Involvement of Matrix Metalloproteinase in Thrombolysis-Associated Hemorrhagic Transformation After Embolic Focal Ischemia in Rats

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Background and Purpose—Thrombolytic therapy with tissue plasminogen activator (tPA) for acute ischemic stroke remains complicated by risks of hemorrhagic transformation. In this study we used a previously established quantitative rat model of tPA-associated hemorrhage to test the hypothesis that matrix metalloproteinases (MMPs) are involved.

Methods—Spontaneously hypertensive rats were subjected to embolic focal ischemia by placing homologous blood clots into the middle cerebral artery. Three groups of rats were studied: (1) untreated controls that received saline at 6 hours after ischemia; (2) rats that received tPA alone (10 mg/kg at 6 hours after ischemia); and (3) rats that received tPA plus the broad-spectrum MMP inhibitor BB-94 (50 mg/kg of BB-94 before ischemia and at 3 and 6 hours after ischemia plus tPA at 6 hours). Gelatin zymography was used to quantify MMP levels. A hemoglobin spectrophotometry method was used to quantify cerebral hemorrhage. Ischemic lesions were measured at 24 hours with tetrazolium staining.

Results—At 6, 12, and 24 hours, pro-MMP-9 and cleaved MMP-9 were upregulated in ischemic brain. At 12 hours, tPA-treated rats showed significantly higher levels of pro-MMP-9 and cleaved MMP-9 than untreated controls. By 24 hours, all rats showed evidence of hemorrhagic transformation in the ischemic territory. Rats treated with BB-94 and tPA showed significantly reduced hemorrhage volumes compared with those that received tPA alone. There was no effect on infarct size.

Conclusions—These results indicate that (1) tPA treatment increases levels of MMP-9 after embolic focal cerebral ischemia, (2) MMPs are involved in the mechanism of tPA-associated hemorrhage, and (3) combination therapies with MMP inhibitors may be useful for decreasing the risk and severity of this dreaded complication of thrombolytic therapy.

Key Words: cerebral hemorrhage ■ extracellular matrix ■ reperfusion injury ■ stroke ■ tissue plasminogen activator ■ rats

Although thrombolytic therapy with tissue plasminogen activator (tPA) may be effective for acute ischemic stroke, there is an elevated risk of cerebral hemorrhage. In part, nonspecific damage to cerebrovascular walls due to free radical generation during reperfusion injury has been implicated. However, the precise molecular mechanisms that underlie this dreaded complication remain to be fully elucidated.

Recently, there has been an emphasis on the possible role of proteases that are upregulated after cerebral ischemia and reperfusion. Specifically, the class of zinc-dependent matrix metalloproteinases (MMPs) has been intensively investigated. In mouse, rat, and baboon models of cerebral ischemia, expression of several MMPs is significantly increased after ischemic onset. MMPs can degrade almost all components of extracellular matrix, and therefore uncontrolled activation of these enzymes can result in significant tissue damage. In the context of hemorrhagic transformation after cerebral ischemia, MMPs may degrade vascular basal lamina, weaken vessels, and predispose them to rupture. In experimental studies, activation of MMP-9 and degradation of critical protein components of cerebral blood vessels have been correlated with the development of hemorrhage and edema.

In a recent study, pharmacological inhibition of MMPs significantly decreased the incidence of hemorrhage in a rabbit model of embolic stroke. However, it remained unclear whether there were any effects on the severity of hemorrhage. Surprisingly, a qualitative assessment of hemorrhage size suggested that it may have actually been worsened by MMP inhibitor treatment. If MMP inhibition is to be validated as a therapeutic approach against tPA-associated thrombolytic therapy, further studies are required to determine the optimal therapeutic strategy.
hemorrhagic transformation, it will be important to carefully assess its effects not only on risks but on severity of hemorrhage as well. We have previously characterized a quantitative model of tPA-associated cerebral hemorrhage in rat embolic stroke.16 In the present study we use this model to assess the effects of tPA on the profiles of MMP-2 and MMP-9 upregulation and the efficacy of the broad-spectrum MMP inhibitor BB-94 (batimastat) for reducing the volume of hemorrhage.

