Matrix Metalloproteinase Expression Increases After Cerebral Focal Ischemia in Rats

Inhibition of Matrix Metalloproteinase-9 Reduces Infarct Size

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Background and Purpose—Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade the extracellular matrix and are implicated in numerous pathological conditions including atherosclerosis, inflammation, and tumor growth and metastasis. In the brain, the endothelial cell wall, strengthened by tight junctions, defines the blood-brain barrier (BBB). The extracellular matrix molecules constitute the basement membrane underlying the vasculature and play a critical role in maintaining the integrity of the BBB. After focal stroke, there is a breakdown of the BBB with an associated increase in vascular permeability, inflammatory cell influx, and neuronal cell death. The present study was designed to investigate the effects of MMP expression after stroke.

Methods—Focal stroke was produced by permanent middle cerebral artery occlusion (MCAO) in the rat, and MMP protein expression was measured by Western blot and zymogram analysis over a time course ranging from 6 hours to 30 days (n=32). Immunohistochemistry at 1 and 5 days (n=8 and 6, respectively) was also utilized to characterize the expression of several MMPs and related proteins after stroke, including their cellular source. To test the hypothesis that early increased MMP-9 expression is involved in ischemic brain injury, a neutralizing monoclonal antibody directed against MMP-9 was administered intravenously (n=7 per group) 1 hour before MCAO, and infarct size was measured 24 hours later.

Results—MMP expression increased progressively over time after stroke. After 12 hours, significant (P<0.05) MMP-9 activity was observed that reached maximum levels by 24 hours (P<0.001), then persisted for 5 days at this level and returned to basal (zero) levels by 15 days. On the basis of morphological criteria, MMP-9 appeared to stain with endothelial cells and neutrophils identified both within and at the periphery of the infarct within 24 hours of focal ischemia. After 5 days, MMP-9 appeared to stain with macrophages present within the infarcted brain. MMP-2 activity was significantly (P<0.001) increased by 24 hours and was maximum after 5 days following MCAO. MMP-2 appeared to stain with macrophages present within the infarcted region. Unlike MMP-9 and MMP-2, tissue inhibitor of metalloproteinase-1 was identified at comparable levels in both control and ischemic tissue after MCAO. MMP-1 and MMP-3 could not be detected in the brain after focal stroke. When an MMP-9–neutralizing monoclonal antibody was administered systemically, animals exhibited significantly reduced infarct size (ie, a 30% reduction compared with non–immune antibody controls; P<0.05).

Conclusions—These results demonstrate that early increased MMP-9 expression in endothelial cells and infiltrating neutrophils is a significant response to cerebral focal ischemia and that selective inhibition of MMP-9 activity can significantly reduce brain injury after stroke.

Key Words: antibody inhibition ■ blood-brain barrier ■ endothelium, vascular ■ leukocytes ■ metalloproteinases ■ neutrophils ■ stroke, ischemic

The ECM is a multifunctional complex of proteins and proteoglycans assembled in a highly organized manner that contributes to the structural integrity of cells and tissue within an organ system. The basement membrane, which provides structural support to the vasculature, is composed of ECM molecules such as type IV collagen, laminin, and fibronectin. Various factors are involved in maintaining the integrity of the ECM and the tissues it supports. However, in certain pathological circumstances the ECM is modulated such that the structure of the tissue becomes damaged or destroyed. MMPs are a group of zinc-dependent enzymes that degrade the molecules of the ECM. Two members of the...
In the brain, the endothelial cell wall, strengthened by tight junctions, defines the BBB. The basement membrane underlying the vasculature plays a critical role in maintaining the integrity of the BBB by providing structural support to the endothelial cell wall. The BBB serves to protect the central nervous system from invasive agents, such as inflammatory cells and bacteria, as well as from chemical agents. After focal stroke there is a breakdown of the BBB with an associated increase in vascular permeability. Damage to the BBB often results in hemorrhage and edema, resulting in neuronal cell death. Brain injury after focal stroke is primarily a result of the decrease in blood flow and of energy depletion due to occlusion of a cerebral blood vessel. The neuronal tissue becomes infarcted as a result of these events, with contributions from excitotoxicity, enzyme activation, edema, and inflammation.

The MMP family, MMP-2 (72-kD gelatinase/gelatinase A) and MMP-9 (92-kD gelatinase/gelatinase B), degrade the ECM components of the basement membrane. Their substrates include types IV and V collagen, fibronectin, elastin, and denatured interstitial collagens. Matrix degradation attributed to these proteinases has been shown to play an important role in the progression of diseases such as atherosclerosis, inflammation, and tumor growth and metastasis.

