Mice Deficient in Mac-1 (CD11b/CD18) Are Less Susceptible to Cerebral Ischemia/Reperfusion Injury

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Background and Purpose—Macrophage-1 antigen (Mac-1) (CD11b/CD18), a leukocyte β2 integrin, facilitates neutrophil adhesion, transendothelial migration, phagocytosis, and respiratory burst, all of which may mediate reperfusion-induced injury to ischemic brain tissue in conditions such as stroke. To determine the role of Mac-1 during ischemia and reperfusion in the brain, we analyzed the effect of transient focal cerebral ischemia in mice genetically engineered with a specific deficiency in Mac-1.

Methods—Transient focal ischemia/reperfusion was induced by occluding the left middle cerebral artery for 3 hours followed by a 21-hour reperfusion period in Mac-1–deficient (n=12) and wild-type (n=11) mice. Regional cerebral blood flow was determined with a laser-Doppler flowmeter. Brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride to determine the infarct volume. Neutrophil accumulation was determined by staining the brain sections with dichloroacetate esterase to identify neutrophils.

Results—Compared with the wild-type cohort, Mac-1–deficient mice had a 26% reduction in infarction volume (P<0.05). This was associated with a 50%, but statistically insignificant, reduction in the number of extravasated neutrophils in the infarcted areas of the brains in the mutant mice. There were no differences in regional cerebral blood flow between the 2 groups.

Conclusions—Mac-1 deficiency reduces neutrophil infiltration and cerebral cell death after transient focal cerebral ischemia. This finding may be related to a reduction in neutrophil extravasation in Mac-1–deficient mice. (Stroke. 1999;30:134-139.)

Key Words: cell adhesion molecules • cerebral ischemia, transient • macrophage-1 antigen • reperfusion injury • stroke

A ccumulating evidence suggests that transient cerebral ischemia elicits an inflammatory response that is augmented by reperfusion. Leukocyte infiltration has been well documented after cerebral ischemia and reperfusion1 and is known to mediate local tissue damage and alterations in microvascular perfusion. Leukocyte adhesion and extravasation are controlled by adhesion molecules present on leukocytes and endothelial cells. The leukocyte adhesion receptor macrophage-1 antigen (Mac-1) (CD11b/CD18) is a β2 integrin that is constitutively expressed on the surface of leukocytes but is transformed to an active conformation, as well as quantitatively upregulated on the cell surface, by inflammatory mediators.2 Mac-1 mediates firm adhesion of neutrophils to the blood vessel by binding to its endothelial ligand, intercellular adhesion molecule-1 (ICAM-1).3 It has several other ligands, including complement, and plays a pivotal role in neutrophil chemotaxis, aggregation, phagocytosis, and respiratory burst.2,4

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A functional blocking monoclonal antibody to Mac-1 reduced infarct volume after transient focal ischemia.5,6 However, administration of this antibody also led to partial peripheral white blood cell depletion. Treatment with an antibody to the CD18 subunit has yielded controversial results; in a feline model, the antibody did not alter cerebral blood flow or infarct volume,7 whereas in a primate model, it improved microvascular patency after cerebral ischemia/reperfusion.8 Antibody-antigen interactions can lead to complex responses, including triggering of signal transduction events and incomplete inactivation of functional binding sites on the target molecule. Therefore, knockout mice are being used as an additional in vivo approach to understand the role of leukocyte adhesion receptors in the pathogenesis of stroke. For example, ICAM-1–deficient mice subjected to transient focal cerebral ischemia followed by reperfusion manifested...
significantly smaller cerebral injury compared with wild-type counterparts that were similarly treated.9,10 ICAM-1 is a well-recognized ligand for Mac-1.1,3 Mice deficient in Mac-1 exhibited a defect in intravascular leukocyte adhesion after leukotriene B4 administration in a cremaster muscle preparation.12 In addition, after acute glomerulonephritis, FcγR-dependent glomerular neutrophil accumulation and complement-dependent proteinuria were significantly reduced in these mice.13 Furthermore, Mac-1–deficient neutrophils were unable to phagocytose complement-opsonized particles and displayed a 60% impairment in oxidative burst.12 To test the hypothesis that a complement-opsonized particles and displayed a 60% impairment in oxidative burst.12 To test the hypothesis that a deficiency in Mac-1 leads to a reduction in neutrophil accumulation and infarct size after stroke, we measured the extent of histopathological damage after transient cerebral ischemia/reperfusion in Mac-1–deficient and wild-type mice.

