Matrix Metalloproteinase-9 and Myeloperoxidase Expression 
Quantitative Analysis by Antigen Immunohistochemistry in a Model of 
Transient Focal Cerebral Ischemia

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Background and Purpose—Expression of matrix metalloproteinases (MMPs), proteolytic enzymes that degrade extracellular proteins, is altered after ischemia/reperfusion injury and may contribute to blood–brain barrier (BBB) breakdown. Neutrophils, a source of reactive oxygen species and MMP-9, infiltrate damaged tissue 6 to 24 hours after ischemia and have also been implicated in delayed secondary tissue damage. Here we examined the spatial–temporal relation between MMP-9 expression and neutrophil infiltration after stroke.

Methods—Knockout mice containing 50% manganese superoxide dismutase activity (SOD2-KOs), which are more susceptible to ischemic damage than wild-type (WT) littermates, underwent quantitative antigen (MMP-9, myeloperoxidase) immunohistochemistry (24 and 72 hours) analysis and protein expression by Western blotting (6, 12, 24, 48, and 72 hours) after transient focal cerebral ischemia. BBB breakdown was determined by Evans blue extravasation.

Results—There was a clear spatial relation between MMP-9 expression and Evans blue extravasation. MMP-9–positive cell and vessel counts for SOD2-KOs (72 hours) were significantly different from SOD2-KO (24 hours, \( P = 0.004 \)), WT (24 hours, \( P = 0.01 \)), and WT (72 hours, \( P = 0.007 \)) mice. In contrast, MMP-9–positive neutrophil counts were comparatively low and did not differ by time or animal type. MMP-9 expression was biphasic in SOD2-KOs but not in WT littermates, with a significant increase observed 6 to 12 hours after ischemic insult and again at 48 to 72 hours. SOD2-KOs showed increased MMP-9 expression compared with WT littermates at all time points studied (\( P \leq 0.05 \)).

Conclusions—in this model, neutrophils are not the primary source of MMP-9 protein and thus are unlikely the key contributor to BBB breakdown observed in SOD2-KOs. (Stroke. 2004;35:1169-1174.)

Key Words: metalloproteinases • inflammation • cerebral ischemia, focal • blood-brain barrier • oxygen radical

Reperfusion after cerebrovascular occlusion increases the risk of intracerebral bleeding and cerebral edema, which can cause extensive damage to neurons, disrupt the extracellular matrix, and increase capillary permeability. Although numerous substrates have been implicated in ischemia/reperfusion (I/R) injury, the mechanisms underlying delayed secondary tissue damage are still poorly understood. Recently, increasing attention has been focused on the possible roles of proteases that are upregulated after cerebral I/R. Participation of zinc-dependent matrix metalloproteinases (MMPs), particularly MMP-9 and MMP-2, is of particular interest because they can degrade components of the extracellular matrix, including collagen and laminin in the basal lamina and zona occludens-1 in endothelial tight junctions. Degradation of these critical proteins leads to disruption of cerebrovascular structural integrity and thus, leakage or rupture of the blood–brain barrier (BBB). Hemodynamic changes, injury, inflammation, and oxidative stress all appear to regulate MMP expression and activity.

The emerging concept that MMPs contribute to secondary brain injury is especially interesting for the potential development of therapeutic interventions aimed at preventing intracerebral hemorrhage after treatment with thrombolytic agents. MMP-9 expression occurs early in the ischemic basal ganglia of nonhuman primates displaying hemorrhagic transformations. Sumii and Lo have shown that treatment with tissue-type plasminogen activator (t-PA) increases MMP-9 levels after embolic focal cerebral ischemia in rodents, suggesting that MMPs are involved in the mechanism of t-PA–associated hemorrhage. Treatment with MMP inhibitors, in contrast, has been demonstrated to reduce the presence and extent of hemorrhagic complications in t-PA–treated animals.

Several studies including mostly Western blotting and reverse zymographic techniques have also documented alterations in MMP expression after stroke and traumatic brain injury. Although these studies have highlighted the contribution of extracellular proteolysis to the progression of
tissue damage after acute brain injury, most have used end points of ≤24 hours and have not addressed the contribution of specific MMP-9–producing or MMP-9–sensitive cell types to infarct development.

