Matrix Metalloproteinase-2 Plays a Critical Role in the Pathogenesis of White Matter Lesions After Chronic Cerebral Hypoperfusion in Rodents

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Background and Purpose—Cerebrovascular white matter (WM) lesions contribute to cognitive impairment and motor dysfunction in the elderly. A disruption of the blood–brain barrier (BBB) is believed to be a critical early event leading to these WM lesions. Previous studies have suggested the involvement of matrix metalloproteinase-2 (MMP-2) in BBB disruptions and the upregulation of MMP-2 after chronic cerebral hypoperfusion in a rat model. In the present study, we asked whether MMP-2 is involved in the BBB disruption and the subsequent WM lesions after chronic cerebral hypoperfusion.

Methods—We compared the severity of white matter lesions in rats after chronic cerebral hypoperfusion with or without an MMP inhibitor. Then, we also induced the chronic cerebral hypoperfusion in wild-type and MMP-2-null mice.

Results—In the rats treated with a relatively selective MMP-2 inhibitor, AG3340, the WM lesions after chronic cerebral hypoperfusion were significantly less severe, and the number of activated astroglia and microglia were also significantly lower as compared with the vehicle-treated rats. Gene knockout of MMP-2 also reduced the severity of the WM lesions and the number of activated astroglia and microglia in a mice system. In both rodents, the disruption of BBB function, as assessed by IgM staining and the Evans blue extravasation test, was less severe when MMP-2 activity was attenuated.

Conclusions—These findings indicate that MMP-2 plays a critical role in the BBB disruption, glial cell activation, and WM lesions after chronic cerebral hypoperfusion and suggest the potential value of MMP-2 inhibitors as a therapeutic tool in cerebrovascular WM lesions. (Stroke. 2006;37:2816-2823.)

Key Words: blood–brain barrier ■ chronic cerebral hypoperfusion ■ MMP inhibitor ■ MMP-2 ■ white matter lesion

Cerebrovascular white matter (WM) lesions, a neurodegenerative condition characterized by hyperintense signals on magnetic resonance images, are frequently associated with aging and cerebrovascular disease and are responsible for the cognitive decline of the elderly. Chronic cerebral ischemia is likely to cause these WM lesions, because cerebral blood flow is decreased in these patients.1 Indeed, similar WM lesions can be induced in rats and mice after chronic cerebral hypoperfusion, the experimental conditions mimicking chronic cerebral ischemia in humans.2,3 Matrix metalloproteinases (MMPs) are a family of endopeptidases that can degrade most of the major constituents of the extracellular matrix.4 MMP-2 and MMP-9 represent a subgroup of the MMP family and degrade several extracellular matrix components, including type IV collagen, fibronectin, and gelatin. Deregulated MMPs have been implicated in the tissue destruction associated with cancer, arthritis, and multiple sclerosis.4 MMPs may also play a role in neurologic disorders. For instance, MMP-9 is increased in human brains after stroke,5 and MMP-2 and MMP-3 are increased in cerebrovascular WM lesions from patients with vascular dementia.6 A reduction in the basement membrane components, including type IV collagen, is associated with the blood–brain barrier (BBB) disruption during cerebral ischemia.7 In our previous study on chronic cerebral hypoperfusion, the BBB disruption was accompanied by an upregulation of MMP-2 but not MMP-9,8 suggesting the specific involvement of MMP-2 in the WM lesions. We hypothesize that the MMP-2 upregulation after chronic cerebral hypoperfusion correlates with BBB damage, which leads to glial activation and subsequent WM lesions. To clarify the cause–effect relationship among MMP-2 upregulation, BBB disruption, and WM lesions, we used 2 strategies to attenuate MMP-2 activity: an MMP inhibitor, AG3340, and MMP-2 inhibitors.
knockout. The results from both experiments strongly supported the idea that MMP-2 plays a critical role in BBB disruption and WM lesions.

Materials and Methods

Chronic Cerebral Hypoperfusion in Rats and Treatment With an Matrix Metalloproteinase Inhibitor

Chronic cerebral hypoperfusion with bilateral common carotid artery occlusion (BCAO) was induced in male Wistar rats (weight 150 to 200 g; Shimizu Experimental Supply; Kyoto, Japan) by double ligation of the common carotid arteries as previously described. After the operation, the rats were kept in animal quarters with food and water ad libitum.

