Tissue Plasminogen Activator Promotes Matrix Metalloproteinase-9 Upregulation After Focal Cerebral Ischemia

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Background and Purpose—Thrombolytic therapy with tissue plasminogen activator (tPA) in ischemic stroke is limited by increased risks of cerebral hemorrhage and brain injury. In part, these phenomena may be related to neurovascular proteolysis mediated by matrix metalloproteinases (MMPs). Here, we used a combination of pharmacological and genetic approaches to show that tPA promotes MMP-9 levels in stroke in vivo.

Methods—In the first experiment, spontaneously hypertensive rats were subjected to 3 hours of transient focal cerebral ischemia. The effects of tPA (10 mg/kg IV) on ischemic brain MMP-9 levels were assessed by zymography. In the second experiment, wild-type (WT) and tPA knockout mice were subjected to 2 hours of transient focal cerebral ischemia, and MMP-9 levels and brain edema during reperfusion were assessed. Phenotype rescue was performed by administering tPA to the tPA knockout mice.

Results—In the first experiment, exogenous tPA did not change infarct size but amplified MMP-9 levels in ischemic rat brain at 24 hours. Coinfusion of the plasmin inhibitor tranexamic acid (300 mg/kg) did not ameliorate this effect, suggesting that it was independent of plasmin. In the second experiment, ischemic MMP-9 levels, infarct size, and brain edema in tPA knockouts were significantly lower than WT mice. Administration of exogenous tPA (10 mg/kg IV) did not alter infarction but reinstated the ischemic MMP-9 response back up to WT levels and correspondingly worsened edema.

Conclusions—These data demonstrate that tPA upregulates brain MMP-9 levels in stroke in vivo, and suggest that combination therapies targeting MMPs may improve tPA therapy. (Stroke. 2005;36:1954-1959.)

Key Words: blood–brain barrier ● brain edema ● metalloproteinases ● mice ● tissue plasminogen activator

Reperfusion is a logical therapeutic approach for ischemic stroke. The National Institute of Neurological Disorders and Stroke (NINDS) and European Cooperative Acute Stroke Study (ECASS) trials suggested that tissue plasminogen activator (tPA) is effective if administered within 3 hours after stroke onset.1 However, use of tPA in stroke remains limited, primarily because of the narrow time-to-treatment windows available for safe and effective therapy.2 Complications may involve increased risks of cerebral hemorrhage and further brain injury.3

Recently, matrix metalloproteinases (MMPs) have been implicated in neurovascular injury after stroke.4–7 MMPs comprise a family of zinc endopeptidases that can modify almost all components of the extracellular matrix.8–10 After stroke, MMPs become upregulated, degrade blood–brain barrier substrates, and promote edema, increased inflammatory infiltration, and parenchymal damage.4–6,9 Combination treatments using MMP inhibitors plus tPA reduce hemorrhage and improve outcomes in animal models of embolic stroke.11,12 Recently, we showed recombinant tPA transcriptionally elevated MMP-9 levels in human cerebral endothelial cells.13 Hence, it is possible that the deleterious induction of hemorrhage and edema after tPA reperfusion may be related in part to MMPs.

In the present study, we tested the hypothesis that tPA promotes MMP-9 dysregulation in stroke in vivo. A combined pharmacological and genetic approach was used in rat and mouse models. In the first set of experiments, we found that addition of exogenous tPA amplified ischemic MMP-9 levels in rats, and this effect was independent of plasmin. In the second set of experiments, tPA gene knockout decreased ischemic MMP-9 and brain edema in mice. Administration of...
Methods and Materials

Rat and Mouse Models of Focal Cerebral Ischemia
All experiments were performed using an institutionally approved protocol following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In the first experiment, male spontaneously hypertensive rats weighing 260 to 280 g were anesthetized with halothane (1% to 1.5%) 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 standard intraluminal method (silicon-coated 4.0 monofilament) was used to induce focal ischemia. Laser Doppler flowmetry confirmed adequacy of ischemia. Three hours later, reperfusion was achieved by withdrawal of the occluding filament. Brain MMP-9 levels were measured using gelatin zymography at various times from 6 to 24 hours after ischemia. To assess the effects of exogenous tPA on ischemic MMP-9 responses, saline, tPA (10 mg/kg, 2 mg/mL in saline, over 20 minutes; Genentech), or tPA plus the plasmin inhibitor tranexamic acid (300 mg/kg; Sigma) was infused intravenously into 3 separate groups of rats on reperfusion.