To understand the relation between reactive oxygen species (ROS), inflammatory processes, and MMP expression in I/R, it is important to ascertain the spatial–temporal distribution and cellular localization of the substrates in question. Manganese superoxide dismutase knockout (SOD2-KO) mice are particularly susceptible to ischemic injury owing to their inability to adequately scavenge superoxide anion, a key player in the oxidative chain reaction.1-3 Thus, we speculated that MMP-9 expression would be increased in SOD2-KOs compared with wild-type (WT) littermates, causing greater BBB disruption and thus, partly explaining the increased neuronal damage observed in SOD2-KO mice. In this investigation, we performed quantitative analysis of protein expression by antigen immunohistochemistry, focusing on expression of MMP-9 and myeloperoxidase (MPO), a marker for neutrophils, during peak leukocyte infiltration periods (24 to 72 hours) after transient focal cerebral ischemia. We also examined the temporal expression of MMP-9 by Western blotting at 6, 12, 24, 48, and 72 hours after ischemic insult to determine whether neutrophil infiltration peak periods coincided with increased levels of MMP-9.

Materials and Methods

Animal Procedures

Animal protocols were approved by the institutional Administrative Panel on Laboratory Animal Care; procedures were followed according to their guidelines.

Focal Cerebral Ischemia

Male CD-1 mice (32 to 35 g; Stanford University) anesthetized with 2% isoflurane (30% oxygen/70% nitrous oxide) and maintained at a surgical plane of anesthesia (1% to 1.5% isoflurane) underwent middle cerebral artery (MCA) occlusion by the intraluminal suture method7 for 30 minutes (body temperature maintained at 37 °C). Animals were allowed to reperfuse for 5.5, 11.5, 23.5, 47.5, or 71.5 hours with free access to food and water and were euthanized with an isoflurane overdose at the desired end point.

Histopathology and Immunohistochemistry

After transcardiac perfusion (heparinized saline followed by 3.7% paraformaldehyde), brains were removed, postfixed (3 days), embedded in paraffin, sectioned (6-μm thick coronal sections), and stained (hematoxylin and eosin). Infarct was evaluated in the hemisphere, cortex, and striatum; histologic criteria included areas of pannecrosis, with shrunken, dark neurons and glial pallor. Infarcted areas (7 coronal levels) were determined with an image analysis system (MCID, Imaging Research). For immunofluorescence, frozen brain sections treated for endogenous peroxidases and blocked for avidin/biotin were used. Sections were incubated with primary antibody (MMP-9 and MMP2, 1:100; NeuN, glial fibrillary acidic protein [GFAP], MPO, and CD11b, 1:200; at 4 °C for 1 hour) followed by a secondary antibody (1 hour for paraffin sections and 10 minutes for frozen sections at 25 °C). In paraffin-embedded sections, MMP-9 incubation lasted 72 hours; antibodies were detected with the Vector-ABC kit and colorized with Vector-VIP (Vector Labs). Vascular structures and microglia were identified with biotinylated isoelectin-B4 (IB4, Vector Labs; 3-hour incubation); reaction products were detected with the Vector-ABC kit and colorized with diaminobenzidine. Both colorizing agents were also used for identification of different cell types or for colocalizing protein expression through double labeling. Fluorescein avidin dCS, Texas red avidin D, or AMCA avidin D was used for frozen sections; the tissue was mounted with 4′,6-diamino-2-phenylindole (DAPI)-containing medium. Negative controls were run in parallel using adjacent sections incubated without primary antibody.

Cell Counting

The anatomic distribution of MPO-positive and MMP-9–positive cells was studied at 7 levels (Figure 1B) from 2 representative animals to identify the coronal level(s) with consistently high numbers of stained cells or vascular structures. Numbers of MPO- and MMP-9–positive cells and/or vascular structures were counted in the entire ipsilateral hemisphere (coronal section level 3, 3.4 mm anterior to the bregma) of 4 representative animals and sham-manipulated controls and in 3 regions of interest (ROIs; Figure 1A). ROIs were defined with reference to unique topographic features of the infarct and anatomic landmarks. Only animals with nearly identical infarct sizes and locations were used. For morphometric image analysis of MMP-9– and MPO-immunostained material, coronal sections (level 3) were divided into 26 image areas (precisely at the same location in every brain) that were digitized in a Zeiss Axiosplan 2 microscope (10-fold objective) with a Zeiss AxioCam and imported into AxioVision. Images were subsequently viewed at 175% of the original 10× image with AdobePhotoshop 5.5 software (Adobe Systems) for counting.