In MMP activity after stroke has been demonstrated. Re-activated studies have shown that laminin, fibronectin, and type IV collagen (all basement membrane components) are lost after cerebral ischemia and reperfusion, contributing to the loss of microvascular integrity. There is also a significant inflammatory response that follows stroke. It has been shown that leukocytes, including neutrophils and macrophages, infiltrate into the ischemic brain tissue and contribute to ischemic brain injury. Furthermore, it has been demonstrated that neutrophils utilize MMPs for their migration.

**Selected Abbreviations and Acronyms**

- **BBB** = blood-brain barrier
- **DAB** = 3,3′-diaminobenzidine
- **ECM** = extracellular matrix
- **MCAO** = middle cerebral artery occlusion
- **MMP** = matrix metalloproteinase
- **MMP-1** = matrix metalloproteinase 1; interstitial collagenase
- **MMP-2** = matrix metalloproteinase 2; 72-kD gelatinase
- **MMP-3** = matrix metalloproteinase 3; stromelysin
- **MMP-9** = matrix metalloproteinase 9; 92-kD gelatinase
- **SDS-PAGE** = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
- **TIMP** = tissue inhibitor of metalloproteinase

**Figure 1.** Western blot analysis of stroke-induced MMP-9 and MMP-2 in ischemic rat brain tissue extracts. A, Tissue extracts were analyzed for stroke-induced MMP activities over a time course of 6 hours to 30 days with the use of SDS-PAGE zymography containing gelatin or casein. Active MMP-9 (molecular weight [MW] = 87 kD) and MMP-2 (MW = 67 kD) as well as the latent “pro” forms (MW = 92 kD and 72 kD, respectively) were detected in the gelatin-containing zymogram shown here. Lanes marked with A indicate samples from the ischemic cortical region of the rat brain. Lanes marked with B indicate samples from the contralateral control cortical region of the rat brain. MW markers are as indicated on the left. Data are representative examples; similar analyses were conducted in other animals with identical results (n = 4 animals per time point studied).

**Figure 2.** SDS-PAGE zymographic analysis of stroke-induced MMP activity in ischemic rat brain tissue extracts. A, Tissue extracts were analyzed for stroke-induced MMP activities over a time course of 6 hours to 30 days with the use of SDS-PAGE zymograms containing either gelatin or casein. Active MMP-9 (MW = 87 kD) and MMP-2 (MW = 67 kD) as well as the latent “pro” forms (MW = 92 kD and 72 kD, respectively) were detected in the gelatin-containing zymogram shown here. Lanes marked with A indicate samples from the ischemic cortical region of the rat brain. Lanes marked with B indicate samples from the contralateral control cortical region of the rat brain. MW markers are as indicated on the left. Data are representative examples; similar analyses were conducted in other animals with identical results (n = 4 animals per time point studied).

It has been demonstrated that MMP-2 injections directly into rat brain disrupt the BBB and are associated with the increased expression of MMP-9. More recently, an increase in MMP activity after stroke has been demonstrated. Related studies have shown that laminin, fibronectin, and type IV collagen (all basement membrane components) are lost after cerebral ischemia and reperfusion, contributing to the loss of microvascular integrity. There is also a significant inflammatory response that follows stroke. It has been shown that leukocytes, including neutrophils and macrophages, infiltrate into the ischemic brain tissue and contribute to ischemic brain injury. Furthermore, it has been demonstrated that neutrophils utilize MMPs for their migration.
These studies suggest a role for MMPs and basement membrane degradation in the breakdown of the BBB and in the leukocyte infiltration that occurs with stroke.

The cellular source(s) of MMP expression and its contribution to focal ischemic injury have not previously been studied. The present series of experiments were performed to define the time course, isozyme subtype, and cellular sources of increased MMP expression that occurs after cerebral focal ischemia in the rat. In addition, a selective MMP-9 inhibitor was administered systemically to rats receiving cerebral ischemia to determine whether selective, early blockade of increased MMP-9 activity could reduce brain injury after focal stroke.