Materials and Methods

Animals and Induction of Transient Focal Cerebral Ischemia

Mac-1–deficient mice (Mac-1<sup>−/−</sup>) generated by gene targeting12 and wild-type mice (Mac-1<sup>+/+</sup>) were bred and maintained in a virus antibody–free facility at the Longwood Medical Research Center of the Harvard Medical School. Mac-1–deficient and wild-type mice were of a mixed C57Bl/129Sv strain and were generated as follows. Wild-type and Mac-1–deficient progeny were obtained from the breeding of mice heterozygous for Mac-1. The wild-type mice were also bred to each other to produce wild-type mice, and the Mac-1–deficient mice were bred to produce Mac-1–deficient mice, avoiding brother-sister matings. To prevent the 2 genotypes from straying in their background genes, new breedings with mice derived from heterozygous breedings were routinely set up. Mice from both the heterozygous and homozygous breedings were used in the experiments with similar results. These mice were maintained in a 12-hour light/dark cycle and had access to water and food at libitum. Only 8-week-old male mice were used for the study.

With approval of the institutional review board, mice weighing 25 to 30 g were anesthetized with isoflurane (1% to 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 right femoral artery was cannulated in 3 wild-type and 3 Mac-1–deficient mice to determine arterial blood pressure and sample arterial blood gas and glucose.

Arterial blood pressure was recorded before ischemia, during ischemia, and at reperfusion continuously with a computerized data acquisition system (Mac Labs 8s, ADInstruments). Arterial blood gases and glucose were measured 10 minutes after reperfusion with a blood gas and glucose analyzer (Stat Profile Ultra C, Nova Biomedical). The left internal carotid artery was exposed through a midline cervical incision under a dissecting microscope. All of the extracranial branches of the left internal carotid artery were ligated. A 6.0 monofilament nylon suture (Ethicon Inc) with a flame-rounded tip coated with silicon was inserted into the lumen of the external carotid artery and advanced distally into the internal carotid artery ∼10 mm to the base of the middle cerebral artery (MCA).9,14 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.

Assessment of Cerebral Blood Flow

To determine changes in regional cerebral blood flow (rCBF), we used a laser-Doppler flowmeter (BPM, Vasamedics) with a 0.7-mm probe (P433, Vasamedics). The analog signal of the laser Doppler was collected with a computerized data acquisition system (MacLab 8s, ADInstruments, on a Macintosh LC 475 computer). The skull was exposed through a midline sagittal incision, and the probe tip was placed on the skull surface 3 mm lateral to midline and 2 mm posterior to the bregma. These cortical coordinates represented the ischemic core of the infarct. rCBF was recorded in wild-type (n=3) and Mac-1–deficient mice (n=3) over 15 minutes, before and immediately after middle cerebral artery occlusion (MCAO) and before and immediately after reperfusion, as previously described.9,16 Data were presented as percentage of the preischemic rCBF.

Detection and Quantification of Cerebral Infarction

After the reperfusion period, the mice were killed with a lethal dose of pentobarbital (150 mg/kg IP). The brains were immediately removed, and 1.5-mm coronal sections were cut with a tissue cutter. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C for 30 minutes.14 These sections were fixed in 4% parafomaldehyde in phosphate buffer for digital photography. The digitized image of each brain section and the infarcted area was measured by a masked observer using a computerized image analysis program (SigmaScan, Jandel Corp.). To minimize the effect of brain edema, calculation of the infarcted volume was indirectly determined by subtracting the volume of the noninfarcted ipsilateral hemisphere (left) from the contralateral hemisphere (right), as previously described.19,20

Histological Examination for Leukocyte Accumulation

A cohort of wild-type and Mac-1–deficient mice were subjected to the same ischemia and reperfusion protocol (n=8 for each group). 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 subjected to reaction with dichloroacetate esterase to identify extravasated neutrophils31 and counterstained with nuclear fast red. To assess the neutrophil infiltrate, the masked observer then counted the number of dichloroacetate esterase–positive cells in 10 high-powered fields (×40). The fields for observation were selected to assess neutrophils present only in the periphery of the infarct. On sections with clusters of neutrophils, the observer started at the edge of the infarct, where the highest numbers of neutrophils were present, and the neutrophils were then followed in 10 nonoverlapping contiguous fields. On sections with low numbers of neutrophils, the observer started at the recognizable edge of the infarct and tracked the neutrophils along the edge of the infarct. In all cases, only neutrophils in the parenchyma, and not within blood vessels, were counted.

Statistical Analysis

Infarct volumes and neutrophil counts were compared by an unpaired t test. Data were reported as mean±SEM. Mortality rates were compared with Fisher’s exact test. A P value <0.05 was accepted as statistically significant.