Evans’s Blue Extravasation

Six hours before euthanasia, 2.5 mL/kg of 4% Evans blue (Sigma) in 0.9% saline was injected into every animal. Animals were anesthetized and perfused (200 mL heparinized saline). For qualitative examination of Evans blue extravasation, brains were fixed in formaldehyde and sectioned at 20 μm. Sections were incubated (10 minutes) with 2 μg/mL Hoechst 33258 (Molecular Probes) for nuclear counterstaining, mounted, and observed with a fluorescence microscope.

Western Blot Analysis

One-millimeter-thick samples from each hemisphere (coronal levels 3 and 4) were homogenized in suspension buffer (1 mol/L KOH, 1 mol/L. KCl, 1 mol/L. MgCl2, 0.5 mol/L. EDTA, 0.5 mol/L. EGTA, sucrose, protease cocktail) and centrifuged (14,000 rpm, 10 minutes, 4 °C), and supernatants were transferred into equal volumes of sodium dodecyl sulfate buffer with β-mercaptoethanol and stored (−20 °C). Aliquots were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% to 20% Tris-glycine gel; In Vitrogen). Polyvinylidene difluoride membranes were incubated in methanol and then transfer buffer (5 minutes each). Transfer (25 V, 1.5 hours) was followed by incubation in 5% milk in phosphate-buffered saline–TWEEN (60 minutes). Membranes were incubated overnight (4 °C) with MMP-9 antibody (1:100 dilution, 1% milk in phosphate-buffered saline–TWEEN) and then in peroxidase-conjugated secondary antibody (1 hour, 25 °C). Signals were detected with a chemiluminescence kit ECL plus a Western blotting detection system (Amersham Biosciences). To confirm consistent protein loading for each lane, membranes were stained for β-actin. Films were scanned with a GS-700 imaging densitometer (Bio-Rad); quantitative analyses were performed with Multi-Analyst software (Bio-Rad).

Statistical Analysis

MMP-9 and MPO data were analyzed by generalized linear models for a nested factorial design (2-tailed; SAS software version 8). Model main effects were animal type, time, and area. All data are expressed as mean±SEM; a value of P<0.05 was considered significant.

Results

The spatial distribution of MMP-9 and MPO expression is shown in Figure 1B. MMP-9–positive cells and vascular
structures were observed throughout the affected tissue at all 7 coronal levels. Neutrophils were also visible throughout the ischemic tissue, even outside the infarct border, but appeared particularly concentrated in ROI 3, near the MCA base. Sham-manipulated animals (suture advanced 5 mm) showed virtually no MMP-9 immunostaining and no MPO-positive cells (not shown). Animals subjected to 30 minutes of MCA occlusion showed a clearly delineated area of MMP-9– and MPO-positive immunostaining in the infarcted hemisphere (Figure 1A) at 24 and 72 hours after the ischemic insult. MMP-9–positive vessels, including capillaries, were clearly delineated by the dark blue reaction product in both WT and SOD2-KOs, although immunoreactivity was more intense in the latter (Figure 2A and 2B). MMP-9–immunopositive vessels were found throughout the infarcted hemisphere but most prominently in the peri-infarct zone, where Evans blue extravasation was still clearly visible 72 hours after the ischemic insult (Figure 2C and 2D). MMP-9-/IB4-positive microglia were also observed in the infarcted hemisphere, but not all IB4-positive cells showed MMP-9 immunoreactivity (Figure 2E). In contrast, all MPO-positive cells were immunoreactive for MMP-9 antibody (Figure 2F). MMP-9 immunofluorescence was easily discerned in vessels showing Evans blue extravasation (Figure 2F and 2G). Antigen specificity and MMP-9 protein levels were confirmed by Western blotting. Typical temporal expression of MMP-9 is shown in Figure 3A. MMP-9 increased significantly 6 to 12 hours after the insult but decreased at 24 hours in SOD2-KOs and WTs; MMP-9 increased again at 48 to 72 hours of reperfusion in SOD2-KOs only. SOD2-KOs showed increased levels of MMP-9 expression at all time points compared with WT animals. Figure 3B shows variability in MMP-9 expression among animals. Relative to WTs, SOD2-KOs had significantly greater MMP-9 levels at all time points (n=6 per group, per time point): 14% (6 hours), 20% (12 hours), 20% (24 hours), 50% (48 hours), and 47% (72 hours).