AG3340 (Ago-311 Pharmaceuticals) was dissolved at 75 mg/mL in 50% DMSO in propylene glycol. The rats were treated twice a day with an intraperitoneal injection of AG3340 (100 mg/kg) or vehicle (DMSO/propylene glycol) from just before the operation until 14 days after the operation. Similar doses and treatment paradigms have been shown to be effective in inhibiting MMP activity in gliomas in model animals. Because our previous study demonstrated that the number of microglia peaked on 3 days and WM lesion started to become evident on 14 days after BCAO, the animals were subjected to the analyses described subsequently.

Mice

The generation of C57BL/6J mice carrying the MMP-2-null allele has been described elsewhere. In this mutant allele, a region containing the promoter and the first exon of the MMP-2 gene is replaced by the pgk-neo cassette. MMP-2 parents were mated to obtain both wild-type and MMP-2–/– (MMP-2-null) littermates. Genotyping was performed by polymerase chain reaction using the following primers: wild-type forward, CAACGATGGAGGACAGGATG; wild-type reverse, GCCCCGGGAACTTGTGGAGG; mutant forward, CTTGGGTGGAGGCTTCT; and mutant reverse, AGGTGAGGTACAGGATGC.

Chronic Cerebral Hypoperfusion in Mice and Cerebral Blood Flow Measurement

Adult male mice (weight 20 to 25 g) were subjected to bilateral common carotid arteries stenosis (BCAS) by applying the microcoils with an inner diameter of 0.18 mm to both common carotid arteries as previously described. The cerebral blood flow (CBF) was recorded by laser Doppler flowmetry with placing a straight probe (OmegafLO-N1: Neuroscience Inc) on 1 mm posterior and 2 mm lateral from bregma perpendicular to the skull bone through the guide cannula. The baseline CBF recordings were obtained just before and at 2 hours and 3, 7, 14, and 30 days after the surgery. The CBF values were expressed as a percentage of the baseline value.

Histochemical Evaluation of White Matter Lesions and Glial Activation

Under deep anesthesia, the animals were perfused with 10 mmol/L phosphate-buffered saline (300 mL for rats, 100 mL for mice) and then with a fixative consisting of 4% paraformaldehyde, 0.2% picric acid, and 0.1 mol/L phosphate buffer at pH 7.4 (300 mL for rats, 100 mL for mice). The brains were removed and postfixed for 24 hours in 4% paraformaldehyde in 0.1 mol/L phosphate buffer and then stored in 15% sucrose in 0.1 mol/L phosphate buffer. The fixed brains were embedded in paraffin and sliced into 2-μm-thick coronal sections. Klüver-Barrera staining and Bielschowsky staining were used to visualize the myelin sheaths and axons, respectively. As previously described, the severity of the WM lesions was semiquantitatively graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and disappearance of myelinated fibers (grade 3) by an investigator blind to the experimental condition. For immunohistochemistry, serial sections (20-μm-thick) were cut in a cryostat and incubated overnight with a primary antibody at 4°C followed by incubation with the appropriate biotinylated secondary antibody (1 hour, room temperature), treatment with an avidin–biotin complex (diluted 1:200; Vector Laboratories), and visualization with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H2O2 in 50 mmol/L Tris-HCl (pH 7.6). The primary antibodies used were as follows: monoclonal anti-rat glial fibrillary acidic protein (GFAP) (diluted 1:5000; Sigma-Aldrich; Mo, USA), polyclonal rabbit anti-mouse GFAP (diluted to 1:5000); Dako Cytomation, Denmark), polyclonal rabbit anti-MMP-2 (diluted to 1:1000, Chemicon International, Inc), monoclonal rat anti-mouse MHC class II antigen antibodies (diluted to 1:5000; Dako Cytomation), and rabbit anti-Iba-1 antibody (1 μg/mL; Wako Pure Chemical Industries, Ltd; Osaka, Japan). Some sections were incubated with a biotinylated goat anti-rat IgM (μ), biotinylated goat anti-mouse IgM (μ) (diluted 1:1000; Kirkegaard & Perry Laboratories; Md, USA), or biotinylated Ricinus communis agglutinin-1 (diluted 1:1000; Vector Laboratories; Calif, USA) and were incubated directly with the avidin–biotin complex. To confirm the cellular source of IgM, sections were labeled by biotinylated anti-mouse IgM and rabbit anti-mouse GFAP followed by fluorescein isothiocyanate-labeled avidin (diluted 1:100; Dako Cytomation) and rhodamine-labeled goat anti-rabbit IgG (2.5 μL/mL; Dako). In the sections immunostained for Ricinus communis agglutinin-1, MHC class II antigen, Iba-1, GFAP, and IgM, we counted the number of immunopositive cells in at least 6 representative fields (per 0.25 mm2) in the corpus callosum, the caudoputamen, and the optic tract for the quantitative analysis.

