Intercellular Adhesion Molecule-1–deficient Mice Are Less Susceptible to Cerebral Ischemia–Reperfusion Injury

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Neutrophil emigration is mediated by adhesion proteins that are highly expressed on the endothelial surface during inflammatory processes in the brain. Intercellular adhesion molecule-1 (ICAM-1) is an inducible adhesion molecule that binds to leukocyte integrins and facilitates neutrophil adhesion and transendothelial migration. To study the role of ICAM-1 during ischemia and reperfusion in the brain, we analyzed the effect of transient focal cerebral ischemia in ICAM-1–deficient mice generated by gene targeting in embryonic stem cells. Transient focal ischemia was induced by occluding the left middle cerebral artery for 3 hours followed by a 21- or 45-hour reperfusion period. When compared with their wild-type littermates, ICAM-1–deficient mice were less susceptible to cerebral injury as demonstrated by a 5.6- or 7.8-fold reduction in infarction volume, respectively. These data support the premise that neutrophil adhesion in ischemic areas may be deleterious and that ICAM-1 deficiency reduces neurological damage after transient focal cerebral ischemia.

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Intercellular adhesion molecule-1 (ICAM-1) is an inducible protein ligand that is up-regulated on microvascular endothelium and glial cells during inflammatory processes in the brain [1, 2]. This adhesion molecule serves as a counterreceptor for leukocyte integrins and facilitates firm adherence and transendothelial migration [3]. There is accumulating evidence that cerebral ischemia elicits an inflammatory response that is augmented by reperfusion. Leukocyte infiltration has been well documented after cerebral ischemia and reperfusion [4, 5]. Inflammatory reactions that involve leukocyte activation and transmigration appear to be controlled by adhesion molecules present on endothelium and leukocytes. These leukocytes mediate local tissue damage and alterations in microvascular perfusion. Since ICAM-1 is either not constitutively expressed or expressed at low levels, reoxygenation and proinflammatory cytokines produced after transient cerebral ischemia increase ICAM-1 expression [1, 6, 7]. Since ICAM-1 plays a pivotal role in leukocyte activation and transmigration, the potential for ICAM-1 inactivation has become a fertile area for cerebral protection.

ICAM-1 has been a target for monoclonal antibody (mAb) blockade in several models of ischemia–reperfusion. Specific blockade of leukocyte integrins (Mac-1) and ICAM-1 by mAbs has been shown to reduce cerebral damage after transient focal ischemia [8, 9]. Since antibody–antigen interactions are complex, the full implications of mAb blockade cannot be completely delineated. mAbs can trigger receptor-mediated alterations of the target cell. Furthermore, mAbs are constructed to bind to a single epitope and may not completely inactivate all functional binding sites. Therefore, the optimal way to rigorously study the role of adhesion molecules is through the use of models devoid of these receptors rather than through attempted receptor blockade. We hypothesized that deficiency or "knockout" of the ICAM-1 gene would minimize post–ischemic reperfusion injury in the brain. To test this hypothesis, we measured the extent of functional and histopathological damage after cerebral ischemia and reperfusion in ICAM-1–deficient mice.

Materials and Methods

Generation of ICAM-1–Deficient Mice

ICAM-1–deficient mice and their wild-type littermates were generated by standard gene targeting in embryonic stem cells [10]. The murine ICAM-1 gene consists of seven different

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exons [11] and was inactivated by inserting a neomycin resistance gene (*neo*^{r}) into the fourth exon of the gene in embryonic stem cells [10]. Heterozygotes were intercrossed on a murine C57BL/6 × SV129 background to produce homozygous progeny that were ICAM-1 deficient. Seven-week-old male mice were used for the study. As previously reported, we found no ICAM-1 expression in any tissue in the ICAM-1–deficient mice [10]. The wild-type littermates served as control subjects. We confirmed transmission of the disrupted ICAM-1 gene by Southern blot analysis. DNA was isolated from mouse tails, digested with the restriction enzyme *Bam*HI, and hybridized with a full-length murine ICAM-1 cDNA.