The results of quantitative analysis for MMP-9 and MPO antigen immunoreactivity in cells and/or vascular structures are shown in the Table. The data show that results from the evaluation of MMP-9 counts are not consistent by ROI. The

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**Figure 1.** (A) Anatomic ROIs from which MMP-9- and MPO-positive cell and vascular structure counts were measured, superimposed on a schematic diagram of coronal level 3 (0.4 mm anterior to the bregma). Photomicrographs show typical MMP-9 (blue)/IB4 (brown) immunoreactivity in ROI 2 and MPO-positive cells (neutrophils) in ROI 3. Scale bars = 50 μm. Insert shows infiltration of neutrophils from a microvessel 72 hours after ischemic insult. Scale bar = 20 μm. (B) Coronal sections from a representative SOD2-KO mouse 72 hours after MCA occlusion (30 minutes), showing typical distribution of MMP-9 (blue)/IB4 (brown; top row) and MPO (bottom row) areas. MMP-9–positive cells and vascular structures were found scattered throughout the ischemic area but were most prominent in the infarct border (higher magnification depicted in ROI 2 above). MPO-positive cells were also found throughout the affected tissue but in slightly higher numbers in ROI 3, near the MCA base.
most consistent results were obtained for counts from multiple areas or entire levels, such as total MMP-9–positive counts for cells or vascular structures. The least-squares means for normalized cell counts from ROIs were obtained from linear models that included type of animal (WT vs SOD2-KO) and time (24 vs 72 hours) as main effects and the interaction between animal type and time. The data support an association between time and MMP-9 count. Although MMP-9 was clearly present at high levels in neutrophils, the counts were comparatively low and did not differ by time or animal type. Because there was considerable variability in these counts (as shown by the large SEs relative to the adjusted means), the use of regions that included more cells or vessels (such as the entire coronal level) increased the power to detect a difference. Our results highlight the fact that the spatial-temporal expression of MMP-9 is variable within the infarcted hemisphere, with various cell types contributing differently, depending on the selected ROI.

Discussion

The pathophysiologic mechanisms of reperfusion injury are not well understood, but recent studies implicate excess production of ROS, BBB disruption, and postischemic brain inflammation as key components of this process.13–16 ROS can be released by injured tissues to trigger activation of the immune response. The vascular endothelium and activated leukocytes are also sources of ROS. As the levels of ROS increase, they participate in activation of various pathophysiologic responses, such as attenuation of vasodilatory mechanisms and promotion of adhesion protein expression. When the cellular antioxidant systems become overwhelmed, ROS become activators of apoptotic or necrotic cellular injury.17 MMPs, which are activated by proteases and free radicals, are another set of substrates that participate in cellular damage after cerebral ischemia. MMPs also contribute to the neuroinflammatory response in many neurologic diseases, thus sup-

Figure 2. Photomicrographs from representative animals after MCA occlusion (30 minutes) and reperfusion (72 hours). MMP-9–immunopositive vessels were found throughout the infarcted hemisphere but most prominently in the peri-infarct zone in WT (A) and SOD2-KO (B) mice. MMP-9 immunoreactivity was significantly stronger in SOD2-KO compared with WT animals in large vessels and capillaries (inserts; scale bar=10 μm). (C)–(H) Representative SOD2-KOs 72 hours after ischemic insult. (C) Evans blue (red) and Hoechst (blue) staining in the contralateral cortex. Evans blue extravasation (diffuse red staining) in the infarct border was still clearly visible 72 hours after insult (D). (E) MMP-9-/IB4-positive microglia were observed throughout the infarcted hemisphere, but not all IB4-positive cells showed MMP-9 immunoreactivity. However, all MPO-positive cells were immunoreactive for MMP-9 (F). MMP-9 immunofluorescence (green), Evans blue, and Hoechst staining in the cortical ischemic penumbra were clearly discerned in both large vessels (G) and capillaries (H).