For the second series of experiments, a mouse model of focal ischemia was used. Male tPA knockout mice were compared with matching male C57BL/6 WTs. tPA knockout mice have been backcrossed for ≥10 generations into the C57BL/6 background. Under halothane anesthesia (1 to 1.5%), focal ischemia was induced using a standard intraluminal approach using silicon-coated 7.0 monofilaments. Core temperature was maintained at 37±0.5°C with a thermostat-controlled heating pad. Consistent ischemia was confirmed with laser Doppler flowmetry. After 2 hours of ischemia, reperfusion was induced by withdrawing the filament. Three groups were studied: WT mice that received saline, tPA knockout mice that received saline, and tPA knockout mice that received tPA (10 mg/kg, 2 mg/mL in saline, over 20 minutes) infused intravenously on onset of reperfusion.

Hydrogen Clearance Measurement of Cerebral Blood Flow
Hydrogen (H₂) clearance was used to measure resting cerebral blood flow in WT and tPA knockout mice. The femoral artery was catheterized for monitoring blood pressure. Platinum H₂-sensitive electrodes were inserted through a burr hole into the caudate putamen. Reference Ag–AgCl electrodes were attached to the base of the tail. H₂ (2.5% in air) was added to anesthetic gaseous mixture via the respirator for 60 seconds before H₂ containing gas was added to the base breathing gas and the washout H₂-curves were recorded for blood flow calculations. Absolute values of cerebral blood flow (mL×100 g⁻¹×min⁻¹) were calculated by the initial slope method.

SDS-PAGE Gelatin Zymography
Gelatin zymograms were used to measure the levels of MMP-2 and MMP-9 in ischemic brain homogenates following previously described techniques. Briefly, rats or mice were deeply anesthetized and then transcardially perfused 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. Samples were homogenized in lysis buffer including protease inhibitors on ice. After centrifugation, supernatant was collected, and total protein concentrations were determined using the Bradford assay (Bio-Rad). Prepared protein samples were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. MMP activity was quantified via standard densitometry.

Measurement of Infarction and Edema
Rats and mice were killed 24 hours after induction of focal cerebral ischemia. Coronal brain sections were stained with 2,3,5-triphenyl-tetrazolium chloride (Sigma). Infarct volume was quantified with a standard computer-assisted image analysis technique. Brain water content was measured using the standard wet–dry method. Edema was calculated as the net increase in water content in ipsilateral versus contralateral hemispheres.

Reverse Transcription–Polymerase Chain Reaction
RT-PCR was used to analyze levels of MMP-9 mRNA in sham-operated mice and WT mice at 8 hours after 2 hours of transient ischemia. Mice were killed, perfused with ice-cold PBS, and brains were removed and frozen in liquid nitrogen. Total RNA was isolated using RNAeasy mini kit (Qiagen) according to manufacturer instructions. Forward and reverse primers were 5'-GCATACTTGTACCGTATGG-3' and 5'-TAACCCGGAGGTGCCAAGCTTG-3' for MMP-9 (amplified length was 294 bp), and 5'-TGGATCACTGTGGCATCCATGAAA-3' and 5'-AAAAGCGCATGCATACGTCCAATGTCGG-3' for β-actin (amplified length was 349 bp).

