Cerebral Protection in Homozygous Null ICAM-1 Mice after Middle Cerebral Artery Occlusion

Role of Neutrophil Adhesion in the Pathogenesis of Stroke

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Abstract

Acute neutrophil (PMN) recruitment to postischemic cardiac or pulmonary tissue has deleterious effects in the early reperfusion period, but the mechanisms and effects of neutrophil influx in the pathogenesis of evolving stroke remain controversial. To investigate whether PMNs contribute to adverse neurologic sequelae and mortality after stroke, and to study the potential role of the leukocyte adhesion molecule intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of stroke, we used a murine model of transient focal cerebral ischemia consisting of intraluminal middle cerebral artery occlusion for 45 min followed by 22 h of reperfusion. PMN accumulation, monitored by deposition of [111In]-labeled PMNs in postischemic cerebral tissue, was increased 2.5-fold in the ipsilateral (infarcted) hemisphere compared with the contralateral (noninfarced) hemisphere (P < 0.01). Mice immunodepleted of neutrophils before surgery demonstrated a 3.0-fold reduction in infarct volume (P < 0.001), based on triphenyltetrazolium chloride staining of serial cerebral sections, improved ipsilateral cortical cerebrovascular integrity, with immunohistochemistry localizing increased ICAM-1 expression on cerebral microvascular endothelium. The role of ICAM-1 expression in stroke was investigated in homozygous null ICAM-1 mice (ICAM-1 –/–) in comparison with wild-type controls (ICAM-1 +/+) and transgenic ICAM-1 null mice are relatively resistant to cerebral ischemia/reperfusion injury. While strategies to block each of these mechanisms of neutrophil recruitment are protective in various models of ischemia and reperfusion injury, their effectiveness in cerebral ischemia/reperfusion injury remains controversial. There is considerable evidence that in the brain, as in other tissues, an early PMN influx follows an ischemic episode (12–17). Immunohistochemical studies have described increased expression of the PMN adhesion molecules P-selectin and ICAM-1 in the postischemic cerebral vasculature (12, 18–20). The pathogenic relevance of adhesion molecule expression in the brain remains controversial; however, data from a trial of a monoclonal anti–ICAM-1 antibody in stroke in humans are not yet available (Rothlein, R., personal communication). In animal models, there is conflicting experimental evidence regarding the effectiveness of anti–adhesion molecule strategies in the treatment of experimental stroke (21–23). To determine whether ICAM-1 participates in the pathogenesis of postischemic cerebral injury, the experiments reported here were undertaken in a murine model of focal cerebral ischemia and reperfusion so that the role of a single, critical mediator of PMN adhesion (ICAM-1) could be determined. These studies demonstrate that enhanced ICAM-1 expression and neutrophil influx follow an episode of focal cerebral ischemia. Furthermore, these studies show that both neutrophil-deficient and transgenic ICAM-1 null mice are relatively resistant to cerebral infarction after ischemia and reperfusion, providing strong evidence for an exacerbating role of ICAM-1 in the pathophysiology of stroke.

Key words: cerebral ischemia • stroke • neutrophil • ICAM-1

Introduction

Neutrophils (PMNs) are critically involved in the earliest stages of inflammation after tissue injury, initiating scavenger functions which are later subsumed by macrophages. However, there is a darker side to neutrophil influx, especially in postischemic tissues (1–7), where activated PMNs may augment damage to vascular and parenchymal cellular elements. Experimental evidence points to a pivotal role for endothelial cells in establishing postischemic PMN recruitment, in that hypoxic/ischemic endothelial cells synthesize the proinflammatory cytokine IL-1 (8) as well as the potent neutrophil chemoattractant and activator IL-8 (9). Firm adhesion of PMNs to activated endothelium in a postischemic vascular milieu is promoted by translocation of P-selectin to the cell surface (10) as well as enhanced production of platelet activating factor (PAF) and intercellular adhesion molecule-1 (ICAM-1) (11).