Materials and Methods

Focal Brain Ischemia

Cerebral focal ischemia or sham surgery was performed in male spontaneously hypertensive rats (Taconic Farms, Germantown, NY) at 16 to 18 weeks of age weighing 250 to 330 g by permanent MCAO, as described in detail previously20,21,24, and in compliance with the guidelines for the care and use of laboratory animals approved by The Animal Care and Use Committee at SmithKline Beecham Pharmaceuticals. Briefly, the animals were anesthetized with pentobarbital (60 mg/kg IP), and the MCA was occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein with the use of electrocoagulation (Force 2 Electrosurgical Generator, Valley Laboratory Inc). In sham-operated rats the dura was opened over the MCA, but the artery was not occluded. Body temperature was maintained at 37°C until recovery from anesthesia. Rats were later killed with an overdose of pentobarbital, and the forebrains were removed for cortical dissection at various times after MCAO. In some cases the ischemic frontoparietal cortex was dissected from the ipsilateral hemisphere and the contralateral hemisphere of the rat brain. Results are representative of the total number of animals evaluated (n=6 for MCAO and 4 for sham surgery).

Preparation of Tissue Extracts

To analyze protein expression patterns in control and ischemic brain tissue, protein extracts of the tissues were prepared. The forebrains were removed from each animal at various times after surgery, and cortical samples were dissected as described above. Immediately after dissection, the tissues were stored at −80°C until all the samples from the time course were collected. To prepare the tissues for extraction, they were first weighed and then minced into 1-mm³ pieces. The minced tissues were incubated in an extraction buffer consisting of 0.5% Triton X-100 (Sigma) in PBS containing 0.5 U/mL aprotinin (Sigma) and 0.01% sodium azide while gently rotating at 4°C for 18 hours. The concentration of the initial extraction mixture for each tissue sample was normalized to 500 µL/mL of tissue extracts. After the extraction was complete, the samples were centrifuged at 14 000 rpm for 10 minutes at 4°C, and the supernatants were collected, aliquoted into 10-µL volumes, and stored at −20°C. To check the quality and uniformity of each extraction throughout the study (ie, to demonstrate that the extractions were consistent between time points and animals evaluated), samples of each extract prepared were analyzed by SDS-PAGE (10% polyacrylamide) in which the gel was stained with 0.25% Coomassie brilliant blue R-250 (Sigma).

Western Blot Analysis

To investigate the protein expression of various MMPs in control and ischemic tissue extracts, equal volumes (10 µL) of tissue extracts normalized for protein concentration were prepared for Western blot analysis. Briefly, extracts were resolved by electrophoresis through a
10% polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane. Unoccupied binding sites were blocked overnight at 4°C with 5% nonfat powdered milk in a 0.1 mol/L Tris-HCl buffer, pH 8.0, containing 1.5 mol/L NaCl and 0.5% Triton X-100 (TBST buffer). A primary antibody, diluted in TBST, was then added to the membrane and allowed to incubate for 1 hour at 25°C. The membrane was washed three times, 15 minutes each, with TBST and then incubated for 30 minutes with a secondary antibody conjugated to horseradish peroxidase (Sigma). The membrane was washed as above, and the blot was developed with the use of the enhanced chemiluminescence method (Amersham) according to the manufacturer's instructions. The primary antibodies used included the following: mouse monoclonal antibodies directed against MMP-1 (clone 41-IE5, Oncogene Science), MMP-2 (clone 42–5D11, Oncogene Science), and MMP-3 (clone 55–2A4, Oncogene Science); rabbit polyclonal antibodies directed against MMP-3 (Biogenesis); and MMP-9, a gift from Dr Hideaki Nagase, University of Kansas Medical Center.

SDS-PAGE Zymography

MMP enzyme expression was assayed by zymography as described by Herron et al. Briefly, equal volumes (10 μL) of tissue extracts normalized for protein concentration were subjected to electrophoresis, without boiling or reduction, through a 10% polyacrylamide gel copolymerized with gelatin (0.5 mg/mL) or casein (0.5 mg/mL) at 4°C. After electrophoresis was complete, the gel was incubated for 1 hour at 25°C in a 2.5% Triton X-100 solution, washed two times, 20 minutes each, with water, and then incubated overnight at 37°C in a 0.05 mol/L Tris-HCl buffer, pH 8.0, containing 5 mmol/L CaCl₂. As a control, duplicate samples were loaded onto another gel that was then incubated in a 0.05 mol/L Tris-HCl buffer, pH 8.0, containing 10 mmol/L EDTA to inhibit MMP activity. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie blue R-250, and then destained with 10% methanol and 7% acetic acid. Enzyme activity attributed to MMP-1, MMP-2, and MMP-9 can be visualized (on the basis of molecular weight) in the gelatin-containing zymograms as clear bands against a blue background. Similarly, casein-containing zymograms can be used to determine MMP-3 activity. To quantify the relative levels of MMP expression as detected by SDS-PAGE zymography, the gels were digitized, and the area of lysis for each band detected was quantified by computer-assisted planimetry of the lytic zone area in square millimeters (Amersham RAS 3000 Image Analysis System, Loats Associates, Inc).

