or 6 hours (n = 8) after MCAO. All injections were intravenously administered via the dorsal penile vein.

**Measurement of Cerebral Blood Flow**

Transcranial measurements of cerebral blood flow were made by laser-Doppler flowmetry (Perimed, Inc), as previously described. With a 0.7-mm straight laser-Doppler probe (model PF 303, Perimed) and previously published landmarks (2 mm posterior to the bregma, 6 mm to each side of the midline), relative cerebral blood flow measurements were made as follows: after anesthesia, immediately after occlusion, before reperfusion, immediately after reperfusion, and at time of death. Data are expressed as the ratio of the Doppler signal intensity of the ischemic compared with the nonischemic, contralateral hemisphere. Although this is a ratiometric and not absolute measure of blood flow per gram of tissue, it allows for the comparison of cerebral blood flow in the same animal over time. The surgical procedure was considered technically adequate if a ≥70% reduction in cerebral blood flow was observed immediately after placement of the intraluminal occluding suture. These methods have been used in previous studies. Neurological Examination

After both 90 minutes and 24 hours of MCAO and reperfusion, mice were assessed for neurological deficit with a 4-tiered grading system. A score of 1 was given if the animal demonstrated normal spontaneous movements; a score of 2 was given if the animal was circling clockwise when viewed from above while receiving a noxious stimulus; a score of 3 was given if the animal was observed to spin clockwise on a longitudinal axis including the tail; and a score of 4 was given if the animal was crouched on all fours unresponsive to noxious stimuli. This scoring system has been described previously and has been shown to correlate with infarct volume.

**Calculation of Infarct Volumes**

After neurological examination, mice were anesthetized, and final cerebral blood flow measurements were obtained. The animals were decapitated, and brains were removed intact and placed in a mouse brain matrix (Activational Systems Inc) for 1-mm sectioning. Sections were immersed in 2% triphenyltetrazolium chloride (Sigma) in 0.9% saline and incubated for 12 minutes at 37°C. Infarcted brain was identified as an area of unstained tissue. Infarct volumes were calculated from planimetric analysis of digitized images of serial cerebral sections and expressed as the percentage of infarct in the ipsilateral hemisphere. This method of calculating infarct volumes has been used previously by our group.

**Polymerase Chain Reaction and E-Selectin Expression**

Total cellular RNA was prepared from cortical samples from the following: normal (n = 2), sham-operated (n = 4), 4-hour reperfusion (n = 4), 10-hour reperfusion (n = 4), and 24-hour reperfusion (n = 4) groups. Total RNA was extracted from the ipsilateral and contralateral hemispheres at the indicated time points after MCAO with the use of TRIZOL reagent (GIBCO BRL). Total cellular RNA (2 μg) from each sample was reverse transcribed with 200 U of RNAse H-SuperScriptII reverse transcriptase (GIBCO BRL) for 50 minutes at 42°C primed with 0.5 μg of oligo(dT) 12-18 (GIBCO BRL) at conditions recommended by the manufacturer. Reverse transcription products were then digested with RNase H at 37°C for 20 minutes to remove the RNA template from the cDNA:RNA hybrid molecule and stored at −20°C until ready for polymerase chain reaction (PCR) amplification. PCR primers used for amplification of E-selectin and β-actin were synthesized according to published sequences (Table 1). The predicted lengths for E-selectin and β-actin PCR fragment are 1408 and 540 bp, respectively. The positions are those given in the published cDNA sequences for mouse E-selectin and β-actin. According to our experiment, the β-actin mRNA expression has been shown to be constant throughout the time course after MCAO. We used it as an internal PCR control for normalizing the degree of expression in a quantitative manner. To do so, the control experiment was performed to identify the optimal amount of cDNA and the number of cycles for both genes to be in the linear portion of amplification. The optimal amounts of primers for both genes were added to adjust the relative intensity for the coamplification. On the basis of these results, the PCR condition for our experiment is as follows: in 50 μL reaction mixture containing reverse transcription product from 0.2 μg RNA; 1 μmol/L each of sense and antisense primers for E-selectin and 20 nmol/L of each for β-actin; 2.5 U of platinum Taq DNA polymerase (GIBCO BRL); initial denaturation, 3 minutes at 94°C followed by 30 cycles of denaturation, 30 seconds at 94°C; annealing, 30 seconds at 60°C; and extension, 2 minutes at 72°C. PCR product (10 μL) was loaded and electrophoresed through a 1% agarose gel and photographed.