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While strategies to block each of these mechanisms of neutrophil recruitment are protective in various models of ischemia and reperfusion injury, their effectiveness in cerebral ischemia/reperfusion injury remains controversial. There is considerable evidence that in the brain, as in other tissues, an early PMN influx follows an ischemic episode (12–17). Immunohistochemical studies have described increased expression of the PMN adhesion molecules P-selectin and ICAM-1 in the postischemic cerebral vasculature (12, 18–20). The pathogenic relevance of adhesion molecule expression in the brain remains controversial; however, data from a trial of a monoclonal anti–ICAM-1 antibody in stroke in humans are not yet available (Rothlein, R., personal communication). In animal models, there is conflicting experimental evidence regarding the effectiveness of anti–adhesion molecule strategies in the treatment of experimental stroke (21–23). To determine whether ICAM-1 participates in the pathogenesis of postischemic cerebral injury, the experiments reported here were undertaken in a murine model of focal cerebral ischemia and reperfusion so that the role of a single, critical mediator of PMN adhesion (ICAM-1) could be determined. These studies demonstrate that enhanced ICAM-1 expression and neutrophil influx follow an episode of focal cerebral ischemia. Furthermore, these studies show that both neutrophil-deficient and transgenic ICAM-1 null mice are relatively resistant to cerebral infarction after ischemia and reperfusion, providing strong evidence for an exacerbating role of ICAM-1 in the pathophysiology of stroke.
These methods have been used in previous studies (26).

A 33% increase in flow over baseline occlusion was observed immediately after removal of the occluding suture. A 50% reduction in relative cerebral blood flow immediately after reperfusion, and at 24 h just before euthanasia. A score of 1 was given if the animal demonstrated normal spontaneous movements; a score of 2 was given if the animal was noted to be turning to the right (clockwise circles) when viewed from above (i.e., toward the contralateral side); a score of 3 was given if the animal was observed to spin longitudinally (clockwise when viewed from 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 in mice (26) and is based upon similar scoring systems used in rats (28, 29) which are based upon the contralateral movement of animals with stroke; after cerebral infarction, the contralateral side is “weak” and so the animal tends to turn toward the weakened side. Previous work in rats (28) and mice (26) demonstrates that larger cerebral infarcts are associated with a greater degree of contralateral movement, up to the point where the infarcts are so large that the animal remains unresponsive.

Calculation of infarct volume. After neurologic examination, mice were given 0.3 ml of ketamine (10 mg/ml) and xylazine (0.5 mg/ml), and final cerebral blood flow measurements were obtained. Humane killing was performed by decapitation under anesthesia, and brains were removed and placed in a mouse brain matrix (Activational Systems, Inc., Warren, MI) for 1-mm sectioning. Sections were immersed in 2% 2,3,5-triphenyl, 2H-tetrazolium chloride (TTC; Sigma Chemical Co., St. Louis, MO) in 0.9% PBS, incubated for 30 min at 37°C, and placed in 10% formalin (26, 30–32). Infarcted brain was visualized as an area of unstained tissue, in contrast to viable tissue, which stains brick red. Infarct volumes were calculated from planimetered serial sections and expressed as the percentage of infarct in the ipsilateral hemisphere.

RNA extraction and Northern blot analysis. 24 h after focal ischemia and reperfusion, brains were obtained and divided into ipsilateral (infarct) and contralateral (noninfarct) hemispheres. To detect ICAM-1 transcripts, total RNA was extracted from each hemisphere using an RNA isolation kit (Stratagene, La Jolla, CA). Equal amounts of RNA (20 μg/lane) were loaded onto a 1.4% agarose gel containing 2 M formaldehyde for size fractionation and then transferred overnight to nylon (Nytren) membranes with 10× SSC buffer by capillary pressure. A murine ICAM-1 cDNA probe (33) (1.90 kb; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) was preadsorbed to red blood cells as a daily intraperitoneal injection (0.3 ml of 1:12 solution) for 3 d. Experiments in these mice were performed using a 0.7-mm straight laser Doppler probe (Perimed, Inc., Piscataway, NJ) after reflection of the skin overlying the calvarium, as described previously (27) (transcranial). Measurements of cerebral blood flow were made using laser Doppler flow measurements (Perimed, Inc., Piscataway, NJ) after reflection of the skin overlying the calvarium, as described previously (26).

Measurement of cerebral cortical blood flow. Transcranial measurements of cerebral blood flow were made using laser Doppler flow measurements (Perimed, Inc., Piscataway, NJ) after reflection of the skin overlying the calvarium, as described previously (27) (transcranial). Measurements of cerebral blood flow were made using laser Doppler flow measurements (Perimed, Inc., Piscataway, NJ) after reflection of the skin overlying the calvarium, as described previously (26).
paired variables. $^{111}$In-neutrophil deposition was evaluated as paired data (comparing contralateral [noninfarct] with ipsilateral [infarct] hemisphere), to control for variations in injected counts or volume of distribution. Survival differences between groups were tested using contingency analysis with the $\chi^2$ statistic. Values are expressed as means±SEM, with a $P < 0.05$ considered statistically significant.