In Situ Zymography

Although SDS-PAGE zymograms are useful in identifying the presence of latent ("pro") and active forms of various MMPs, they cannot indicate the actual net proteolytic activity because of the presence of TIMPs. TIMPs are generally coexpressed with MMPs in tissue samples and can inhibit MMP activity. However, the presence of SDS in SDS-PAGE zymograms displaces TIMPs from the MMPs and also activates latent enzymes. Therefore, to analyze net endogenously active MMP expression within the brain tissue after MCAO, in situ zymography was conducted. With this method, endogenous MMP activity could be identified and correlated to a particular region of the tissue. Brain tissue from animals that had undergone focal cerebral ischemic or sham surgery was removed after 24 hours and immediately placed on ice. Coronal sections (1 mm) were made
through the forebrain and rinsed briefly in PBS. The sections were then directly overlaid on top of a gel consisting of 10% polyacrylamide copolymerized with gelatin (0.5 mg/mL) in 50 mmol/L Tris, 5 mmol/L CaCl₂, pH 7.4. The tissue sections were incubated on top of the gel for 8 hours at 37°C, after which the sections were removed and the gel was incubated further for a total of 18 hours. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie blue R-250, and then destained with 10% methanol and 7% acetic acid. Gelatinase activity was visualized as a zone of clearing against a blue background.

**Immunohistochemistry**

Forebrains were removed from animals that had undergone permanent focal cerebral ischemia after 24 hours (n=8) or 5 days (n=6) and immediately placed on ice. Sham-operated rats killed after 24 hours (n=5) or 5 days (n=5) were used as controls. Coronal sections (2 mm) were made through the forebrain, after which the sections were fixed with 10% (wt/vol) phosphate-buffered formalin (Baxter Scientific Products) for 18 to 20 hours. After standard histological processing and embedding in paraffin, 6-μm-thick sections were prepared for immunoperoxidase staining with the Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. Briefly, endogenous peroxidase was quenched with 0.3% H₂O₂ in methanol for 30 minutes. Nonspecific immunoglobulin binding sites were blocked with normal goat serum for 1 hour, and then the sections were incubated with a primary monoclonal antibody for 1 hour at room temperature. As a negative control, serial sections were incubated with mouse IgG (Vector Laboratories) instead of the primary antibody. The sections were then incubated for 30 minutes with a biotinylated goat anti-mouse IgG secondary antibody (1:200, Vector Laboratories) followed by 30 minutes of incubation with the Vectastain Elite ABC reagent solution. Immunoglobulin complexes were visualized on incubation with DAB (Vector Laboratories) at 0.5 mg/mL in 50 mmol/L Tris-HCl (pH 7.4) and 3% H₂O₂. DAB staining was enhanced by treating the sections for 10 seconds with DAB Enhancing Solution (Vector Laboratories). Sections were washed, counterstained with Gill’s hematoxylin, cleared, mounted with Aquamount (Polysciences), and then examined by light microscopy with an Olympus IX70 microscope. The primary monoclonal antibodies used for these studies were as follows: anti–MMP-1 (clone 41-IE5, Oncogene Science), anti–MMP-2 (clone 42–5D11, Oncogene Science), anti–MMP-3 (clone 55–2A4, Oncogene Science), anti–MMP-9 (clone 56–2A4, Oncogene Science), anti–TIMP-1 (clone 7–6C1, Oncogene Science), anti–neurofilament-200 (N52, Sigma), and an antibody that recognizes monocytes and macrophages (clone ED1, BioSource).

**In Vivo Studies**

Spontaneously hypertensive rats (Taconic Farms), weighing 300 to 330 g, were treated with a murine neutralizing monoclonal antibody previously demonstrated to inhibit MMP-9 activation. The IgG monoclonal antibody (clone 6–6B, Oncogene Science) was dis-
solved in sterile PBS, and an estimated inhibitory dose was administered intravenously (3 mg/kg) 1 hour before MCAO. As an appropriate control for comparison, nonimmune mouse IgG (Vector) was administered (3 mg/kg) in the same manner to another group of animals.