Immunohistochemistry
Mice were transcardially perfused at 24 hours after ischemia. Brains were removed, immersed with 4% paraformaldehyde in PBS overnight at 4°C, and cryoprotected in 30% sucrose in PBS at 4°C. Immunohistochemistry was performed on 20-μm frozen sections using an MMP-9 rabbit polyclonal antibody (1:200; Robert Senior, Washington University, St. Louis, Mo). Negative controls were examined without primary antibody. Double staining was performed using a rat anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1) monoclonal antibody (1:50; Pharmingen).

Statistical Analysis
Quantitative data were expressed as mean±SD. Statistical comparisons were conducted using ANOVA followed by Tukey–Kramer tests for intergroup comparisons. Differences with P<0.05 were considered statistically significant.

Results

tPA Promotes MMP-9 After Focal Ischemia in Rats
In the first set of experiments, we aimed to show that tPA amplified MMP-9 in a rat model of 3 hours of transient focal cerebral ischemia. MMP-9 but not MMP-2 levels in the ischemic hemisphere increased over time after stroke onset (Figure 1A). Multiple bands corresponding to what is likely to be pro-MMP-9 and cleaved MMP-9 forms (80 to 92 kDa) were observed on gelatin zymography. To be conservative, total MMP-9 levels (ie, all bands together) were quantified together. Administration of exogenous tPA (10 mg/kg IV) at the onset of reperfusion did not appear to change infarct volumes at 24 hours (303±36 mm³ in saline-treated rats versus 326±23 mm³ in tPA-treated rats). However, tPA significantly amplified MMP-9 levels (P<0.05), approximately doubling the response of this extracellular matrix protease in ischemic brain tissue by 24 hours (Figure 1A and 1B). This phenomenon may be independent of plasmin because cotreatment with the potent plasmin inhibitor tranexamic acid did not alter tPA-amplified MMP-9 responses (Figure 1B).

MMP-9 Is Reduced in tPA Knockout Mice After Focal Ischemia
In the second set of experiments, the effect of tPA gene knockout on ischemic MMP-9 regulation was examined in mouse brain. Because MMP-9 responses could potentially be influenced by cerebral blood flow and different degrees of ischemia in the various mouse strains and conditions used.
here, we initially checked baseline and ischemic perfusion levels. H2 clearance electrodes demonstrated that cerebral blood flow was similar in WT (73 ± 10 mL/100 g per minute) and tPA knockout mice (78 ± 19 mL/100 g per minute). Resting arterial blood pressures were also similar: 89 ± 4 mm Hg in WTs and 87 ± 3 mm Hg in tPA knockout mice. On onset of middle cerebral artery occlusion, cortical perfusion rapidly dropped <15% of preocclusion baselines in all mice; levels of cerebral ischemia were similar in all groups (Table).

In nonischemic WT brain, baseline MMP-9 levels were very low. After 2 hours of transient focal ischemia, MMP-9 was upregulated; RT-PCR showed increased MMP-9 mRNA levels (Figure 2A), consistent with the findings of increased MMP-9 protein. To assess the spatial distribution of MMP-9 after transient focal cerebral ischemia in our mouse models, immunohistochemistry was performed. MMP-9 upregulation in all mice was restricted to the ischemic hemisphere coinciding with the occluded middle cerebral artery territory comprising cortex and striatum. Immunoreactive MMP-9 signals appeared mainly to be associated with vascular-like structures that stained positive for PECAM-1, a marker for endothelial cells (Figure 2B). Overall, the degree of MMP-9 staining appeared lower in tPA knockout mice compared with WTs. To quantitate these MMP-9 profiles, gelatin zymography was performed. At 24 hours after ischemic onset, brain MMP-9 protein levels were markedly increased, as expected. However, compared with WT mice, tPA knockouts had significantly reduced MMP-9 levels (P < 0.05; Figure 3A and 3B). Because it has been reported that tPA knockout mice may have smaller ischemic infarcts under some conditions, it is possible that the reduction in MMP-9 may be an indirect effect attributable to changes in infarction and severity of tissue damage. In the present study, infarct volumes were indeed smaller in our tPA knockouts (88 ± 9 mm³) compared with WT mice (116 ± 7 mm³). However, when the data were normalized to calculate “MMP-9 per cubic mm of infarct,” the ischemic MMP-9 responses were still significantly lower in tPA knockouts versus WT mice (P < 0.05; Figure 3C). Finally, brain edema at 24 hours after ischemia was also significantly lower in tPA knockouts compared with WT mice (P < 0.05; Figure 3D).