Results

Neutrophil accumulation in stroke. Previous pathologic studies have shown neutrophil accumulation after cerebral infarction (15–17, 34–36). To determine whether neutrophils accumulate in our murine model of focal cerebral ischemia and reperfusion, neutrophil accumulation after transient (45 min) ischemia and reperfusion (22 h) was quantified by measuring the deposition of $^{111}$In-labeled neutrophils given to wild-type mice before the ischemic event. These experiments demonstrated significantly greater neutrophil accumulation (2.5-fold increase) in the ipsilateral (infarcted) compared with the contralateral (noninfarcted) hemispheres ($n = 7, P < 0.01$; Fig. 1). Similar results were obtained when neutrophil influx was monitored by myeloperoxidase assays, though low levels of activity were recorded in the latter assay (data not shown).

Effect of neutrophil depletion on stroke outcome. To determine the effect of neutrophil influx on indices of stroke outcome, mice were immunodepleted of neutrophils beginning 3 d before surgery. When surgery was performed on the fourth day, nearly complete agranulocytosis was evident on smears of peripheral blood. Neutropenic mice ($n = 18$) were subjected to 45 min of cerebral ischemia and 22 h of reperfusion, and indices of stroke outcome were determined. Infarct volumes were threefold smaller in neutropenic animals compared with wild-type controls (11.1±1.6% vs. 33.1±6.4%, $P < 0.001$; Fig. 2 A). The decrease in infarct volumes in neutropenic mice was paralleled by reduced neurologic deficit scores (Fig. 2 B), increased postreperfusion cerebral cortical blood flows (Fig. 2 C), and a trend toward reduced overnight mortality (22% mortality in neutropenic mice vs. 50% mortality in controls, Fig. 2 D).

ICAM-1 expression in murine stroke. To establish the effect of cerebral ischemia/reperfusion in our murine model, ICAM-1 mRNA levels were evaluated after cerebral ischemia and reperfusion in wild-type mice. Ipsilateral (infarcted) cere-
Role of ICAM-1 in stroke. To explore the role of ICAM-1 in stroke, transgenic mice which were homozygous ICAM-1 deficient (24) were studied in the murine model of focal cerebral ischemia and reperfusion. Because variations in cerebrovascular anatomy have been reported to result in differences in susceptibility to experimental stroke in mice (37), indium ink staining was performed on the circle of Willis in homozgyous null (ICAM-1 −/−) and ICAM-1 +/+ mice. These experiments (Fig. 5) demonstrated that there were no gross anatomic differences in the vascular pattern of the cerebral circulation. To determine the role of ICAM-1 in neutrophil influx after focal cerebral ischemia and reperfusion, neutrophil accumulation was measured in homozgyous null ICAM-1 mice (ICAM-1 −/−) mice (n = 14) and wild-type controls (n = 7) infused with 111In-labeled neutrophils. Relative neutrophil accumulation (ipsilateral counts per minute/contralateral counts per minute) was diminished (39% reduction) in the ICAM-1 −/− mice compared with ICAM-1 +/+ controls (1.70±0.26 vs. 2.9±0.52, P < 0.05).

Experiments were then performed to investigate whether expression of ICAM-1 has a pathophysiologic role in outcome after stroke. ICAM-1 −/− mice (n = 13) were significantly protected from the effects of focal cerebral ischemia and reperfusion, based on a 3.7-fold reduction in infarct volume (P < 0.01) compared with ICAM-1 +/+ controls (Figs. 6 and 7A). This reduction in infarct volume was accompanied by reduced neurologic deficit (Fig. 7B) and increased postreperfusion cerebral cortical blood flow (Fig. 7C). Given these results, it was not surprising that mortality was also significantly decreased in the ICAM-1 −/− mice compared with ICAM-1 +/+ controls (15% vs. 50%, P < 0.05; Fig. 7D).