MCAO was performed as described above; 24 hours later the animals were killed with an overdose of pentobarbital, and the forebrains were sectioned into seven coronal slices and immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride in a 1.0 mol/L phosphate buffer, pH 7.0, at 37°C for 20 minutes.28 Stained tissue slices were digitized and analyzed as described previously 20,24 with the use of computerized assisted planimetry (Optimus Image Analysis, Inc and customized software). Infarct measurements were adjusted for brain swelling due to edema, which is known to contribute to and overestimate the degree of infarction.29,30 Infarct area in square millimeters was expressed as the percent infarcted tissue relative to the contralateral normal hemisphere area in square millimeters. In addition, the total volume of forebrain infarction was calculated from the infarct areas on the individual forebrain slices.

Statistical Analysis
Data were expressed as mean±SEM. For statistical analysis of gel lytic zone data, an ANOVA was followed up with the Fisher’s least significant difference test. For percent hemispheric infarct and infarct volume measurements, the t test for unpaired data was used. Statistical significance was accepted at P<0.05.

Results
MMP-9 and MMP-2 Are Expressed in Ischemic Brain Tissue Extracts
The time course of MMP protein expression after occlusion of the MCA was assessed in tissue extracts prepared from control and ischemic regions of rat brain. The proteins extracted from the tissues were evaluated by SDS-PAGE before further analysis, and the quality of the extracts was demonstrated to be satisfactory (data not shown). MMP-1, MMP-2, MMP-3, and MMP-9 protein expression were evaluated by Western blot. The results demonstrated that MMP-9 was detected in the ischemic tissue within 24 hours after occlusion and was still observed at the 5-day time point (Figure 1A). By day 15, however, MMP-9 protein expression was no longer observed. MMP-9 expression was absent in the contralateral control cortex samples at each time point analyzed (Figure 1A). MMP-2 expression was also detected in the ischemic tissue but predominantly at the 5-day time point (Figure 1B). MMP-9 and MMP-2 were not detected in the sham-operated animals (data not shown). Western blot analysis was conducted for MMP-1 and MMP-3 protein expression, but these proteins were not detected in any of the samples assayed (data not shown).
MMP expression was also evaluated by SDS-PAGE zymography with the use of gels containing either gelatin or casein. The results of these experiments showed that MMP-9 activity was detected 6 hours after stroke, was significantly increased 12 hours after stroke, was markedly (maximally) expressed at 24 hours, and remained maximally expressed after 5 days (Figure 2A). The expression of MMP-9 returned to basal (zero) level at the 15-day time point. On the basis of molecular weight, the results suggested that MMP-9 was predominantly in the active form (molecular weight \( \approx 87 \text{kD} \)) with only a modest amount present as the latent "pro" form (molecular weight \( \approx 92 \text{kD} \)) (Figure 2A). Furthermore, it was demonstrated that MMP-2 occurred in both the latent and active forms and was identified in the ischemic cortical samples (Figure 2A). However, while MMP-9 activity was detected after 6 hours and significantly expressed 12 hours after stroke, MMP-2 expression was detected after 24 hours but was most pronounced (ie, maximal) 5 days after injury and was still detectable at nonsignificant low levels for up to 30 days after MCAO (Figure 2A). Figure 2B illustrates these quantified results for MMP-9 and MMP-2 after sham surgery and at various times after MCAO compared with the contralateral control and sham samples in which MMP expression was not detected. MMP-1 expression was not detected in the gelatin-containing zymograms. Likewise, MMP-3 expression was not detected in the casein-containing zymograms (data not shown). For Western blot analysis and SDS-PAGE zymography, four different sets of tissue samples collected over the entire time course were analyzed (ie, four animals per time point). The results shown in Figures 1 and 2A are representative of all the sets evaluated.