**Exogenous tPA Reinstates Ischemic MMP-9 Response in tPA Knockout Mice**

To determine the specificity of our findings, a “phenotype rescue” experiment was performed. Administration of tPA in tPA knockout mice did not affect infarct volumes (88 ± 9 mm³) structures that stained positive for PECAM-1, a marker for endothelial cells (Figure 2B). Overall, the degree of MMP-9 staining appeared lower in tPA knockout mice compared with WTs. To quantitate these MMP-9 profiles, gelatin zymography was performed. At 24 hours after ischemic onset, brain MMP-9 protein levels were markedly increased, as expected. However, compared with WT mice, tPA knockouts had significantly reduced MMP-9 levels (P < 0.05; Figure 3A and 3B). Because it has been reported that tPA knockout mice may have smaller ischemic infarcts under some conditions, it is possible that the reduction in MMP-9 may be an indirect effect attributable to changes in infarction and severity of tissue damage. In the present study, infarct volumes were indeed smaller in our tPA knockouts (88 ± 9 mm³) compared with WT mice (116 ± 7 mm³). However, when the data were normalized to calculate “MMP-9 per cubic mm of infarct,” the ischemic MMP-9 responses were still significantly lower in tPA knockouts versus WT mice (P < 0.05; Figure 3C). Finally, brain edema at 24 hours after ischemia was also significantly lower in tPA knockouts compared with WT mice (P < 0.05; Figure 3D).
in knockouts versus 76±8 mm³ in knockouts treated with tPA intravenously). But adding exogenous tPA back into tPA knockout mice reinstated the MMP-9 response back up to WT levels (Figure 3A and 3B), even when the data were normalized as “MMP-9 per cubic mm of infarct” (Figure 3C). Correspondingly, brain edema at 24 hours after ischemia was significantly increased in tPA-treated tPA knockout mice compared with saline-treated tPA knockout mice (P<0.05; Figure 3D).

Discussion

Properly titrated use of tPA is beneficial in reperfusing ischemic brain tissue. However, under some circumstances, use of tPA in delayed times after stroke onset induces brain hemorrhage and injury. Many potential mechanisms have been proposed, including tPA-mediated N-methyl-D-aspartate excitotoxicity and tPA-mediated microglial inflammation. Data from experimental models also suggest the involvement of the extracellular protease family of MMPs. MMPs can degrade basal lamina and blood–brain barrier substrates, thus leading to edema and vascular rupture. An emerging hypothesis states that neurovascular complications of tPA reperfusion are attributable to tPA-induced MMP-9 dysregulation in the neurovascular unit. This hypothesis has been indirectly supported by data showing that combination therapies using broad-spectrum MMP inhibitors reduce tPA-induced hemorrhagic conversion and improve outcomes in experimental clot embolic models of stroke.

In the present study, we used rat and mouse model of focal cerebral ischemia to investigate the relationship between tPA and MMP-9 in vivo. Administration of exogenous tPA doubled the “normal” MMP-9 response after ischemia in rats, tPA gene knockout significantly decreased ischemic MMP-9 levels compared with WT mice, and exogenous tPA reinstated the MMP-9 response back up to WT levels. Together, these pharmacological and genetic data show that tPA can amplify MMP-9 in stroke in vivo. Insofar as MMP-9 may mediate neurovascular injury, this may account for some of the neurotoxic side effects of tPA therapy.