Zymography and Matrix Metalloproteinases-2 Activity Assay

Minced forebrain tissues were incubated with gentle rotation at 4°C for 20 hour in an extraction buffer consisting of 0.5% Triton-X 100, 0.5 U/mL aprotinin, and 0.01% sodium azide in 0.01 mol/L phosphate-buffered saline. The samples were then centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatants were collected. The protein content was adjusted to 10 mg/mL. The gelatinolytic activity of these samples was detected by SDS-PAGE zymography as described elsewhere, although MMP-2 activity in the gray matter may interfere a sensitive detection of the activity in the WM. Equal amounts of tissue extract (50 μg) were then subjected to electrophoresis. To restore the activity of the protein, sample gels were agitated at 0.01 mol/L Tris-HCl (pH 8.0) containing 2.5% Triton-X-100 (30 minutes×2). After washed in 0.05 mol/L Tris-HCl (pH 8.0) for 30 minutes, the gels were incubated overnight twice at 37°C in 0.01 mol/L Tris-HCl (pH 8.0) containing 0.5 mmol/L CaCl2 and 1.0 mol/L ZnCl2. After incubation, the gels were stained with Coomassie blue R-250. The amount of activated and latent forms of MMP-2 in the whole forebrain extracts were also assessed using the Matrix Metalloproteinase-2 Biotrak Activity Assay System (Amersham Biosciences), which is based on a 2-site enzyme-linked immunosorbent assay “sandwich” format and recognizes both the proform and active form of MMP-2.

Evans Blue Extravasation

The mice were killed at 3 hours and 1, 3, 5, 7, and 14 days after BCAS. One hour before each time point, 1 mL of 4% Evans blue (EB; Nakalai Chemicals Ltd) in normal saline was injected intraperitoneally. The animals were anesthetized and then perfused transcardially with 200 mL of 10 mmol/L phosphate-buffered saline. The brains were snap-frozen, sectioned into 20-μm-thick slices, and examined by fluorescence microscopy. For quantitative measures, the images were analyzed within 4 structurally similar areas (2 paramedian portions of the corpus callosum on each hemisphere) in each mouse and digitally level-adjusted by Adobe Photoshop (Adobe Systems) so that intravascular EB would be reported as white (pixel value 255) on a black background (pixel value 0). Using the public domain NIH Image 1.61 program (National Institutes of Health), the images were then binarized with intensity threshold set at pixel value 50 so that the white pixels represent intravascular and extravasated EB. The number of white pixels was divided by the total pixel
number in the selected area to estimate percent area containing intravascular and extravasated EB as an approximate index of BBB breakdown. Image analysis was focused on the paramedian portion of the corpus callosum facing the dorsal part of the lateral ventricle, because WM lesions were most intense in this region.\textsuperscript{3}

**Statistical Analysis**

All data are presented as means±SE. A one-factor ANOVA followed by Fisher protected least significant difference procedure was used to compare the differences between groups. \( P < 0.05 \) were considered to be statistically significant.