Examination of Functional Cerebrovascular Anatomy

A cohort of ICAM-1–deficient (n = 4) and wild-type (n = 4) mice were examined for differences in cerebrovascular anatomy. The mice were anesthetized and a sternotomy was performed to access the heart. After making a drainage incision in the right atrium, the left ventricle was perfused with 10 ml of 0.9% NaCl followed by 5 ml of India ink. The brains were removed and fixed in 10% paraformaldehyde for 24 hours. The brains were compared for differences in vascular pattern.

Anesthesia and Induction of Transient Focal Cerebral Ischemia

With approval of the institutional review board, mice weighing 20 to 30 gm were anesthetized with isoflurane (1–2%) and a 2:1 mixture of nitrous oxide and oxygen by nose cone. Body temperature was maintained by a water blanket, which was servo controlled at 37 ± 1°C by a rectal temperature probe. The surgeon was unaware of the mouse genotype. The left internal carotid artery (ICA) was exposed through a midline cervical incision under a dissecting microscope. All of the extracranial branches of the left ICA were ligated. A 6.0 monofilament nylon suture (Ethicon Inc.) with a flame-rounded tip was inserted into the lumen of the external carotid artery and advanced distally into the ICA approximately 10 mm to the base of the middle cerebral artery (MCA) [12]. Anesthesia was maintained for the duration of the surgical procedure, which typically lasted 30 minutes. Ischemia was induced for 3 hours by leaving the tip of the filament at the origin of the MCA. After the 3-hour occlusion period, the mice were reanesthetized. Reperfusion was accomplished by withdrawing the intraluminal filament. There were four groups as follows: group 1 = wild type with 21-hour reperfusion (n = 8); group 2 = ICAM-1–deficient with 21-hour reperfusion (n = 9); group 3 = wild type with 45-hour reperfusion (n = 12); and group 4 = ICAM-1–deficient with 45-hour reperfusion (n = 13).

Assessment of Cerebral Blood Flow

To determine changes in cerebral blood flow (CBF), we used a laser Doppler flowmeter (Vasamedics, BPM) with a 0.7-mm probe (Vasamedics, P433). The skull was exposed through a midline sagittal incision and the probe tip was placed over the left hemisphere, on the skull surface, 3 mm lateral to midline and 2 mm posterior to the bregma. CBF was recorded in wild-type (n = 3) and ICAM-1–deficient (n = 3) mice over 15 minutes, prior to and immediately after MCA occlusion (MCAO), and prior to and immediately after reperfusion.

Neurological Assessment

The functional effects of ischemia were assessed by an observer blinded to the mouse genotype. We evaluated the severity of the neurological deficit by using a modified five-point scale (0 = no deficit; 1 = failure to extend right paw; 2 = circling to the right; 3 = falling to the right; and 4 = unable to walk spontaneously) [13].

Detection and Quantification of Cerebral Infarction

After the reperfusion period, the mice were killed with a lethal intraperitoneal dose of pentobarbital (150 mg/kg). The brains were removed immediately, and 1.5-mm coronal sections were cut with a tissue slicer. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium (TTC) in phosphate buffer at 37°C for 30 minutes [14]. These sections were fixed in 4% paraformaldehyde in phosphate buffer for digital photography. The image of each brain slice was digitized and the infarcted area was measured by a blinded observer (NIH 1.55 image analysis software on a Macintosh Centris 660 computer). To minimize the effect of brain edema, calculation of the infarcted volume was determined indirectly by subtracting the volume of the noninfarcted ipsilateral hemisphere (left) from the contralateral hemisphere (right) [15, 16]. Infarction volume was represented as the percentage of the contralateral hemisphere.

Northern Blot Analysis

A cohort of ICAM-1–deficient (n = 2) and wild-type (n = 2) mice was subjected to 3 hours of ischemia followed by 21 hours of reperfusion. A sham-operated wild-type mouse was also included in the northern blot analysis. The brains were removed and frozen quickly in liquid nitrogen. Total mRNA was isolated from the brain by using the guanidinium-thiocyanate method of Chomczynski and Sacci [17] with a commercially available total RNA isolation reagent (Life Technologies, TRIzol). RNA (15 μg) was loaded into each lane of a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with an ICAM-1 cDNA probe. All samples were hybridized simultaneously with a γ-actin probe serving as an internal control.