Materials and Methods

Embolic Model of Focal Cerebral Ischemia

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male spontaneously hypertensive rats (Taconic, Germantown, NY) were used. Animals were anesthetized with halothane (1% to 1.2%) under spontaneous respiration in a 30% O2/70% N2O mixture. Rectal temperatures were maintained at 37±0.5°C with a thermostat-controlled heating pad. The right femoral artery was cannulated, and physiological parameters, including rectal temperature, mean arterial blood pressure, pH, Pco2, and Po2, were monitored throughout all experiments. The right femoral vein was cannulated for drug administration. Focal ischemia was induced with the use of homologous blood clots following methods that have been previously described.19-21 Briefly, femoral arterial blood from a donor rat was withdrawn into 50 cm of polyethylene tubing (PE-50), kept in the tube for 2 hours at room temperature, and subsequently retained for 22 hours at 4°C. Five centimeters of the PE-50 tubing containing the clot was cut and connected to a syringe filled with saline with a 23-gauge needle. The clot was transferred into a dish filled with saline and washed 5 times with a 30-cm PE-10 tube. Then the clot was shifted to a modified PE-50 catheter with a 0.3-mm outer diameter filled with saline. Under a surgical microscope (Carl Zeiss, Inc), a modified PE-50 catheter with a 5-cm-long blood clot was gently inserted into the external carotid artery until the tip was positioned just proximal to the origin of middle cerebral artery. Then the clot in the catheter was injected into the internal carotid artery along with small amount of saline. After 5 minutes, the catheter was withdrawn from the external carotid artery. Reperfusion was achieved with the use of tPA. Continuous laser-Doppler flowmetry (Perimed AB) was used to monitor regional cerebral perfusion to ensure adequacy of embolic occlusions (perfusion decreased to <15% of preischemic baselines). For placement of the laser-Doppler flowmetry probe, a burr hole 2 to 3 mm in diameter was created in the right parietal bone (2 mm posterior and 6 mm lateral to bregma).

Experimental Groups and Drug Treatments

The goals of this study were (1) to assess the profiles of MMP upregulation after rat embolic stroke with and without tPA treatment and (2) to assess the effects of the broad-spectrum MMP inhibitor BB-94 on tPA-associated hemorrhage. Three groups of rats were studied: (1) untreated controls that received saline at 6 hours after ischemia; (2) rats that received tPA alone (10 mg/kg at 6 hours after ischemia); and (3) tPA plus BB-94–treated rats that received 50 mg/kg of BB-94 before ischemia and at 3 and 6 hours after ischemia plus tPA at 6 hours. tPA (Activase, Genentech) was administered intravenously (10 mg/kg, 2 mg/mL concentration in saline, over 30 minutes) according to previously established protocols that have been demonstrated to effectively induce clot lysis and cerebral reperfusion in rat embolic stroke.22-25 The relatively high dose of tPA was chosen on the basis of the approximately 10-fold difference in fibrin-specific activity between human and rodent systems.26 BB-94 was dissolved in PBS with 0.01% Tween-80 (3 mg/mL) and given intraperitoneally. Timing for the 3 doses of BB-94 was based on the estimated 3-hour plasma half-life of BB-94 in rodents.27 Similar doses have been shown to be effective inhibitors of MMP activity in rodent tumor models.28 Furthermore, the intraperitoneal route of BB-94 administration has been previously demonstrated to effectively penetrate brain,29 and we have previously shown that this dose of BB-94 worked in a mouse model of focal cerebral ischemia.7 BB-94 was a kind gift from British Biotech.