Figure 3. (A) Western blot and optical density measurements (B) of MMP-9 at 6, 12, 24, 48, and 72 hours after 30 minutes of MCA occlusion (n=1 per time point). MMP-9 expression was biphasic in SOD2-KOs, with an initial peak at 6 to 12 hours after insult and a second increase at 48 to 72 hours into reperfusion. (C) and (D), Typical MMP-9 expression variability in SOD2-KOs and WT littermates. Overall (n=6 independent studies per time point), SOD2-KOs had significantly greater MMP-9 levels at all time points studied compared with WT animals.
MMP-9 Counts

<table>
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<tr>
<th>ROIs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Sum of 1–3</th>
<th>Coronal Level 3</th>
<th>MMP-9+ (Minus Neutrophils)</th>
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<td>WT</td>
<td>458 ± 219</td>
<td>500 ± 201</td>
<td>707 ± 229</td>
<td>1666 ± 324</td>
<td>3703 ± 409</td>
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<tr>
<td>K0</td>
<td>583 ± 203</td>
<td>879 ± 186</td>
<td>742 ± 212</td>
<td>2205 ± 300</td>
<td>4343 ± 379</td>
<td>4108 ± 370</td>
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<td>24h</td>
<td>338 ± 203</td>
<td>284 ± 186*</td>
<td>691 ± 212</td>
<td>1314 ± 300*</td>
<td>2621 ± 379*</td>
<td>2421 ± 370*</td>
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<tr>
<td>72h</td>
<td>703 ± 219</td>
<td>1095 ± 201*</td>
<td>758 ± 229</td>
<td>2557 ± 324*</td>
<td>5424 ± 409*</td>
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<tr>
<td>WT</td>
<td>56 ± 45</td>
<td>68 ± 45</td>
<td>61 ± 56*</td>
<td>186 ± 141</td>
<td>446 ± 484</td>
<td>265 ± 463</td>
</tr>
<tr>
<td>K0</td>
<td>177 ± 42†</td>
<td>143 ± 42</td>
<td>205 ± 52‡</td>
<td>526 ± 130</td>
<td>1427 ± 448</td>
<td>1335 ± 429</td>
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<tr>
<td>24 h</td>
<td>53 ± 42†</td>
<td>61 ± 42</td>
<td>73 ± 52</td>
<td>187 ± 130</td>
<td>430 ± 448</td>
<td>157 ± 429*</td>
</tr>
<tr>
<td>72 h</td>
<td>180 ± 45†</td>
<td>151 ± 45</td>
<td>193 ± 56</td>
<td>525 ± 141</td>
<td>1443 ± 484</td>
<td>1443 ± 463*</td>
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<td>Neutrophils</td>
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<tr>
<td>WT</td>
<td>27 ± 8†</td>
<td>18 ± 6</td>
<td>52 ± 19</td>
<td>98 ± 24</td>
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<tr>
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<td>17 ± 6</td>
<td>52 ± 17</td>
<td>92 ± 22</td>
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<tr>
<td>24 h</td>
<td>20 ± 7</td>
<td>8 ± 6</td>
<td>55 ± 17</td>
<td>84 ± 22</td>
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<tr>
<td>72 h</td>
<td>29 ± 8</td>
<td>27 ± 6</td>
<td>48 ± 19</td>
<td>106 ± 24</td>
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</table>

Values are least-squares means ± SEM for normalized counts from regions of interest. Values were obtained from linear models that included type of animal (WT vs SOD2-KO) and time (24 vs 72 hours) as main effects and the interaction between animal type and time. Abbreviations are as defined in text.

*Mean cell count for 24 hours was different from 72-hour count (P = 0.01 for ROI 2 and sum of ROIs; P = 0.0004 for coronal level 3; P = 0.0003 for all MMP-9+ cells minus neutrophil counts; and P = 0.07 for all MMP-9+ vessels).
†Mean vessel count for WT was somewhat different from SOD2-KO count (P = 0.08), as were mean counts for 24 vs 72 hours (P = 0.06); interaction between animal type and time with SOD2-KO 72-hour counts was significantly different from WT at 24 hours (P = 0.01), WT at 72 hours (P = 0.007), and SOD2-KO at 24 hours (P = 0.004).
‡P = 0.09 for mean vessel count for WT vs SOD2-KO.

Our previous work has shown that activated MMP-9 can be observed 4 hours after ischemic onset, paralleling the increases in BBB permeability as detected by Evans blue extravasation. We have also shown that I/R (1 hour/7 hour)-induced BBB disruption in mice deficient in copper/zinc superoxide dismutase (SOD1) can be reduced by 73% after MMP inhibition and that local metalloproteinase-generated proteolytic imbalance is more intense in ischemic regions of SOD1-KO mice than in WT littermates.