Histological Examination for Neuronal Necrosis and Leukocyte Infiltration

A cohort of wild-type and ICAM-1–deficient mice were subjected to the same ischemia and reperfusion protocol (n = 4 for each of the four groups). Brain sections were fixed in 10% formaldehyde and embedded in paraffin. A 5-μm section from the coronal slice, 4.5 mm from the frontal pole, was stained with hematoxylin and eosin (H&E) for microscopic examination. Neuronal necrosis was identified as exhibiting either pyknotic nuclei, eosinophilic cytoplasm, or ghost neurons [18]. Neutrophils were identified by their size and characteristic nucleus. We quantified neutrophil infiltration in the cortical and striatal areas of the infarcted hemi-

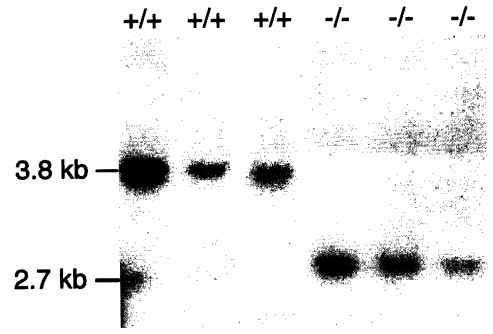

Fig 1. Southern blot analysis of BAM *HI-digested tail DNA from wild-type (+/+) and intercellular adhesion molecule-1 (ICAM-1)-deficient mice (−/−). The probe identified two fragments, of 3.8 and 2.7 kb, corresponding to the wild-type and ICAM-1–deficient mice, respectively.*

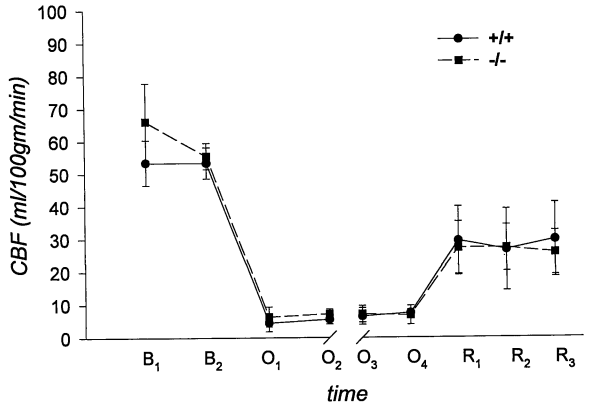

Fig 2. Absolute cerebral blood flow (CBF) measured by laser Doppler in the middle cerebral artery (MCA) region during 3 hours of MCA occlusion followed by reperfusion. Values were measured every 5 minutes and are represented as mean ± SEM. B = baseline; O = middle cerebral artery occlusion; and R = reperfusion.

sphere by counting the total number of extravascular neutrophils in each microscopic field under 400× magnification (total area, 0.05 mm^2).

Statistical Analysis

Infarction volumes and neutrophil counts were compared by an unpaired *t* test, while each coronal brain slice infarction area was compared by analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Data were reported as mean ± SEM values. A p value < 0.05 was accepted as statistically significant.

Results

ICAM-1–deficient mice were indistinguishable from their wild-type littermates and demonstrated normal growth and behavior. Brains of the wild-type and ICAM-1–deficient mice appeared the same on gross examination, and no differences in cerebrovascular anatomy were visible. The genotype of the mice was confirmed by Southern blot analysis, which showed a 3.8-kb band corresponding to the wild-type mice, while the ICAM-1–deficient mice displayed a 2.7-kb fragment (Fig 1).