Discussion

Epidemiologic evidence in humans suggests that neutrophils contribute to the initiation of stroke (38) as well as to cerebral

Figure 3. Expression of ICAM-1 transcripts 24 h after middle cerebral artery occlusion. RNA was prepared from the ipsilateral (infarct) and the contralateral (noninfarct) hemispheres from the same animal. After overnight transfer to a nylon membrane, the Northern blot was probed with a 32P-labeled 1.90-kb murine ICAM-1 cDNA (33) (top). A β-actin probe was used for a control (bottom).

Figure 4. Expression of ICAM-1 antigen in the cerebral microvasculature 24 h after middle cerebral artery occlusion. A coronal section of brain was obtained for ICAM-1 immunostaining, so that the noninfarcted and infarcted hemispheres from the same brain could be compared under identical staining conditions. Staining was performed using a rat anti-murine ICAM-1 antibody, with sites of primary antibody binding visualized by alkaline phosphatase. (A) Cerebral microvessel in the contralateral (noninfarcted) section of a brain obtained 24 h after middle cerebral artery occlusion. (B) Cerebral microvessel from the ipsilateral (infarcted) hemisphere from the same section of brain as shown in A. Endothelial cells from ipsilateral cerebral microvessels demonstrate increased expression of ICAM-1 (bright red staining). ×250.
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Figure 6. TTC-stained serial sections at 24 h from representative wild-type (left) or homozygous null ICAM-1 mice (right) subjected to transient middle cerebral artery occlusion. The pale white area in the middle cerebral artery territory represents infarcted brain tissue, whereas viable tissue stains brick red. Quantification of infarct volumes by planimetry of serial cerebral sections in multiple experiments is shown in Fig. 7A.
Chemic event, cerebral infarcts were smaller, with improved cerebral perfusion after the ischemic event. These data are quite similar to those reported in a rabbit model of thromboembolic stroke, in which immunodepletion of neutrophils resulted both in reduced infarction volume and improved blood flow (35). Because neutrophils contribute to murine postischemic cerebral injury, we were able to pursue a strategy to elucidate the role of ICAM-1 in the pathophysiology of stroke using deletionally mutant ICAM-1 mice (24). Our experiments indicate that homozygous null ICAM-1 mice are relatively resistant to the deleterious effects of cerebral ischemia and reperfusion.

To demonstrate the role of both neutrophils and ICAM-1 in the pathogenesis of tissue injury in stroke, the studies reported here used several methods for assessing stroke outcome. Although numerous investigators have used TTC staining to quantify cerebral infarct volumes (26, 30–32, 37, 53), there has been some controversy as to the accuracy of this method, especially when evaluated early after the ischemic event. In the TTC method, TTC reacts with intact oxidative enzymes on mitochondrial cristae and is thereby reduced to a colored formazan (54). TTC staining is unreliable before 2 h of ischemia have elapsed; beyond 36 h, cells infiltrating into the infarcted tissue can stain positively with TTC, thereby obscuring the clear demarcation between infarcted and noninfarcted tissues seen with earlier staining (31). Although the size of the infarct delineated by TTC staining correlates well with infarct size delineated by hematoxylin and eosin staining (30, 32), direct morphometric measurements tend to overestimate infarct volumes due to cerebral edema, especially during the first 3 d after the ischemic event (32). Even given these limitations, the studies reported here incorporate three additional methods to define the role of neutrophils and ICAM-1 in stroke outcome, including neurologic deficit score, relative cerebral blood flow to the affected area, and mortality. These additional measures, which do not depend upon the accuracy of TTC staining, contribute strongly to our identification of a pathogenic role for both neutrophils and ICAM-1 in stroke.