**Net MMP Activity Is Detected by In Situ Zymography in the Ischemic Hemisphere of the Rat Brain 24 Hours After MCAO**

Since MMPs can be present in either a latent or active form and can be coexpressed with TIMPs, we were interested in identifying the net MMP activity within the rat brain after stroke. To analyze brain tissue samples for net MMP activity, in situ zymography was conducted. Coronal brain slices were prepared from animals 24 hours after undergoing focal cerebral ischemia or sham surgery and then analyzed for gelatinolytic activity by zymography. The 24-hour time point was chosen for these studies to determine the net proteolytic activity resulting from increased MMP expression by the time brain infarction was maximum after stroke. Figure 3A depicts representative zymograms indicating MMP activity detected only in the ischemic hemisphere (closely associated with the cortical infarction characterized in this model previously) in animals that had undergone MCAO (n=6) and not in the contralateral control cortical region. In addition, Figure 3B illustrates that ipsilateral cortical MMP activity was not detected in brain slices prepared from animals that had undergone sham surgery (n=4).
MMP-9 Is Observed With Endothelial Cells and Neutrophils in Ischemic Brain Tissue 24 Hours After MCAO

MMP-9 expression was evaluated by immunohistochemical analysis in brain tissue sections prepared from animals that had undergone surgery to induce focal cerebral ischemia or sham surgery. The tissues were collected 24 hours after surgery (n=8), the time point at which MMP-9 expression was elevated while MMP-2 expression was only moderately expressed as determined by SDS-PAGE zymography (Figure 2). At this time point, animals that had MCAO-induced focal ischemia exhibited significant cortical tissue damage (Figure 4) and contained infiltrated neutrophils (Figures 5 and 6), consistent with earlier observations. Diffuse MMP-9 expression was observed within and at the periphery of the ischemic cortical lesion (Figure 4B). In contrast, there was no detectable MMP-9 expression in either control contralateral regions of the brain (Figure 4A) or in tissue from rats that had undergone sham surgery (n=5) (data not shown).

At sites located both within and at the periphery of the ischemic lesion, immunoreactive MMP-9 appeared to stain with endothelial cells (identified by morphometric criteria) of microvessels (Figure 5). In addition, MMP-9 expression appeared to present with a significant number of neutrophils (identified by morphometric criteria) that had migrated into the damaged cortical region of the ischemic tissue (Figures 5 and 6). No similar labeling of neutrophils or endothelial cells was observed in either cortex after sham surgery (data not shown). Serial sections that were incubated with mouse IgG instead of MMP-9 primary antibody were negative (data not shown).

MMP-9 and MMP-2 Expression Is Observed With Macrophages in Ischemic Brain Tissue 5 Days After Cerebral Infarct

The expressions of MMP-9 and MMP-2 were examined by immunohistochemistry in rat brain isolated 5 days after MCAO. Consistent with the observations made by SDS-PAGE zymography, MMP-9 and MMP-2 expressions were detected 5 days after MCAO (n=6) in the ischemic cortical region of the rat brain (Figure 7B and 7D, respectively) and were absent in the control contralateral region (Figure 7A and 7C, respectively). MMP-9 and MMP-2 were not observed 5 days after sham surgery (n=5, data not shown). In addition, with the use of an antibody that recognizes monocytes and macrophages (ED1), it was demonstrated that MMP-9 and MMP-2 appeared to stain with macrophages (identified by morphometric criteria; identical morphology for MMPs and ED1) resident within the ischemic lesion (Figure 7B, 7D, and 7F). Although the ED1 antibody also stains activated microglial cells, the morphology of the ED1-positive cells observed within the lesion was indicative of macrophages and not microglial cells. Serial sections that were incubated with mouse IgG instead of MMP-9 or MMP-2 primary antibodies were negative (data not shown).

TIMP-1 Expression Is Similar in Both Control and Ischemic Brain Tissue

Immunohistochemical analysis was conducted to investigate the presence of TIMP-1 in control and ischemic rat brain in tissues prepared 24 hours (n=8) and 5 days (n=6) after MCAO. The results of these studies demonstrated that TIMP-1 expression was localized in both the control and ischemic sides of the brain (Figure 8A, 8B, and 8C) after MCAO and that the levels of expression were comparable to...
that observed in brain tissue from animals that had undergone sham surgery (n = 5 per time point) (data not shown). In addition, the levels of TIMP-1 expression remained relatively unchanged at both the 24-hour and 5-day time points (Figure 8B and 8C). Furthermore, TIMP-1 was present not only in the cortical region of the brain but also in the nerve tracks (corpus callosum and striatum [caudate nucleus and putamen]) located within the white matter and, as determined by staining of serial sections, appeared to have a staining pattern similar to that of a marker for neurofilaments, NF-200, indicating axonal labeling within these nerve tracks (Figure 8D, 8E, and 8F).