Effect of ICAM-1 Deficiency on Transient Focal Cerebral Ischemia

All mice had at least a grade 3 deficit after the MCAO, indicating successful placement of the intraluminal suture. CBF was diminished during the period of MCAO and was restored similarly in both groups of mice (Fig 2). ICAM-1 deficiency resulted in a significant reduction in infarction volume after either 21 or 45 hours of reperfusion (Fig 3A and B). The percent infarction after 21 hours of reperfusion for the wild-type and ICAM-1–deficient groups was 22.31 ± 7.03% and 3.96 ± 2.37%, respectively ($p < 0.05$). After 45 hours of reperfusion, the percent infarction for the two groups was 19.01 ± 5.96% and 2.41 ± 0.78%, respectively ($p < 0.05$). Figure 4A and B show the infarction area of each of the five coronal sections. The ICAM-1–deficient mice in the 45-hour reperfusion group demonstrated a significant reduction in infarction area in the distribution of the MCA.

Histology

Microscopic examination of the H&E-stained brain sections revealed differences in the number of necrotic neurons between the wild-type and ICAM-1–deficient mice. Approximately 80% of the neurons appeared necrotic in the wild-type mice for both reperfusion groups (21 and 45 hours). Furthermore, areas of cavitation were more frequent in the wild-type mice. However, less than 30% of the neurons in the ICAM-1–deficient mice exhibited ischemic damage.

Two different patterns of neutrophil infiltration were seen in our groups. Histological examination of the brain slices revealed a difference between the wild-type and ICAM-1–deficient mice at 21 hours of reperfusion. The wild-type mice had diffuse infiltration of the ischemic core by neutrophils (55.4 ± 6.9 neutrophils per 0.05 mm^2). In contrast, the ICAM-1–deficient mice had significantly less neutrophil accumulation (11 ± 2.7 neutrophils per 0.05 mm^2, $p < 0.05$). After 45 hours of reperfusion, the differences between the wild-type and ICAM-1–deficient mice were not statistically significant; 26.3 ± 9.2 and 13.5 ± 3.4 neutrophils per 0.05 mm^2, respectively.

Transcription of mRNA after Transient Focal Cerebral Ischemia

We determined induction of ICAM-1 mRNA after cerebral ischemia–reperfusion by northern blot analysis