**Results**

The amount of total MMP-2 in the forebrain extracts was comparable between the vehicle-treated and AG3340-injected rats after BCAO as assessed using the Biotrak Activity Assay System. The percentage of activated MMP-2 was only 7% on day 3 after the sham operation but was elevated to approximately 80% on day 3 after the BCAO (supplemental Figure I, available online at http://stroke.ahajournals.org). We also confirmed almost complete suppression of MMP-2 activation with AG3340 administration.

The operation was successful in rats (n=40) except 3, which developed convulsions and was killed within 7 days, and in mice (n=62) except 4, which developed cerebral infarction. These animals with unsuccessful operations were excluded from the statistical analysis. In the vehicle-treated animals, severe WM lesions, as shown by an increased number of disarranged nerve fibers and vacuolation, were found on day 14 after the BCAO in the optic nerve, medial part of the corpus callosum (Figure 1B and 1E), the internal capsule, and the fiber bundles of the dopaminaput. In such WM regions, the number of Ricinus communis agglutinin-1-positive microglia and GFAP-positive astroglia increased (2- to 3-fold) on day 3 after the BCAO (Figure 1H and 1K). Both WM lesions and gliosis were less severe in the AG3340-treated animals (Figure 1C, 1F, 1I, 1L, 1P through 1R, and Table 1).

The BBB integrity in rats subjected to BCAO was also assessed by the immunostaining for IgM. IgM-immunoreactive glial cells represent those cells that have taken up the serum proteins, which leaked into the brain parenchyma, and their number serves as an indicator of BBB dysfunction.\textsuperscript{8} Some IgM-immunoreactive glial cells were found in the vicinity of the microvessels in the corpus callosum in the vehicle-treated animals on day 3 after the BCAO (Figure 1R), suggesting BBB dysfunction in this region. In contrast, much fewer IgM-immunoreactive glia were found in the same area of the AG3340-treated animals (Figure 1O and 1R).

These results strengthen the notion that MMPs play a role in BBB impairment and WM lesions. To further elucidate the roles of MMPs in the WM damage after chronic cerebral hypoperfusion, we applied BCAS (the established technique for mice hypoperfusion)\textsuperscript{9} for mice lacking functional MMP-2 gene (MMP-2-null mice), which showed no obvious developmental abnormalities\textsuperscript{10} or brain anomalies\textsuperscript{11} and examined its effects using histochemical methods. The reduction of CBF after BCAS was comparable between wild-type and MMP-2-null mice. The CBF reductions (wild-type versus MMP-2-null; mean±SE %, n=3 each) were 42.5±4.3% versus 39.1±3.2% (2 hours after BCAS), 38.1±4.3 versus 39.4±4.0 (3 days), 35.2±4.6 versus 33.6±6.2 (7 days), 20.8±1.4 versus 26.9±3.1 (14 days), and 11.2±3.0 versus 24.0±4.0 (30 days). In wild-type mice, MMP-2-immunoreactive glial cells increased after BCAS compared with sham-operated mice (Figure 2A and 2B). MMP-9-immunoreactive cells were not induced after BCAS in both wild-type (Figure 2C and 2D) and MMP-2-null mice (Figure 2E). Consistently, zymography using forebrain homogenates revealed only a faint band of MMP-9 in the samples after BCAS for 3 days in both wild-type and MMP-2-null mice (n=4), whereas a robust band was found in the sample from a mouse with an incidental cerebral infarction after BCAS (Figure 2G). A band of MMP-2 was detected in the samples in wild-type mice but not in MMP-2-null mice after BCAS (n=4). However, zymography using such homogenates failed to show the upregulation of MMP-2 after 3 days of BCAS; regional upregulation of MMP-2 in the WM seemed obscured.

Klüver-Barrera staining revealed that WM lesions were predominant in the corpus callosum, caudoputamen, and internal capsule but not in optic tract on day 30 after BCAS in the wild-type mice. The medial part of the corpus callosum adjacent to the lateral ventricles was most severely affected (Figure 3E). In MMP-2-null mice, such WM lesions were far less severe (Figure 3I; Table 2). The mouse model showed little damage to the visual pathway and no difference was found between the wild-type mice and MMP-2-null mice after the operation. This may be attributable to the fact that BCAS in mice induces a milder decrease in the CBF than in the rat model and maintains a residual blood flow within the common carotid arteries and its branch, the ophthalmic artery.