How does tPA upregulate MMP-9? In part, this phenomenon may be related to free radicals induced by reperfusion injury because the MMP-9 promoter contains nuclear factor κB sites. In addition, tPA is now recognized to be more than just a clot buster. tPA induces cell signaling in neurons and glia. Although the precise pathways remain to be fully elucidated, recent studies suggest that the low-density lipoprotein receptor–related protein (LRP) may be involved. Lipoprotein receptors are implicated in vascular actions of apolipoprotein E and amyloid. LRP is enriched in brain, possesses signal transduction properties, and binds tPA, thus making it a candidate mechanism for the tPA-induced MMP-9 hypothesis. We showed previously that exposure of human brain endothelial cells to tPA upregulated MMP-9, and RNA interference suppression of LRP decreased the tPA-induced MMP-9 response. Our present study here extends the in vitro data and demonstrates that the tPA–MMP-9 connection may be relevant for stroke in vivo.

Nevertheless, a few caveats may be worth considering. First, although we show that tPA can amplify ischemic MMP-9 responses, the link with brain injury remains indirect. In our “phenotype rescue” experiment, administration of exogenous tPA back into the tPA knockout mouse significantly increased brain edema. However, the degree of edema did not reach WT levels, suggesting that MMP-9 may account for only part of the edema process in our model, and other mechanisms may operate in parallel. A second related caveat involves direct versus indirect tPA effects. A recent study showed that intravenous injection of tPA into mouse brain triggered blood–brain barrier opening in WT and MMP-9 knockout mice, suggesting that direct tPA actions on the
blood–brain barrier occurs. The relative importance of MMP versus non-MMP pathways remains to be determined. A third caveat involves tPA effects on blood flow. Although we use a mechanical model of arterial occlusion, is it possible that some of our findings are affected by residual thrombosis after filament withdrawal? Others have also proposed that tPA may possess vasoactive actions as well. Our H2 clearance data suggest that resting blood flows were similar in WT and tPA knockout brains. And laser Doppler flowmetry suggests that, at least in our mouse model, ischemic insults were comparable in all groups. Hence, it is unlikely that our MMP-9 and edema data were affected by significant differences in cerebral perfusion. However, we cannot unequivocally exclude the possibility that subtle changes in penumbral perfusion may still be present. Perhaps quantitative MRI may eventually be used to tackle this issue. Indeed, our tPA–MMP-9 hypothesis may be consistent with emerging data showing that early blood–brain barrier leakage occurs in tPA-treated stroke patients. A fourth caveat is related to specific roles of pro-form versus active enzymes, both within blood and brain parenchyma. Although tranexamic acid is a potent plasmin inhibitor, it is not completely specific because it can bind to Kringle 2 domains. How plasminogen, plasminogen activators, and plasmin per se may affect levels of pro-form and active MMPs in vivo remains to be determined.

Finally, our focus here was restricted to MMP-9. At least in rodent models, MMP-9 appears to be the dominant protease because MMP-9 knockout mice were protected against stroke, whereas MMP-2 knockouts were not. However, other MMPs can be activated after cerebral ischemia and trauma. MMP-3 is upregulated after neuroinflammation and may ameliorate neuronal apoptosis induced by its endogenous inhibitor tissue inhibitor of metalloproteinase-3. MMP-12 is upregulated in intracerebral hemorrhage and spinal cord injury, and suppression of this protease improves functional recovery. The overall response of the large MMP protease family will have to be carefully considered after tPA therapy for acute ischemic stroke.

Consistent with our experimental data, a linkage between tPA and MMP-9 is beginning to emerge in clinical stroke. Patients with high plasma levels of MMP-9 experience more brain injury with poor outcomes. Furthermore, administration of tPA may increase active forms of MMP-9, and patients who experience hemorrhagic conversion after tPA had significantly higher levels of plasma MMP-9 compared with those who did not. Further studies are warranted to dissect these tPA–MMP-9 signaling pathways and validate them for possible clinical applications. Targeting these pathways may allow us to lengthen the time-to-treatment window for tPA and improve its safety and efficacy in stroke.

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