SDS-PAGE Gelatin Zymography

Gelatin zymography was used to measure the levels of MMP-2 and MMP-9 following previously described techniques.28,30 One set of rats was used to document the time course of MMP upregulation at 6, 12, and 24 hours after onset of embolic stroke (n=3 rats per group). Another set of rats was used to compare the profiles of ischemic upregulation in MMP between untreated rats and those treated with tPA. These measurements were performed at 12 and 24 hours (n=5 per group). In all cases, rats were killed after transcardiac perfusion with ice-cold PBS (pH 7.4). The brains were quickly removed, divided into ipsilateral ischemic hemispheres and contralateral nonischemic hemispheres, then frozen immediately in liquid nitrogen and stored at ~80°C to await further procedures. The brain samples were homogenized in ×10 volume of lysis buffer (50 mmol/L Tris-HCl [pH 7.4] containing 150 mmol/L NaCl, 1% Igepal CA-630, 0.1% SDS, and 0.1% deoxycholic acid) including protease inhibitors (2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride) on ice with the use of a Teflon glass homogenizer. After centrifugation (12,000 rpm) for 7 minutes at 4°C, the supernatant fluid was collected. The total protein concentration of each sample was determined by the Bradford assay (Bio-Rad Laboratories). Equal volumes of total protein extracts normalized for protein concentration (15 μg/5 μL) were prepared. Then 5 μL of ×2 sample buffer (126 mmol/L Tris-HCl, 20% glycerol, 4% SDS, 0.005% bromophenol blue) was added to each protein extract. Protein samples were loaded on and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. After separation by electrophoresis, the gel was incubated in renaturing buffer (2.7% Triton X-100 in distilled water) at room temperature for 30 minutes with gentle agitation. Then the renaturing buffer was decanted and replaced with developing buffer (50 mmol/L Tris base, 40 mmol/L HCl, 200 mmol/L NaCl, 5 mmol/L CaCl2, 0.2% Brij 35). After 30 minutes of equilibration by the developing buffer, the gel was incubated with fresh developing buffer at 37°C for 24 hours. After developing, the gel was stained with 0.5% Coomassie blue R-250 for 30 minutes and then destained appropriately. Human MMP-2 and MMP-9 standards were purchased from Chemicon. For densitometry, gels were scanned and analyzed with a standard computer-assisted imaging system. Elevations in MMP were calculated as fold increase in optical density versus nonischemic normal brain.9,31

Analysis of Infarct Volumes and Neurological Deficits

At 24 hours after ischemia, rats were assessed with a 4-point neurological deficit scale that has been extensively used for rat models of stroke.17 After neurological assessment, rats were killed with a lethal overdose of sodium pentobarbital and transcardially perfused to remove all intravascular blood. Coronal brain sections (2 mm thick) were stained with 2.3.5-triphenyltetrazolium chloride (TTC) (Sigma). Infarct volumes were quantified according to the indirect method via standard computer-assisted image analysis techniques.

Spectrophotometric Assay of Intracerebral Hemorrhage

Cerebral hemorrhage was quantified with a previously described spectrophotometric assay.19,34 Initially, a standard curve was obtained with the use of a “virtual” model of hemorrhage. Hemispheric brain tissue was obtained from normal rats subjected to complete transcardiac perfusion to remove intravascular blood. Incremental volumes of homologous blood (0, 0.5, 1, 2, 4, 8, 16, 32, 50, 100, 200 μL) were added to each hemispheric sample with PBS to reach a total volume of 3 mL, followed by homogenization for 30 seconds, sonication on ice for 1 minute, and centrifugation at 13,000 rpm for 30 minutes. Drabkin’s reagent (1 mL; Sigma) was added to 0.4-mL
Results

Normal rat brain showed detectable baseline levels of MMP-2 but not MMP-9 (Figure 1). After injection of homologous blood clots to induce focal ischemia, MMP-9 levels increased over time. Both pro-MMP-9 (92 kDa) and cleaved MMP-9 (84 kDa) were observed (Figure 1). Interestingly, the contralateral hemisphere also showed signs of increased MMP-9 levels (Figure 1).

To assess the effects of tPA treatment on these ischemic profiles of MMP upregulation, untreated rats were compared with rats treated with tPA (administered at 6 hours after ischemia). In all rats MMP-9 levels were elevated, as expected. At 12 hours, tPA-treated rats showed significantly higher levels of pro-MMP-9 and cleaved MMP-9 than untreated rats (Figure 2A to 2C). No significant differences were noted for MMP-2 (Figure 2A and 2D). By 24 hours after ischemia, overall MMP levels increased, but there were no longer any significant differences between untreated rats and tPA-treated rats (Figure 2A to 2D).

A separate series of experiments was performed to assess the effects of MMP inhibition on tPA-associated hemorrhage in this rat model of embolic stroke. Ischemic rats treated with tPA alone were compared with rats treated with tPA plus BB-94. All rats showed physiological parameters within normal range (Table 1). As expected, mean arterial blood pressures were high for these spontaneously hypertensive rats. Injection of homologous blood clots resulted in immediate and uniform reductions in cerebral perfusion, as documented by laser-Doppler flowmetry (Table 2). Delayed tPA treatment (6 hours after ischemia) in this model resulted in a mortality rate of 50% (7 of 14 rats). Rats treated with a combination of tPA plus BB-94 had a significantly reduced mortality rate (9.1%; 1 of 11 rats) compared with controls that received tPA alone (P<0.01) (Table 3).