In the wild-type mice on day 14 after BCAS, numerous activated microglia, as visualized by immunostaining with anti-MHC class II antibodies, were found in some WM regions (Figure 3F). In addition, the number of GFAP-immunoreactive astroglia increased in these mice (Figure 3G). In the MMP-2-null mice, the number of microglia and astroglia was much fewer in the WM as compared with the wild-type animals (Figure 3J, 3K, 3P, 3Q). Thus, both WM lesions and glial activation after chronic hypoperfusion were dramatically reduced in the MMP-2-null mice. There was no difference of the number of microglia, astroglia, and IgM-positive cells in optic tract (Figure 3P).

The BBB integrity in mice subjected to BCAS was assessed by the immunostaining for IgM and EB extravasation assay. After BCAS, the number of IgM-positive cells increased in the WM of the wild-type mice (Figure 3H) as compared with the sham-operated wild-type animals (Figure 3D). Intriguingly, the IgM-immunoreactive cells significantly decreased in the WM of MMP-2-null mice after BCAS (Figure 3L and 3R). IgM-immunoreactive cells were identified as astroglia based on their colabeling with GFAP in the perivascular areas (Figure 3L through 3O). Three days after BCAS, EB apparently leaked into the perivascular area in the corpus callosum (Figure 4B) and the cerebral cortex (data not shown). This extravasation was most notable in the paramedian portion of the corpus callosum. At all time points after BCAS, no extravasation of EB could be detected in the MMP-2-null mice (Figure 4C). The estimated percent area...
stained with EB was approximately 8% in wild-type mice after BCAS, which significantly reduced to 2% in MMP-2-null mice after BCAS (Figure 4D). Taken together, these results indicated that loss of MMP-2 alleviated BBB damage after BCAS and suggested a causative role for MMP-2 in the WM lesions after hypoperfusion.

**Table 1.** Histologic Grading of the WM Lesions in Untreated and AG3340-Treated Rats on Day 14 After BCAO

<table>
<thead>
<tr>
<th></th>
<th>Corpus Callosum</th>
<th>Caudoputamen</th>
<th>Optic Tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle, N=5</td>
<td>1.3±0.045</td>
<td>1.4±0.54</td>
<td>2.6±0.55</td>
</tr>
<tr>
<td>AG3340, N=4</td>
<td>0.5±0.4*</td>
<td>0.63±0.25*</td>
<td>1.13±0.63*</td>
</tr>
</tbody>
</table>

*P<0.05.

**Discussion**

The synthetic MMP inhibitor AG3340 is known to inhibit several MMP family members, including MMP-2 (Ki=0.05 nmol/L), MMP-9 (0.26 nmol/L), MMP-13 (0.03 nmol/L), and MT1-MMP (0.33 nmol/L).12 As a lipophilic, low-molecular-weight (Mr 423.5) compound, AG3340 can readily cross the BBB.12 Using this compound, we have demonstrated that AG3340 shows protective effects against the WM lesions after chronic cerebral hypoperfusion in rats. This is consistent with our previous data using the same model, which showed a correlation of WM lesions with MMP-2 upregulation.8 Then, AG3340 may have reduced the severity of WM lesions by inhibiting MMP-2 activation. In support of this notion,
genetic deletion of MMP-2 attenuated the WM lesions after chronic cerebral hypoperfusion in mice. These data jointly suggest that MMP-2 upregulation plays a major role in the WM lesions.

Previous studies have established the importance of the upregulation and activation of MMPs in acute brain ischemia. Among the members of the MMP family, MMP-9 is of particular interest in the context of acute brain ischemia, because the selective upregulation of MMP-9 has been observed in the brains of patients with stroke. More importantly, the neuronal damage after cerebral ischemia was attenuated in the MMP-9-null mice compared with the wild-type mice. Furthermore, Heo et al demonstrated association of MMP-9 upregulation with hemorrhagic transformation in the nonhuman primates. Thus, MMP-9 upregulation may contribute to the BBB damage and infarct size, especially in the acute setting. Although previous study demonstrated the upregulation of MMP-9 in MMP-2-null mice, no upregulation of MMP-9 was observed in our model, which suggested a negligible role of MMP-9 in chronic cerebral hypoperfusion.