Results

Mac-1 Deficiency Partially Protects Against Cerebral Ischemia/Reperfusion Injury

To determine the role of Mac-1 in infarct development, we subjected Mac-1–deficient mice and wild-type cohorts to 3
hours of focal cerebral ischemia and 21 hours of reperfusion. Mortality values, as represented by the dead/alive ratios, of the wild-type and Mac-1–deficient mice during this 24-hour experimental period were 3/14 and 1/13, respectively. These findings were not significantly different. All mice had at least a grade 3 deficit after MCAO, indicating successful placement of the intraluminal suture. Gross examination of the surface cerebral circulation after carbon black injection demonstrated that the vascular pattern in Mac-1–deficient mice was indistinguishable from that in their wild-type cohorts (data not shown). Therefore, differences in vasculature do not explain differential sensitivity of the 2 genotypes to stroke damage. rCBF was diminished during the period of MCAO and was restored, albeit diminished, similarly in mutants and wild-types (Figure 1). This suggests that postreperfusion infarction volumes and neutrophil infiltration were less in the Mac-1–deficient mice, supporting the hypothesis that Mac-1 has a role in mediating neutrophil extravasation and infarct development.

There is evidence that early neutrophil influx follows an ischemic insult and may contribute to ischemia-related neuronal damage. Neutrophils are recruited to tissues by chemokines and adhesion molecules expressed by endothelial cells and are critically involved in mediating inflammatory injury to the brain parenchyma by liberation of reactive oxygen species, proteases, eicosanoids, and cytokine. Neutrophil accumulation in cerebral infarcts of patients has been associated with poor clinical outcomes. Since Mac-1 mediates neutrophil adhesion to the endothelial surface, reperfusion would increase the likelihood of infiltration of these neutrophils into the affected brain parenchyma. However, permanent ischemia also results in neutrophil infiltration of these neutrophils into the affected brain parenchyma. However, permanent ischemia also results in neutrophil infiltration of these neutrophils into the affected brain parenchyma.

Since Mac-1 mediates neutrophil adhesion to the endothelial surface, reperfusion would increase the likelihood of infiltration of these neutrophils into the affected brain parenchyma. However, permanent ischemia also results in neutrophil adhesion to microvessels and extravasation after occlusion of the MCA. Although anti–Mac-1 monoclonal antibodies decreased tissue injury after transient focal cerebral ischemia, Garcia et al demonstrated that similar treatment did not decrease the number of neutrophils or infarct volume after permanent MCAO. Most human strokes represent transient rather than permanent occlusion. Therefore, we believe that transient MCAO with reperfusion is the most pathophysiologically relevant model for this condition.

### Table 1. Mean Arterial Blood Pressure Before, During, and After MCAO in Wild-Type and Mac-1–Deficient Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Arterial Pressure, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Ischemia</td>
</tr>
<tr>
<td>+/+</td>
<td>100±5</td>
</tr>
<tr>
<td>−/−</td>
<td>105±6</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=3 for each genotype. There was no difference in mean arterial pressure between the genotypes.

### Table 2. Arterial Blood Gases and Glucose Determined After Reperfusion in Wild-Type and Mac-1–Deficient Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Arterial pH</th>
<th>PaCO₂, mm Hg</th>
<th>PaO₂, mm Hg</th>
<th>Glucose, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>7.26±0.03</td>
<td>40±1.6</td>
<td>203±39</td>
<td>152±2</td>
</tr>
<tr>
<td>−/−</td>
<td>7.29±0.05</td>
<td>51.5±7.3</td>
<td>177±11</td>
<td>154±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=3 for each genotype. There were no differences in arterial blood gases and glucose between the genotypes.

Discussion

This study demonstrates that Mac-1–deficient mice are less susceptible to focal cerebral ischemia/reperfusion injury than wild-type mice. We found that postreperfusion infarction volumes and neutrophil infiltration were less in the Mac-1–deficient mice, supporting the hypothesis that Mac-1 has a role in mediating neutrophil extravasation and infarct development.

Figure 1. rCBF after ischemia/reperfusion. rCBF was measured by laser-Doppler flowmetry over the ischemic core of the left MCA region in wild-type (+/+ ) and Mac-1–deficient mice (−/− ) before, during 3 hours of MCAO, immediately after reperfusion, and every 5 minutes. The pres ischemic rCBF was assigned a value of 100%. Subsequent values are presented as a percentage of the pres ischemic rCBF (mean±SEM, n=3 for each genotype).