In surviving rats (n=7 tPA only; n=10 tPA plus BB-94), hemorrhagic transformation, ischemic lesion volumes, and neurological deficits were assessed. Spectrophotometric measurement of whole blood showed a linear response between blood volume and hemoglobin absorbance (Figure 3A), thus validating this method for quantifying hemorrhage. By 24 hours after ischemia, all rats showed evidence of hemorrhagic transformation within the ischemic zone, demonstrating the reproducibility of this model of tPA-associated hemorrhage. Treatment with BB-94 significantly reduced hemorrhage volumes by almost 50% compared with vehicle-treated controls (Figure 3B). There were no effects on ischemic lesion volumes (Figure 3C) or neurological deficits (Table 3).

Discussion

Thrombolysis with tPA can elevate risks of cerebral hemorrhage after ischemic stroke.3,4 Thus, it is important to dissect
the mechanisms involved and identify methods that can ameliorate hemorrhage risks after tPA stroke therapy. In this report we used a previously established quantitative model of hemorrhage in spontaneously hypertensive rats to show that (1) pro-MMP-9 and cleaved MMP-9 are upregulated after focal ischemia induced by a blood clot embolus, (2) tPA treatment further increased the levels of pro-MMP-9 and cleaved MMP-9, and (3) combination therapy with the broad-spectrum MMP inhibitor BB-94 significantly reduced the volume of tPA-associated cerebral hemorrhage.

Hemorrhagic transformation after tPA thrombolysis may be broadly related to deleterious events during reperfusion injury. Historically, reperfusion injury has been associated with the generation of oxygen-derived free radicals. Free radicals damage proteins and lipids in cell membranes, thus amplifying cell death. In the context of hemorrhage, it is conceivable that free radical damage to membranes of the cerebrovascular system during reperfusion injury would lead to vascular leakage or rupture. In rodent models of embolic focal ischemia induced with homologous blood clots, delayed treatment with tPA has been observed to result in hemorrhagic transformations. Experimental interventions using free radical scavengers effectively reduce the severity of hemorrhage after tPA-induced reperfusion. These data provide hope that combination therapies that target free radical mechanisms can be designed to ameliorate risks of hemorrhagic transformation after thrombotic reperfusion.

In addition to free radical–mediated reperfusion injury, another emerging candidate mechanism may involve MMPs. It has been shown that MMPs are upregulated after acute central nervous system injury after ischemia, hemorrhage, and trauma. As a family of extracellular proteases, MMPs can degrade almost all components of the extracellular matrix. In the context of hemorrhagic transformation, uncontrolled MMP activation after tPA reperfusion can degrade critical proteins in the cerebrovasculature. These include collagen and laminin in the basal lamina and the blood–brain barrier–associated protein ZO-1. Degradation of these critical components may disrupt vascular structural integrity and lead to leakage and rupture. Interestingly, it has been shown that free radical injury is mechanistically linked to MMP upregulation. Hence, the involvement of MMPs in the pathophysiology of hemorrhage fits well in the context of oxidative damage and reperfusion injury. Recent data also suggest that tPA is biochemically linked to the MMP axis of extracellular proteolysis, with plasmin acting as an upstream activator of the MMP cascade. This provides another potential mechanism for the role of MMP-mediated hemorrhage after tPA thrombolysis. It will be critical for future studies to carefully examine the mechanisms and effects of tPA on all potential sources of MMP activity, including inflammatory cells that may infiltrate through the disrupted blood–brain barrier.

In the present study we showed that pro-MMP-9 and cleaved MMP-9 were both upregulated after embolic focal ischemia in rats. Importantly, delayed treatment with tPA significantly enhanced this increase in pro-MMP-9 and cleaved MMP-9. These data suggest that MMP-9 may be involved in the process of tPA-associated hemorrhage. A recent study demonstrated that cotreatment with the MMP inhibitor BB-94 reduced the incidence of hemorrhage in a rabbit model of embolic stroke. However, the effect of MMP inhibition on the severity of hemorrhage was unclear; the data suggested that the MMP inhibitor may have actually increased the severity of hemorrhage when it occurred. For clinical applications, it will be important to assess the effects not only on risk or incidence but also on severity or volume of hemorrhage. We have previously established a quantitative model of tPA-associated cerebral hemorrhage after clot-based embolic focal ischemia in spontaneously hypertensive rats. Clinically, the relevance of this model may be related both to the presence of hypertension and the concomitant vascular phenotype within this strain of rats. Nevertheless, the incidence of tPA-associated hemorrhage in this model is 100%, and therefore this reproducible model allows us to test the effects of BB-94 on the severity or volume of hemorrhage. In the present study we showed that BB-94 reduced the volume of tPA-associated hemorrhage by almost 50%. These findings implicate a role for MMPs in tPA-associated cerebral hemorrhage and suggest that targeting the MMP cascade may be a useful therapeutic approach.