What then would be the role of MMPs in cerebral ischemia? Hamann et al reported disappearance of the basal lamina around the microvessels during cerebral ischemia and reperfusion. Fukuda et al demonstrated that the ischemic primate brain contained elevated levels of activity enough to digest basal lamina components such as type IV collagen. In fact, Heo et al indicated that MMP-2 upregulated significantly by 1 hour after MCAO and was persistently elevated thereafter in primates, and Chan et al demonstrated the upregulation of activation system for latent MMP-2 after focal cerebral ischemia. These findings support the hypothesis that excessive degradation of the vascular basal lamina is a mechanism by which MMP triggers BBB dysfunction, edema, hemorrhage. The most marked extravasation of Evans blue in the paramedian portion of the corpus callosum facing the lateral ventricle was consistent with a previous report on a rat model of chronic cerebral hypoperfusion and further indicated a vulnerability of the BBB in this area. In the case of chronic hypoperfusion, a previous study suggested the association of MMP-2 but not MMP-9 upregulation with BBB disruption. Consistently, Rosenberg et al showed that the activated
**Figure 3.** Histologic evaluation of the WM lesions in wild-type and MMP-2-null mice after BCAS. A through L, Klüver-Barrera staining 28 days after BCAS (A, E, I) and immunostaining 14 days after BCAS for MHC class II (B, F, J), GFAP (C, G, K), or IgM (D, H, L) of corpus callosum sections from wild-type (Wt) mice (A through H) or MMP-2-null (M2-) mice (I through L) that had undergone either a sham operation (A through D) or BCAS (E through L). Note that MMP-2 gene knockout recover the decrease of Klüver-Barrera staining in the WM after BCAS (compare E with I) and glial activation (compare F with J for microglia and G with K for astroglia). Scale bar, 50 μm. M through O, Double staining with GFAP and IgM of the WM lesions in wild-type mice after BCAS. IgM was observed on endfeet of GFAP-positive glia (O). Scale bar, 10 μm. P through R, A histogram representing the density of cells immunoreactive for MHC-class II or Iba-1 (P), GFAP (Q), or IgM (R) in sections from the corpus callosum (CC), caudoputamen (CP), and optic tract (OT) of mice that had undergone BCAS (n=6 each; *P<0.05, **P<0.01). For the microglial count, anti-MHC-class II antibodies were used for mice with BCAS operation, whereas anti-Iba-1 antibodies were used for mice with sham operation (P). Note that glial activation was not observed in the optic tract, being consistent with the absence of rarefaction of this structure.
astroglia and microglia/macrophages around the arterioles expressed MMP-2 and MMP-3, but not MMP-9, in the brains of patients with vascular dementia.

Caplan proposed that the major pathologic features of WM lesions such as demyelination and gliosis may result from a BBB dysfunction, which allows the leakage of proteins and fluid through the compromised barrier of the penetrating arteries. This hypothetical pathway is consistent with our present findings. Given the overlapping substrate specificity between MMP-2 and MMP-9, in the case of chronic cerebral hypoperfusion, MMP-2 may contribute to the BBB disruption through the excessive digestion of the vascular basal lamina and activation of glia. In addition, MMP-2 may be directly involved in demyelination associated with WM lesions, because MMP-2 can digest myelin more efficiently than MMP-9.

In conclusion, the present study has provided direct evidence that MMP-2 is involved in the pathogenesis of WM lesions in the mouse model. Although the species difference between rodents and humans should be taken into consideration, our data also suggest the potential value of MMP inhibitors in preventing subcortical ischemic vascular dementia resulting from BBB dysfunction and chronic cerebral ischemia in humans. Activation of MMP-2 is reported to participate in matrix injury during focal cerebral ischemia. An elucidation of the exact roles of MMP-2 in BBB disruption may also provide information useful in developing strategies for controlling neuroinflammation in general.

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Disclosures
None.

References


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