**Table 1. PCR Primers Used for Amplification of E-Selectin and β-Actin**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>S/A</th>
<th>Position</th>
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<tr>
<td>E-selectin</td>
<td>5'-GTGGCCTGTAGGGGCTCTCTGG-3'</td>
<td>S</td>
<td>794-813</td>
</tr>
<tr>
<td></td>
<td>5'-CTCTCTGCTGGCACGTGTTTC-3'</td>
<td>A</td>
<td>2183-2202</td>
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<tr>
<td>β-Actin</td>
<td>5'-GTGGCCCTGCTTAGGCACCAA-3'</td>
<td>S</td>
<td>25-45</td>
</tr>
<tr>
<td></td>
<td>5'-CTTTTGATGTCCAGCGATTTTC-3'</td>
<td>A</td>
<td>541-564</td>
</tr>
</tbody>
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Brain Myeloperoxidase Assay
Myeloperoxidase (MPO) (a leukocyte granule–specific lysosomal enzyme) activity was measured in brain tissue homogenates in sham-operated mice (n=5) and in mice that received either vehicle (n=4), rat nonimmune IgG (n=4), or antibody to E-selectin (n=4) as a marker of leukocyte influx. Brains were isolated, separated by hemisphere, and bathed in 5 mL of hexadecyltrimethylammonium bromide per gram of tissue. Brains were homogenized for 30 seconds, frozen at −80°C, and then thawed at room temperature for 30 minutes. Brains were then centrifuged at 40,000g for 15 minutes at 4°C. The supernatant (33 μL) was added to 970 μL of substrate buffer containing o-dianisidine dihydrochloride and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm over 3 minutes was measured spectrophotometrically. One unit of MPO activity was defined as the degradation of 1 μmol of hydrogen peroxide per minute. Hemispheric results are expressed as units of MPO activity per gram of tissue.

Results
Physiology
There were no differences in mean arterial blood pressure, heart rate, pH, Pco2, Po2, hematocrit, and white blood cell count for the control and treatment cohorts that received anti–E-selectin antibody at 10, 35, or 50 μg (Table 2).

E-Selectin Expression in Murine Stroke
The temporal expression of E-selectin mRNA was studied at 4, 10, and 24 hours after reperfusion with reverse transcription and PCR. Compared with nonoperated and sham-operated control animals, there was increased contralateral E-selectin expression at all 3 time points. Mild contralateral E-selectin expression was likewise noted at all 3 time points.

Role of E-Selectin in Cerebrovascular No-Reflow Phenomenon
Serial measurements of relative cerebral blood flow were obtained by laser-Doppler flowmetry in mice treated with vehicle, rat nonimmune IgG, and anti–E-selectin antibody (10, 35, or 50 μg). For a representative dose of anti–E-selectin antibody (35 μg) before initiation of ischemia (Figure 1, top panel, time point A), baseline relative cerebral blood flows were nearly identical between groups, as were the reductions immediately after MCAO (Figure 1, top panel, time point B). Immediately before withdrawal of the intraluminal occluding suture after 45 minutes of ischemia (Figure 1, top panel, time point C), cerebral blood flows remained similar between groups. Immediately after withdrawal of the occluding suture to initiate reperfusion (Figure 1, top panel, time point D), cerebral blood flows in the anti–E-selectin IgG group were greater than those in the saline control group, which remained flat. These early differences became even more pronounced after 24 hours of reperfusion, when cerebral perfusion was greater in the animals treated with anti–E-selectin IgG than in cohorts treated with either nonimmune IgG or saline (Figure 1, top panel, time point E; P<0.005).

Cerebral perfusion at 24 hours of reperfusion, immediately before the animals were killed, was markedly improved by anti–E-selectin IgG treatment before the ischemic injury (Figure 1, bottom panel; before MCAO for 10-, 35-, and 50-μg doses: 43±2%, 47±2%, and 45±4% versus saline at 18±2% and nonimmune IgG controls at 30±4%; P<0.05). Even when anti–E-selectin IgG treatment was administered after stroke with a 3- or 6-hour delay, cerebral blood flow was increased by 53% (Figure 1, bottom panel; 3 hours after MCAO: control IgG 36±1% versus 55±1%; P<0.0001) and 58% (Figure 1, bottom panel; 6 hours after MCAO: control IgG 31±1% versus 50±2%; P<0.0005).