There are a few caveats in this study worth discussing. First, we administered BB-94 before and during ischemia. Our purpose here was to obtain proof that MMPs were involved in mediating the occurrence of hemorrhagic transformation after tPA. For clinical purposes, it will be important to determine whether delayed treatment after tPA administration will also reduce hemorrhage volume. Second, we did not directly assay for BB-94 effects on brain MMP activity. However, others have previously shown that this class of hydroxamate-based inhibitors can indeed decrease zymographic MMP activity in brain inflammation. Third, it was

### TABLE 2. Regional Cerebral Blood Flow

<table>
<thead>
<tr>
<th>Group</th>
<th>Immediately After Ischemia, %</th>
<th>10 min After Ischemia, %</th>
</tr>
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<tbody>
<tr>
<td>Vehicle (n=7)</td>
<td>6.13±1.32</td>
<td>4.93±0.82</td>
</tr>
<tr>
<td>BB-94 (n=10)</td>
<td>3.95±1.07</td>
<td>3.09±0.85</td>
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Values are mean±SEM.
interesting to note that BB-94 had no effects on ischemic lesion volumes. This stands in contrast to our previous study, in which BB-94 reduced infarct volumes in a mouse model of focal ischemia. However, the previous study used a mechanical method of arterial occlusion, whereas a clot-based embolic approach was used here. There may be significant differences in neuroprotective responses between mechanical versus embolic models of focal ischemia, and further studies are needed to resolve these issues. Fourth, despite the reduction in hemorrhage volumes, we did not detect any improvements in neurological deficits using a relatively simple scoring system in rats. It will be useful for future studies to use more sensitive behavioral tests to define possible benefits in functional outcomes. Fifth, we used BB-94, a broad-spectrum MMP inhibitor, to ameliorate hemorrhage severity. It will be critical to ultimately define the specific MMP members and pathways involved. For example, we have shown that MMP-9 but not MMP-2 may be important for amplifying tissue infarction after ischemia in mouse brain. Targeting specific MMPs may lead to more optimal outcomes for preventing tPA-associated hemorrhage. Finally, in this model tPA was administered in a delayed fashion, ie, 6 hours after ischemic onset. Admittedly, tPA would not be given in such a delayed time frame in clinical stroke. Our purpose here, however, was to use a model in which hemorrhagic transformation occurred reproducibly so that we could study the mechanisms involved. The effects of MMP inhibition on the much lower rates of hemorrhage after early tPA therapy will have to be determined carefully.

In conclusion, this study showed that ischemic upregulation in MMP-9 is significantly enhanced by tPA treatment after embolic stroke, and cotreatment with the broad-spectrum MMP inhibitor BB-94 significantly reduced tPA-associated cerebral hemorrhage. Further studies are warranted to dissect specific MMP pathways involved, assess therapeutic windows, and translate these findings into clinical applications.

Acknowledgments

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References


<table>
<thead>
<tr>
<th>Group</th>
<th>Neurological Deficit Score</th>
<th>Mortality at 24 h (Dead Animals/Surgical Animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n=7)</td>
<td>2.71±0.18</td>
<td>2.00±0.22</td>
</tr>
<tr>
<td>BB-94 (n=10)</td>
<td>2.30±0.15</td>
<td>2.50±0.17</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P<0.01.

Figure 3. A, The linear relationship between blood volume and hemoglobin absorbance in the spectrophotometric assay allowed this to be used as a standard curve for calculating hemorrhage volumes in tPA-treated brains. B, tPA-associated hemorrhage volumes (mean±SEM) were significantly reduced by cotreatment with BB-94 (*P<0.05). C, Infarct volumes (mean±SEM) were not significantly different in tPA only vs tPA plus BB-94 groups.
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