There has been a recent profusion of scientific studies exploring the mechanistic basis for neutrophil recruitment to postischemic tissues. Endothelial cells appear to be the chief regulators of neutrophil traffic, regulating the processes of neutrophil chemoattraction, adhesion, and emigration from the vasculature (55). When exposed to a hypoxic environment as a paradigm for tissue ischemia, endothelial cells synthesize the potent neutrophil chemoattractant and activator IL-8 (9), the blockade of which appears to be beneficial in a lung model of ischemia and reperfusion (6). In addition, hypoxic endothelial cells synthesize the proinflammatory cytokine IL-1 (8), which can upregulate endothelial expression of the neutrophil adhesion molecules E-selectin and ICAM-1 in an autocrine fashion (8, 9, 56). Other neutrophil adhesion mechanisms may also be activated in the brain after ischemia, such as release of P-selectin from preformed storage pools within Weibel-Palade body membranes (10). In a primate model, P-selectin expression was rapidly and persistently enhanced after focal middle cerebral artery ischemia and reperfusion (18). Although P-selectin–dependent neutrophil recruitment appears to be deleterious after cardiac ischemia and reperfusion (57), its pathophysiologic relevance in the setting of stroke has not yet been determined. While hypoxia induces de novo synthesis of the bioactive lipid PAF (11), in a spinal cord ischemia reperfusion paradigm for tissue ischemia, endothelial cells synthesize the potent neutrophil chemoattractant and activator IL-8 (9), the blockade of which appears to be beneficial in a lung model of ischemia and reperfusion (6). In addition, hypoxic endothelial cells synthesize the proinflammatory cytokine IL-1 (8), which can upregulate endothelial expression of the neutrophil adhesion molecules E-selectin and ICAM-1 in an autocrine fashion (8, 9, 56). Other neutrophil adhesion mechanisms may also be activated in the brain after ischemia, such as release of P-selectin from preformed storage pools within Weibel-Palade body membranes (10). In a primate model, P-selectin expression was rapidly and persistently enhanced after focal middle cerebral artery ischemia and reperfusion (18). Although P-selectin–dependent neutrophil recruitment appears to be deleterious after cardiac ischemia and reperfusion (57), its pathophysiologic relevance in the setting of stroke has not yet been determined. While hypoxia induces de novo synthesis of the bioactive lipid PAF (11), in a spinal cord ischemia reperfusion model, PAF antagonism offered no incremental benefit when given simultaneously with antibody to CD11/CD18 (48).

Understanding the role of ICAM-1 in the pathophysiology of stroke appears to be of particular relevance in humans for several reasons. Increased cerebrovascular ICAM-1 expression has been demonstrated in primates by 4 h of ischemia and reperfusion, particularly in the lenticulostriate microvasculature (18). An autopsy study of recent cerebral infarcts in humans also demonstrated increased ICAM-1 expression (20). Since rats also express cerebral vascular ICAM-1 within 24 h
in both a photochemically induced model of rat cerebral ischemia (19) and a middle cerebral artery occlusion model (12), these data suggested the potential usefulness of transgenic ICAM-1–deficient mice in elucidating the pathophysiologic significance of increased postcerebral ischemic ICAM-1 expression. In particular, the time frame of ICAM-1 expression (increased by 4–24 h) in these models suggests that ICAM-1–mediated neutrophil–endothelial interactions may be targeted in future pharmacologic strategies to improve human stroke outcome, as this time frame represents a realistic clinical window for therapeutic intervention.

Although neutropenic animals demonstrated increased regional cerebral blood flow compared with controls, compared with neutropenic animals, ICAM-1–deficient mice tended to have even higher ipsilateral cerebral blood flows at 24 h. This observation may relate to the no-reflow phenomenon, wherein blood flow fails to return to preobstruction levels even after release of a temporary vascular occlusion. A significant body of previous work has implicated neutrophil plugging of capillary microvascular beds in this process (58), although in a model of global cerebral ischemia, an 85% reduction in the circulating leukocyte count did not decrease the incidence or severity of reflow failure (49). Our data suggest that non–neutrophil–dependent mechanisms, which nevertheless involve ICAM-1, may contribute to cerebrovascular postischemic no-reflow. Since macrophages and lymphocytes both express LFA-1, which mediates an adhesive interaction with endothelial cell ICAM-1 (51), it is possible that ICAM-1–deficient mice have diminished recruitment of these mononuclear cells, a possibility which is currently the subject of further investigation in our laboratory. This hypothesis is supported by multiple pathologic observations demonstrating macrophage and lymphocyte accumulation by 1–3 d after cerebral infarction (12, 17, 19, 34, 59).

Taken together, our studies indicate that, in a murine model of focal cerebral ischemia and reperfusion, neutrophils accumulate in the infarcted hemisphere and that neutropenic animals demonstrate cerebral protection. Increased expression of ICAM-1 on cerebral endothelial cells appears to be an important mechanism driving this neutrophil recruitment, and mice which are unable to express ICAM-1 demonstrate improved postischemic blood flows, reduced infarct volumes, and reduced mortality. These data suggest that pharmacologic strategies targeted at interfering with neutrophil–endothelial interactions may improve the outcome after stroke in humans.

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