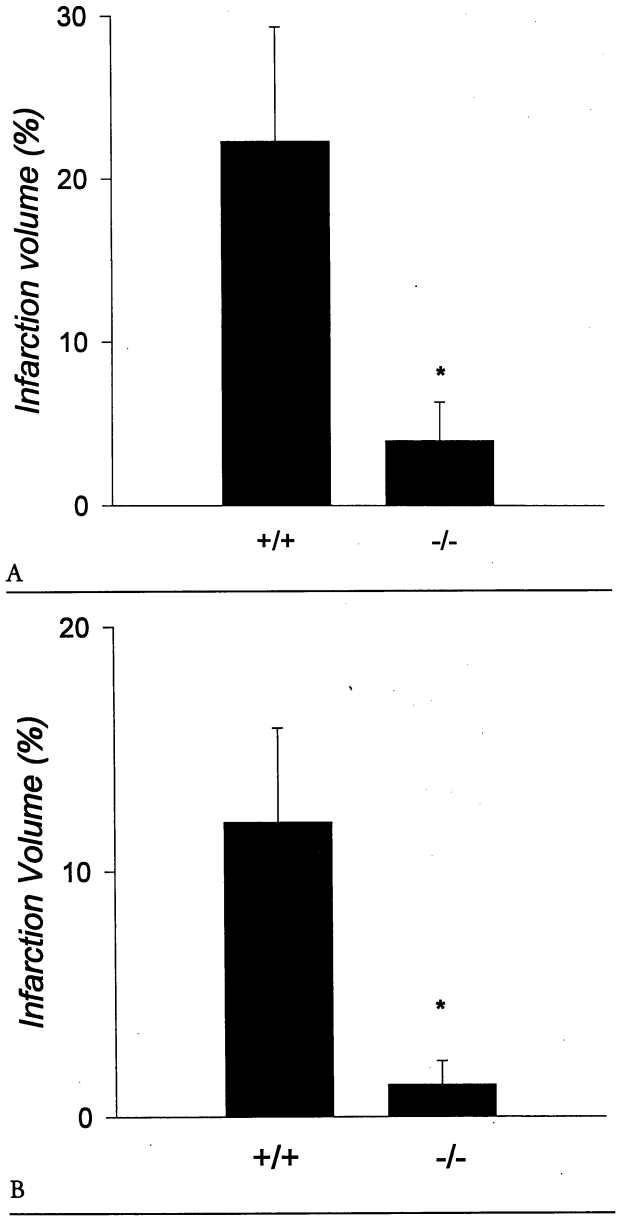

*Fig 3. Percent infarction volume of wild-type (+/+) and intercellular adhesion molecule-1–deficient mice (−/−) after 3 hours of middle cerebral artery occlusion and 21 hours (A) and 45 hours (B) of reperfusion (mean ± SEM). *p < 0.05.*

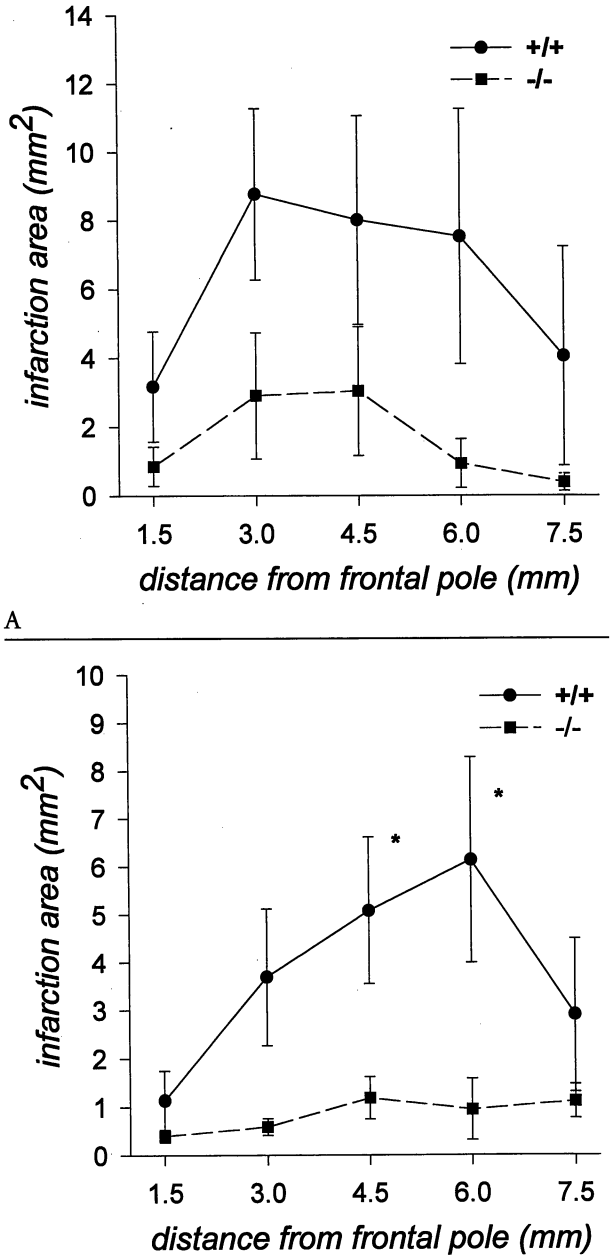

*Fig 4. Infarction area of each coronal section for wild-type (+/+) and intercellular adhesion molecule-1–deficient (−/−) mice at 24 (A) and 48 hours (B) after the onset of ischemia (mean ± SEM). *p < 0.05.*

of the brain after a 21-hour period of reperfusion. There was strong expression of a 2.5-kb mRNA species in both hemispheres of the wild-type mice. Although no 2.5-kb mRNA band was detected in the ICAM-1–deficient mice, we found a strong 3.1-kb and a weak 1.4-kb mRNA species in both hemispheres. These bands correspond to aberrant transcription for the combined *neo*^{r}/ICAM-1 fragments (Fig 5). There was no mRNA signal in the sham-operated mouse. Equal