Effect of E-Selectin Blockade
The therapeutic efficacy of E-selectin blockade was evaluated by comparing the following indices of stroke outcome: neurological deficit (Figure 2, top panel), mortality (Figure 2, middle panel), and infarct volume (Figure 2, bottom panel) in mice pharmacologically treated with vehicle, rat nonimmune IgG, or anti–E-selectin antibody. Because of the relatively brief half-life of the monoclonal anti–E-selectin antibody, agents were administered immediately before the surgery and 30 minutes after reperfusion. Mice treated with anti–E-selectin antibody were significantly protected from the effects of focal cerebral ischemia and reperfusion injury in a dose-dependent fashion (Figure 2, bottom panel, Pre-MCAO). Infarct volume was reduced to 13±4% by anti–E-selectin IgG at the 35-μg dose, which represents a reduction of 76% compared with vehicle-treated (53±4%) and a reduction of 70% compared with nonimmune IgG–treated mice (42±9%; P<0.005 for both controls). The reduction in infarct volume to 6±2% was even greater with a higher dose of 50 μg.
namely, 89% compared with vehicle and 86% compared with nonimmune IgG \((P < 0.01\) for both controls). This decrease in infarct volume was accompanied by increased survival in the mice treated with E-selectin antibody at these doses and decreased neurological deficit scores (saline, 2.6 ± 0.2; control IgG, 3.1 ± 0.3 versus 35 μg, 2.1 ± 0.1 \([P < 0.05]\) and 50 μg, 1.5 ± 0.2 \([P < 0.01]\)). Although a low dose of anti–E-selectin antibody tended to diminish the deleterious effects of ischemic injury, the reductions in neurological deficit, mortality, and infarct volume were not significant at a dose of 10 μg.

E-selectin blockade was determined to be a relevant strategy for the timely treatment of clinical stroke, since delayed administration of anti–E-selectin IgG at 3 hours after ischemic insult resulted in 50% reductions in neurological deficit \((P < 0.05)\) and mortality and a 4-fold reduction in infarct volume (control IgG, 44 ± 12% versus anti–E-selectin IgG, 11 ± 6%; \(P < 0.05\)). Although lengthening the treatment delay to 6 hours improved survival and neurological function
endothelial cells express a family of molecules termed selective. After activation by local and humoral factors, vascular of leukocyte-endothelial interactions appears ever more crit-

MAC-1 may have been responsible. In the absence of P- or E-selectin being every bit as stroke-prone as the deletionally mutant strain also suffered the same degree of no-reflow phenomenon as the intact mice, leading the authors to suggest that compensatory upregulation of ICAM-1 and Mac-1 may have been responsible.

Against this backdrop, we examined whether the sialyl-le^4^-mediated binding^{22,33} of E-selectin induces the same degree of deleterious leukocyte sludging induced by P-selectin, using a highly specific blocking antibody against the murine E-selectin domain. To guard against the possibility that our stroke model was substantially different in terms of the time course, degree, and anatomic location of E-selectin expression, we began by confirming its early expression between 2 and 24 hours, as has been demonstrated for other experimen-
al and clinical stroke models. As a result of these undesirable pathophysiological consequences, the targeting of adhesion molecules has been advocated as a potential treatment of stroke. Unfortunately, despite early experimental work demonstrating a protective effect for anti-ICAM, anti-CD11b, and anti–Mac-1 strategies in laboratory models of reperfusion injury,^{22,24–26} a recently completed human trial of an anti–ICAM-1 strategy failed miserably.^{27} Some have pointed to the complement fixing and proinflammatory properties of the antibody administered in this failed trial for the increase in infectious complications and the lack of cerebral protection.^{28} Nonetheless, despite the hope of investigators that a humanized antibody alternative will meet with better results, concern has been raised as to the biological redundancy controlling leukocyte trafficking and the role these processes play in perpetuating flow failure in both minimally and partially reperfused stroke.^{6}

As a result, investigators have explored the role of the selectins, a related group of leukocyte adherence molecules with distinct structure and function.^{23} The 3 selectin subtypes, L-selectin, P-selectin, and E-selectin, are structurally quite similar, with L-selectin being constitutively expressed on leukocytes and P-selectin being stored in preformed Weibel-Palade bodies before constitutive and inducible translocation to the surface of activated platelets and endothelial cells. In contrast to P- and L-selectin, E-selectin expression is slower, requiring de novo synthesis, but like P-selectin it occurs on activated endothelium. While investigators have been successful in demonstrating a convincing functional role for P-selectin in the pathophysiology of reperfused stroke,^{5} no such data exist for E-selectin. E-selectin has been linked to leukocyte recruitment in other models of tissue inflammation^{15} and has been shown to be induced by cytokine upregulation common to the setting of cerebral ischemia (ie, tumor necrosis factor-α, interleukin-1); however, the functional redundancy of the selectins remains incompletely elucidated in the setting of stroke.^{29–31} For example, prior studies have employed both a sialyl-le^4^- oligosaccharide analogue, CY-1503, and a synthetic N-terminal E-selectin oligopeptide. Unfortunately, conclusions regarding the functional significance of E-selectin blockade alone were obscured by the use of controversial end points and nonselective agents.^{12,13} In addition to these data, recent murine knockout data raise further questions, with mice expressing neither P- nor E-selectin being every bit as stroke-prone as wild-type littermate cousins.^{11} In these latter experiments, the deletionally mutant strain also suffered the same degree of no-reflow phenomenon as the intact mice, leading the authors to suggest that compensatory upregulation of ICAM-1 and Mac-1 may have been responsible.