Additional support for free radical–induced MMP expression and activation has been provided by Gursoy-Ozdemir et al., who have shown that after 2-hour MCA occlusion, the infarct volume is significantly decreased in animals treated with the nonselective nitric oxide synthase inhibitor N-nitro-L-arginine at reperfusion, along with a significant reduction in MMP-9 expression and vascular damage.

The I/R-induced increase in vascular permeability has also been linked to inflammatory events that involve ROS production and leukocyte–endothelial cell adhesion and migration. This concept is supported by studies showing that agents targeting either the generation of ROS by endothelial cells (eg, SOD) and/or the adhesion of leukocytes to vascular endothelium are generally effective in blunting the I/R increase in microvascular permeability. Recently, Ishibashi and colleagues have shown that transgenic mice overexpressing the antioxidant enzyme glutathione peroxidase have reduced I/R damage. Moreover, the protective mechanism in these glutathione peroxidase overexpressers involves modulation of the inflammatory response, as well as reduced sensitivity of neurons to cytotoxic cytokines.

Because neutrophils themselves are a source of ROS and MMPs (MMP-9 and MMP-8) and their peak infiltration period (24 to 72 hours after I/R) coincides with Evans blue leakage, it is reasonable to assume that these cells play a critical role in MMP-mediated BBB disruption and secondary brain damage. Results from our studies show that SOD2-KOs had a greater increase in MMP-9 expression compared with their WT littermates, which was qualitatively reflected by Evans blue extravasation. However, our analyses also showed that sample size and the selected ROIs critically determined the study outcome and thus, the conclusions drawn, relative to MMP-9 and MPO expression. Overall, our data support an association between time and MMP-9–positive counts but do not support an association between animal type and MMP-9 count (based on adjusted means for all WT and all SOD2-KO animals). Although neutrophils showed strong MMP-9 immunostaining, their counts were comparatively low and did not differ by time (24 vs 72 hours) or animal type (WT versus SOD2-KO). This suggests that neutrophils are not the primary source of MMP-9, even at the peak of leukocyte infiltration. Furthermore, the spatial distribution of neutrophils does not always correlate with MMP-9 expression. In our model, neutrophil infiltration 3 days after ischemic insult was very prominent in ROI 3, but these inflammatory cells were also widely distributed throughout the ischemic tissue. MMP-9 expression, however, was very prominent in vascular structures (particularly in peri-infarct areas), microglia, and astrocytes, in addition to being present in neutrophils. Finally, our Western blot analyses showed a biphasic peak of MMP-9 expression that did not necessarily coincide with the peak periods of neutrophil infiltration, particularly in WT animals. Our data are supported by work on activated MMP-2 and MMP-9 in aortic aneurysms, whose authors were unable to...
find a significant correlation between the level of MMP-9 expression in the affected vessels and the score of infiltrating inflammatory cells, presumably because MMP-9–positive cells represented only a very small subset of the inflammatory cells.

The present study does not rule out a significant role for neutrophils in BBB disruption and secondary brain damage after I/R. However, our findings suggest that other cell types (eg, microglia, astrocytes, and endothelial cells) may be the effective targets of MMP inhibition. To develop appropriate therapeutic strategies, it is critical to determine which cell types are the key players in the evolution of brain injury. As with most pharmacologic treatments, MMP inhibition has the potential to be both effective and detrimental, depending on compound specificity and the therapeutic window used. Additional studies are needed to further clarify the role of MMPs and inflammatory cell responses in BBB breakdown and secondary brain damage.

Acknowledgments
This work was supported by American Heart Association Postdoctoral Fellowship No. 0120142Y (to C.M.M.); National Institutes of Health grants NS 14534, NS 25372, NS 36147, and NS 38653; and the American Heart Association Bugher Foundation Award (to P.H.C.). P.H.C. is a recipient of the Javits Neuroscience Investigator Award (to M.M.C.). M.M.C. is supported by National Institutes of Health grants NS 14534, NS 25372, NS 36147, and NS 38653; and the American Heart Association Postdoctoral Fellowship No. 0120142Y (to C.M.M.); National Institutes of Health grants NS 14534, NS 25372, NS 36147, and NS 38653; and the American Heart Association Bugher Foundation Award (to P.H.C.).

References
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Stroke. 2004;35:1169-1174; originally published online April 1, 2004;
doi: 10.1161/01.STR.0000125861.55804.f2

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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World Wide Web at:
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