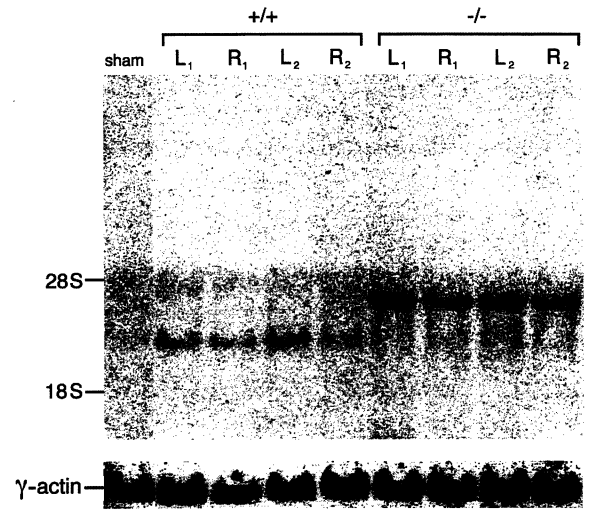

Fig 5. Northern blot analysis of brain mRNA transcription after 3 hours of middle cerebral artery occlusion and 21 hours of reperfusion. Lane 1 is mRNA from a sham-operated mouse. The wild-type (+/+) mice had a 2.5-kb band, while the intercellular adhesion molecule-1 (ICAM-1)–deficient (−/−) mice displayed signals at 3.4 and 1.7 kb, representing aberrant transcription of the combined neo^r/ICAM-1 fragment. Hybridization with γ-actin demonstrates equal loading of the lanes.

loading of mRNA in each lane was verified by the isointensity of the γ-actin bands.

Discussion

This study demonstrates that ICAM-1 mutant mice are less susceptible to focal cerebral ischemia–reperfusion injury. We found that post–reperfusion infarction volumes were lower in the transgenic mice, supporting the hypothesis that ICAM-1 deficiency minimizes post–ischemic–reperfusion injury.

There is mounting evidence that reperfusion plays a significant role in the progression of cellular and vascular injury after cerebral ischemia. During the reperfusion period, cytokines and reoxygenation induce expression of adhesion molecules [19]. These receptors and ligands mediate recruitment and activation of leukocytes during inflammation [3]. Neutrophil binding to the endothelium is the part of a multistep process that activates neutrophils during inflammation. The major adhesion families that promote neutrophil–endothelial interaction are the carbohydrates and integrins on the neutrophil surface, and the selectin and immunoglobulin superfamilies on the endothelial side. Mac-1 (CD11b/CD18) is a member of the integrin superfamily that is constitutively expressed on neutrophils. ICAM-1(CD54) is an inducible protein ligand expressed on the endothelial surface. After selectin-induced neutrophil margination and rolling, Mac-1 and ICAM-1 mediate direct tethering of the neutrophil to the endothelium. This Mac-1/ICAM-1 network firmly links the neutrophil to the endothelial surface and facilitates neutrophil migration into the perivascular space, leading to tissue inflammation and injury.

mAbs directed against adhesion molecules have been shown to attenuate inflammation and tissue destruction. In the central nervous system, mAb against ICAM-1 produced a significant reduction in neurological deficits after reversible, but not after permanent, ischemia in rabbit spinal cords and rat brains [20, 21]. These findings suggest that ICAM-1 mediates the extension of neurological injury after the onset of reperfusion. Anti–ICAM-1 mAb resulted in a 41% reduction in infarction size and neutrophil accumulation after transient MCAO in rats [9]. Here, we are able to demonstrate directly the deleterious effects of ICAM-1 in focal stroke by utilizing "knockout" mice.

In our study, we applied a similar model of transient focal ischemia on ICAM-1–deficient transgenic mice and demonstrated a dramatic reduction in infarction volumes when compared with the wild-type mice. Our data demonstrate the utility of genetic knockout mice in the study of adhesion molecules and also provide evidence that absolute inactivation of ICAM-1 during inflammatory reactions in the brain can lessen reperfusion injury after cerebral ischemia.