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previous reports, our data show that this expression is most intense in postischemic, reperfused tissue and only mildly elevated in contralateral, nonischemic cerebral tissue, suggesting autocrine regulation.8,10 When this expression was pharmacologically blocked with the highly specific antibody, this result was not only in marked, dose-dependent improvement in outcome as measured by neurological examination, periprocedural mortality, and infarct volume, but it resulted in reduced PMN infiltration and improved hemispheric cerebral blood flow. Furthermore, a therapeutic window of 3 hours was demonstrated with delayed treatment.

Together, these results underscore the importance of E-selectin in the pathophysiology of stroke and indicate that the specific elimination of the effects of E-selectin is cerebroprotective in transient focal cerebral ischemia. Given our previous, similar findings with P-selectin and given the functionally redundant nature of selectin activity, we propose that a combined blockade of both P- and E-selectin might provide more therapeutic benefit than blockade of either molecule separately, despite the contradictory findings presented by groups working with combined knockout strains. While these strains may develop compensatory mechanisms, these mechanisms in all likelihood take time to develop, allowing acute therapeutic blockade to remain a viable strategy. 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The article by Huang et al describes the preventive and therapeutic action of an E-selectin antibody in a mouse model of stroke. The data seem to be straightforward: the treatment reduced histological damage, improved functional deficits, and increased survival. The positive outcome, by these key end points, is mechanistically supported by the ability of the anti–E-selectin antibody to reduce influx of neutrophils into the ischemic brain (less myeloperoxidase) and also by improved cerebral blood flow into the injured brain tissue. This study joins several other reports (largely cited by the authors) in which interference in the neutrophil-endothelium interaction resulted in “improved” histological and functional outcomes. The implied mechanism of action of these anti–adhesion molecule strategies is facilitation of blood flow into the ischemic zone by reducing “clogging” of the cerebral capillaries with leukocytes and possibly diminishing the impact of leukocyte-derived toxic factors. The authors point out the partial efficacy of the E-selectin antibody in combating injury and point to the redundancy associated in regard to adhesion molecules that enable leukocyte traffic into ischemic brain tissue. However, the authors did not take issue with an apparent discrepancy: complete inhibition of leukocyte accumulation was achieved by the anti–E-selectin treatment, whereas functional outcome and mortality were substantially less affected. One key issue, therefore, is that at best leukocytes may contribute to the injury, but they by no means mediate the major histological and functional deficiencies induced by the ischemic insult.

The authors suggest that the cerebroprotective effect of anti–E-selectin, fortified by combination with anti–P-selectin, might provide therapeutic benefits in stroke. The authors are aware of the inconsistency of the results obtained with E/P-selectin null mutation mice and, even more so, the failure of a clinical trial with anti–ICAM-1 antibody in stroke patients. Sensible explanations have been raised to “disqualify” these “failures”: genetic models allow for developmental adaptation not feasible in adults; and the anti–ICAM-1 antibody trial might have been plagued by complications due to a “murine” product, hence immune complications or unfortunate inflammatory/infectious conditions. However, one point must be made: neither E- nor P-selectins are adhesion molecules that mediate “nonreversible,” committed leukocyte binding, while ICAM-1 is believed to exercise such a function. For anti–E/P-selectin strategies to prove superiority over ICAM-1 blockade, one must assume that “clogging” of the microcirculation via E/P-selectin interaction is not alleviated by the ICAM-1 antagonists, and therefore such strategy was doomed to fail a priori; evidence to support such a possibility could be very useful to sort out this problem.

Should the clinical community adopt the author’s suggestions and consider anti–E/P-selectin inhibitors for clinical trials in stroke? This commentator takes a cautionary position to such a possibility for the following reasons: (1) The model utilizes brief ischemia and reperfusion paradigm, a condition not common in human stroke (spontaneous reperfusion is a relatively infrequent and late phenomenon). (2) The study has not been carried out to more chronic time points; it has been shown that early “positive” outcome can “wash out” few weeks later. (3) It is questionable whether a therapeutic window of only 3 hours is sufficient; a 6- to 8-hour time window is much more attractive. (4) It is imperative that a gyroencephalic model of stroke be used to confirm at least some of the key findings in the mouse. (5) The need for a humanized agent for clinical trials might be important, in view of the anti–ICAM-1 trials.

Taken together, this interesting and well-rounded study in a rodent model of focal stroke provides important data on the possible therapeutic efficacy of a readily available agent, anti–E-selectin antibody. It is hoped that these studies are further pursued in view of addressing the issues and concerns prior to commencing with clinical trials.

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Postischemic Cerebrovascular E-Selectin Expression Mediates Tissue Injury in Murine Stroke

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