Figure 2. Neutrophil accumulation in the periphery of the infarct after injury in the Mac-1–deficient mice (Figure 2).
ICAM-1–deficient mice have smaller infarct volumes than the Mac-1–deficient or neutropenic mice. Thus, ICAM-1 deficiency may confer protection by mechanisms other than the reduction of neutrophil accumulation in tissues. It is known that infarcts are initiated during the ischemic period because of deprivation of oxygen, and therefore any mechanism that reduces the ischemic time, such as a decrease in the no-reflow phenomenon during the reperfusion period, would be protective. In fact, ICAM-1–deficient mice and neutrophil-depleted animals were shown to have an increase in rCBF compared with the contralateral (nonischemic) hemisphere after a 45-minute period of cerebral ischemia. The lack of no-reflow may be the result of a reduction in neutrophil-neutrophil or neutrophil-platelet interactions with subsequent vessel occlusion. In our study the ischemic period was 4-fold longer, and we measured rCBF in the ischemic hemisphere and compared subsequent values with the preischemic rCBF. The rCBF during the MCAO was 5% to 10% of the preischemic values, and restoration of flow resulted in rCBF equal to 50% of baseline in both genotypes (Mac-1–deficient and wild-type). This finding is consistent with previous studies using a 3-hour ischemic period. The 50% decrease from the baseline preischemic rCBF may be due to a combination of perivascular edema and non–Mac-1–mediated microvascular plugging. This prolonged period of ischemia can lead to perivascular edema, which would result in external compression of blood vessels and a subsequent decrease in rCBF compared with preischemic values. Therefore, we would expect some baseline tissue injury due to reduced perfusion of ischemic tissue in both genotypes. Furthermore, microvascular plugging occurs during reperfusion, and relevant receptors in this phenomenon may be ICAM-1 and LFA-1, a β2 integrin present on both neutrophils and platelets. Differences in tissue injury and neutrophil infiltration between wild-type and Mac-1–deficient mice are most probably due to events beyond microvascular plugging, since rCBFs were comparable during both the ischemic and reperfusion periods.

In both the ICAM-1 and Mac-1–deficient mice, the effects of reperfusion-induced injury at time points >24 hours after the onset of ischemia were not assessed. It is possible that the lack of Mac-1 or ICAM-1 may only delay the conversion of the ischemic lesion to infarction. On the other hand, 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. Both monocytes and activated microglia residing in the brain may play a role in the progression of cerebral ischemic injury. Notably, Mac-1 and ICAM-1 are expressed and upregulated on activated microglia and may play a role in microglia- and monocyte-mediated maturation of ischemic lesions. Therefore, the role of the microglia in the development of stroke injury is a fertile area for further investigation. In summary, our data suggest an important role for Mac-1 in the evolution of ischemic injury after transient cerebral ischemia. Our findings demonstrate that selective inhibition of Mac-1 is a promising therapeutic option for the acute treatment of transient focal cerebral ischemia.

Acknowledgments
This study was supported by the William F. Milton Fund and William Randolph Hearst Fund (Dr Soriano), National Institutes of Health grant NS33296 (Dr Mayadas), and a postdoctoral fellowship from the Lady Tata Memorial Trust (Dr Coxon). The authors wish to thank Michael Goodman (Department of Pathology, Brigham and Women’s Hospital, Boston, Mass) for his technical assistance.

Figure 3. Effect of Mac-1 on neutrophil accumulation after transient focal cerebral ischemia. Extravascular neutrophils (arrows) in the brain parenchyma were identified in coronal sections stained with dichloroacetate esterase and nuclear fast red. Representative sections from wild-type mice (a) and Mac-1−deficient mice (b) are shown. Bars=50 μm. c, Number of neutrophils in the ischemic hemisphere in wild-type (+/+) and Mac-1−deficient mice (−/−) (mean±SEM; n=8 for each genotype).

References
Inflammatory response and leukocyte infiltration after cerebral ischemia have been known to play key roles in ischemic brain injury, particularly during the reperfusion phase. Previous experimental approaches used chemical methods to produce leukopenia or to use selective antibodies specific for adhesion molecules that are associated with the binding of leukocytes to endothelial cells. These approaches may have unwarranted side effects and toxicity. One current experimental strategy to prove the principle of the involvement of leukocyte infiltration is to use animals that lack the expression of the key molecule involved in the binding process between leukocytes and endothelium.

Using this unique approach, Soriano and colleagues now provide strong evidence that the ischemic infarction is significantly reduced in mice deficient in Mac-1 (CD11b/CD18), an adhesion molecule that mediates adhesion of neutrophils to ICAM-1, an endothelial ligand, after transient focal cerebral ischemia. The study appears to be carefully done, and the findings are independent of the alteration of cerebral blood flow but are closely related to the reduced infiltration of neutrophils. Although the findings provide an impetus for future pharmacological developments and therapeutic approaches in stroke research, the acute nature (ie 21 hours after reperfusion) may preclude a definite conclusion as to whether the neuroprotection in the mutant mice is long-lasting. Additional studies during the long reperfusion/recovery period may be helpful to address this important issue.

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Stroke. 1999;30:134-139
doi: 10.1161/01.STR.30.1.134

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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