Neutrophil infiltration of reperfused tissue is the hallmark of ischemia–reperfusion injury [22]. Leukocytic responses in rats undergoing transient MCAO reveal that neutrophils initiate the inflammatory response at 6 hours and peak in numbers at 48 hours [23]. Transcription ICAM-1 mRNA occurs 2 hours after the ischemic insult and peaks at 10 hours, while maximal expression of ICAM-1 protein on vascular endothelium occurs at 46 hours after the onset of reperfusion [24]. In vitro studies of the brain demonstrated that cytokines induce ICAM-1 expression after 4 to 72 hours [25]. We previously demonstrated that ICAM-1 mutant mice have a two- to three-fold increase in circulating neutrophils compared with wild-type mice [10] and therefore expected a substantial increase in extravasation of neutrophils of the ICAM-1–deficient mice. At 21 hours of reperfusion, we demonstrated a significant increase in neutrophil accumulation in the wild-type mice over the ICAM-1–deficient mice. However, at 48 hours, there was no significant difference between the neutrophil counts of the two groups. Leukocytosis in the ICAM-1–deficient mice should have favored greater neutrophil extravasation. We attribute the reduction of extravascular neutrophils at 48 hours in the wild-type mice to neutrophil clearance by disintegration or macrophage uptake [26]. Furthermore, neutrophil infiltration seen in the ICAM-1–deficient mice also suggests that P-, E-, or L-selectin–mediated neutrophil recruitment may also be involved.

Cessation of CBF was verified in both groups of mice by laser Doppler flowmetery during the period of MCAO. Reperfusion also resulted in restoring blood flow in the affected areas in both the wild-type and knockout mice. This finding demonstrates that there were no flow-related differences between the wild-type and mutant mice. Production of cytokines such as tumor necrosis factor-α and interleukin-1 was normal in the ICAM-1–deficient mice [10]. Therefore, we believe that ICAM-1–mediated inflammation was largely responsible for the increased neurological injury observed in wild-type mice compared with the knockouts.

One likely mechanism for the reduction in neurological injury in our study is that the lack of ICAM-1–mediated leukocyte binding to endothelial and glial cells may lead to decreased ischemic damage. Histological examination of the brain sections verified that neuronal damage was present in all groups, but more so in the wild-type mice. Previous studies have shown that glial ICAM-1 may play a role in inflammatory processes in the brain [2, 27, 28]. Since ICAM-1 is expressed on glial cells, we speculate that the absence of ICAM-1 on the mutant glial cells attenuated direct leukocyte and lymphocyte binding to postischemic glial tissue [29].

In our study, we cannot assess the effect of ICAM-1 deficiency more than 48 hours after the onset of ischemia. It is possible that the lack of ICAM-1 ligand may only delay the conversion of the ischemic lesion to infarction. However, anti-ICAM-1 mAb blockade has been shown to reduce ischemic cell damage in rats 7 days after transient MCAO [21]. Administration of the same mAb during permanent MCAO had no effect. Despite the lack of reperfusion during permanent ischemia, leukocytes continue to infiltrate ischemic brain parenchyma [5]. Leukocyte subtypes differ at 1 and 7 days after the onset of MCAO, with neutrophil predominance in the former and monocytes and macrophages in the latter. Monocytes and macrophages, which both express Mac-1, can potentially bind to ICAM-1 ligands on glial cells and further worsen cerebral ischemic injury. Therefore, the role of monocyte- and macrophage-mediated maturation of ischemic lesions merits further investigation.

In summary, our data confirm the significance of ICAM-1 in the evolution of ischemic injury after transient cerebral ischemia. Our observations are limited to the first 48 hours after transient ischemia and do not reflect long-term outcome. However, our findings and those of others demonstrate that selective inhibition of ICAM-1 is a promising therapeutic option for the treatment of transient focal cerebral ischemia [9, 21]. Further characterization of ICAM-1 in cerebral tissues is essential to maximize antiadhesion therapy. Additional studies on the mechanism of ICAM-1 up-regulation during inflammatory processes in the brain are needed to devise new strategies to minimize neurological morbidity and improve outcome after transient focal cerebral ischemia.

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