**Administration of an MMP-9 Inhibitor Reduces Infarct Size**

Intravenous treatment with the neutralizing monoclonal antibody inhibitor of MMP-9 (3 mg/kg) 1 hour before MCAO significantly reduced percent hemispheric infarct size by 28.3% (Figure 9A; P < 0.05) and infarct volume by 29.9% (Figure 9B; P < 0.05) compared with the mouse IgG control. Previous studies have demonstrated that in using an IgG antibody therapeutic agent against tumor necrosis factor-α, administration of an immune or nonimmune IgG antibody into this animal model does not exhibit any effects on blood pressure, body temperature, blood gases, pH, or blood glucose levels.

**Discussion**

The present studies indicate that after focal ischemia there are marked differences in the distribution and temporal expression of MMP-9 and MMP-2 at the ischemic site. MMP-9 expression was detected much earlier after focal ischemia and appeared to stain with many endothelial cells and infiltrating neutrophils. While not all neutrophils appeared to stain for MMP-9 after 24 hours, it is possible that most individual neutrophils might express MMP-9 associated with their migration at some point after stroke. The later (ie, 5 days after stroke) expression of MMP-9 and MMP-2 appeared to stain predominantly with macrophages. Expression of MMP-9 by endothelial cells within and at the periphery of the ischemic lesion appears to be a key mechanism by which the cerebral vascular walls become compromised, leading to edema and leukocyte infiltration. It has been demonstrated that endothelial cells secrete MMPs, including MMP-9 in the basal direction, thereby facilitating the degradation of the basement membrane. Proteolysis of the ECM components of the basement membrane underlying the vasculature has been associated with an increase in vascular permeability and the loss of vascular integrity. Additionally, degradation of the vascular basal lamina allows the blood vessels to become permissive to neutrophil extravasation into the brain tissue. In addition, as neutrophils migrate from the circulation into the tissue, it has been reported that they employ MMP-9 for their invasion. The later expression of MMP-2 with MMP-9 by macrophages may aid in their migration into the ischemic lesion as well and might also contribute to the clearing of cellular debris later during the later wound-healing/resolution phase after focal stroke.

MMP expression can be modulated by various mechanisms. Regulation of MMP expression can occur not only at the level of transcription but also at the level of enzymatic activation. MMPs are secreted in a zymogen form containing a propeptide that renders the enzyme inactive. Notably, active MMP-9 was identified in ischemic tissue in the present study. The results of the in situ zymography clearly demonstrated this. Activation of MMP-9 has been demonstrated to occur on proteolytic cleavage by MMP-3, or stromelysin, although other unknown mechanisms of activation are still possible. We speculate that since we were unable to detect MMP-3 in the ischemic tissue using two different antibodies directed against MMP-3, MMP-9 is activated by another unknown mechanism. However, another possibility is that we were unable to identify a transient expression of MMP-3 in the early phase of the time course.

MMP expression can also be regulated by enzyme inhibitors. The endogenous inhibitors of MMP-9 and MMP-2 are the TIMPs, with TIMP-1 having preferred selectivity for inhibiting MMP-9, and TIMP-2 having preferred selectivity for inhibiting MMP-2. In diseases in which excessive matrix degradation occurs, the balance between MMPs and TIMPs is often offset, resulting in an overall net increase in MMP activity. The results of the immunohistochemical...
analysis demonstrated that TIMP-1 was expressed at comparable levels in both the control and ischemic cortical regions of the brain. Interestingly, TIMP-1 expression was also present within the white matter of the corpus callosum bordering the infarcted cortex. The expression pattern was similar to that of a marker for neurofilaments in the nerve tracks present within this white matter. These same regions were completely devoid of MMP expression. It could be hypothesized that TIMP-1 provides an endogenous means of protecting the nerve tracks from structural damage due to MMP proteolysis.

The role of MMP-9 in stroke was examined further by administering a neutralizing anti–MMP-9 monoclonal antibody to animals before MCAO in the present study. The results of this experiment demonstrated that the selective, early inhibition of MMP-9 significantly reduced the degree of brain infarction, clearly demonstrating the importance of this enzyme in contributing to focal ischemic brain injury. Breakdown of the BBB after stroke can result in hemorrhage and edema and ultimately, because of lack of blood flow, neuronal cell death.13–16 The protection provided by MMP-9 inhibition can be due to blocking the early actions of MMP-9 secreted by the endothelial cells. However, MMP-9 expression by neutrophils can also contribute to their migration and exacerbation of ischemic brain injury.22,30 It is unlikely that the neuroprotective effects are mediated by IgG antibody effects on vascular or blood parameters since previous thorough evaluation of these parameters demonstrated no effects of IgG antibodies.31

In summary, increased MMP-9 and MMP-2 expressions were induced in ischemic regions of the rat brain and were not detected in nonischemic control samples. Given the potential complexity of central nervous system–associated MMPs, it may be premature to focus on MMP-9 and MMP-2 in ischemic stroke. However, the present data substantiate the importance of these two enzymes in early and late phases of ischemia, respectively. The data also corroborate previous studies demonstrating an increase of these two MMPs after stroke and extend beyond those studies by identifying the cellular sources of MMP-9 and MMP-2 and demonstrating neuroprotection produced by interfering with MMP-9 activation. In our assays, MMP-9 expression was markedly expressed within 12 to 24 hours after MCAO, and its expression appeared to stain with endothelial cells and neutrophils identified both within and at the periphery of the ischemic lesion. After 5 days, MMP-9 expression was also detected with macrophages within the infarct. Compared with MMP-9, MMP-2 expression was detected later after MCAO, perhaps associated with the resolution (ie, the remodeling and healing processes that occur after stroke). Like MMP-9, MMP-2 was detected with macrophages within the infarct at the 5-day time point. Finally, the expression of TIMP-1, an inhibitor of various MMPs, was evaluated and was shown to be similar in both the control and ischemic brain tissue. This stroke-induced MMP expression can be expected to shift the protease-protease inhibitor balance toward proteolytic activity and ECM degradation. In particular, the expression of MMP-9 within the first 24 hours after stroke suggested that it is involved in the initial tissue destruction and contributes to the rapidly developing brain injury that occurs after stroke. Systemic pretreatment with an anti–MMP-9 neutralizing antibody produced a significant decrease in brain injury observed 24 hours after stroke. Although additional studies need to be performed to evaluate dose-response relationships and poststroke treatments for anti–MMP-9 treatment, the present data clearly demonstrate that MMP-9 plays a significant role in brain injury produced by focal stroke.

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References

MMP Expression After Cerebral Focal Ischemia

In the central nervous system inflammatory mediators are activated in a manner similar to but different from peripheral inflammatory responses after brain injury or during neurological disease. The current focus of the research on inflammatory response in the central nervous system largely consists of cytokine-induced factors. MMPs are a family of calcium-dependent, zinc-dependent endopeptidases that are induced by cytokines. MMPs contribute to the enzymatic cascade responsible for degradation of ECM proteins such as collagen, proteoglycan and laminin. In the central nervous system, endothelial cells, microglia, and astrocytes can express MMPs in response to injury resulting, in part, in the breakdown of the BBB and facilitation of leukocyte infiltration and other inflammatory responses. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are primary MMPs whose levels increase after stroke. Opposing the actions of metalloproteinases are the TIMPs.

Recent studies indicate that MMP-2, MMP-9, and TIMP increase after focal ischemia. Thus, these proteins might play critical roles in the disruption of the BBB and facilitation of leukocyte infiltration and inflammatory responses following stroke. As reported in the accompanying article, using an antibody to MMP-9, Romanic and colleagues observed a decrease in infarct size after focal ischemic insult in rats, thus identifying MMP-9 as a potential therapeutic target for the treatment of stroke. In this study the authors observed MMP-2 and MMP-9 expression in endothelial cells, neurotrophs, and macrophages but not in astrocytes or microglia, as other studies have reported. This is likely due to differences in model systems and mechanism of injury. Romanic and colleagues also did not observe an increase in TIMP by immunohistochemistry following ischemic insult, also in contrast to previous studies. The discrepancies between the work described by Romanic and colleagues and other reports likely reflect the complexity of the response of MMPs and TIMPs to injury. MMPs and TIMPs are translated in latent form and then activated by cellular response to injury. Transcriptional, translational, and posttranslational regulation of these enzymes is complex, and the pathways for these events in brain are not known. This is a new and developing field, and it is anticipated that relatively selective and specific inhibitors for each MMP will be forthcoming. These agents might provide significant therapeutic benefit without deleterious side effects. The current work indicates that selective modulation of this class of enzymes might have significant positive benefit for patients suffering from stroke.

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References
Matrix Metalloproteinase Expression Increases After Cerebral Focal Ischemia in Rats: Inhibition of Matrix Metalloproteinase-9 Reduces Infarct Size
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