Extension of the Thrombolytic Time Window With Minocycline in Experimental Stroke

Yoshihiro Murata, MD; Anna Rosell, PhD; Robert H. Scannevin, PhD; Kenneth J. Rhodes, PhD; Xiaoying Wang, PhD; Eng H. Lo, PhD

Background and Purpose—Thrombolysis with tPA is the only FDA-approved therapy for acute ischemic stroke. But its widespread application remains limited by narrow treatment time windows and the related risks of cerebral hemorrhage. In this study, we ask whether minocycline can prevent tPA-associated cerebral hemorrhage and extend the reperfusion window in an experimental stroke model in rats.

Methods—Spontaneously hypertensive rats were subjected to embolic focal ischemia using homologous clots and treated with: saline at 1 hour; early tPA at 1 hour, delayed tPA at 6 hours; minocycline at 4 hours; combined minocycline at 4 hours plus tPA at 6 hours. Infarct volumes and hemorrhagic transformation were quantified at 24 hours. Gelatin zymography was used to measure blood levels of circulating matrix metalloproteinase-9 (MMP-9).

Results—Early 1-hour thrombolysis restored perfusion and reduced infarction. Late 6-hour tPA did not decrease infarction but instead worsened hemorrhagic conversion. Combining minocycline with delayed 6-hour tPA decreased plasma MMP-9 levels, reduced infarction, and ameliorated brain hemorrhage. Blood levels of MMP-9 were also significantly correlated with volumes of infarction and hemorrhage.

Conclusion—Combination therapy with minocycline may extend tPA treatment time windows in ischemic stroke. (Stroke. 2009;40:000-000.)

Key Words: cerebral ischemia ■ hemorrhagic transformation ■ edema ■ tPA ■ neuroprotection

Tissue plasminogen activator (tPA) is an effective therapy for acute ischemic stroke, but its use remains limited to patients who are treated within 3 hours of ischemic onset. In part, this may be related to increased risks of brain edema and hemorrhagic conversion with delayed thrombolysis. Therefore, any way to reduce tPA-associated blood-brain barrier (BBB) injury may extend the time window for safe and effective reperfusion therapy.

Studies in experimental models and stroke patients suggest that the neurovascular protease matrix metalloproteinase-9 (MMP-9) may contribute to this phenomenon of tPA-associated BBB injury. Activated MMP-9 degrades neurovascular matrix, and tPA amplifies total levels of MMP-9 after ischemia. Recently, the tetracycline antibiotic minocycline has been found to be a potent MMP inhibitor that can effectively penetrate brain and target MMPs. The translational attractiveness of this approach lies in the fact that minocycline can be easily used in stroke patients.

In this study, we tested the hypothesis that minocycline can reduce tPA-associated hemorrhage in our rat embolic stroke model and thus extend the tPA treatment window up to 6 hours. We also assessed the secondary hypothesis that reductions in hemorrhage and infarction volumes were correlated with amelioration of blood MMP-9 biomarker levels.

Materials and Methods

Animal Model
All experiments were performed following an institutionally approved protocol in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Spontaneously hypertensive male rats (Charles River Laboratories, Wilmington, Mass) were anesthetized with isofluorane (1 to 1.2%) in a 30% oxygen and 70% nitrous oxide mix. Temperature was maintained at 37°C. Femoral arteries were cannulated to monitor pressure, pH and gases, and to draw blood samples. The embolic stroke model was adapted from Zhang et al. Briefly, 3 cm of homologous clots was injected via a modified PE-50 catheter to occlude the middle cerebral artery. Only rats that showed sustained ischemia to less than 20% of preischemic baselines were included. Animals with spontaneous recanalization before tPA thrombolysis were excluded (13 of a total of 81 rats). Exclusion takes place before assignment into the various treatment groups: saline injected at 1 hour after ischemia (n=9); tPA injected at 1 hour after ischemia (n=9); tPA at 6 hours after ischemia (n=9); minocycline injected at 4 hours plus tPA at 6 hours after ischemia (n=11); and minocycline alone at 4 hours after ischemia (n=7). Minocycline (Sigma) was injected intravenously at 3 mg/kg over 5 minutes. tPA (Genentech) was administered intravenously at 10 mg/kg with a 10% bolus and 90% continuous infusion over 30 minutes. Laser-doppler flowmetry (2 mm posterior, 5 mm lateral to bregma) was used to monitor cerebral perfusion.

Measurement of Infarction and Intracranial Hemorrhage
Rats were euthanized at 24 hours after ischemia, brains were perfused with saline, and 7 coronal sections (2 mm thick) were

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Table 1. Physiological Variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Rectal temperature, °C</th>
<th>MABP, mm Hg</th>
<th>pH</th>
<th>Po2, mm Hg</th>
<th>Po2, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Saline at 1 Hour)</td>
<td>283±7.54</td>
<td>37.0±0.12</td>
<td>184.2±5.25</td>
<td>7.38±0.05</td>
<td>39.2±3.25</td>
<td>125.2±6.22</td>
</tr>
<tr>
<td>2 (tPA at 1 Hour)</td>
<td>290±7.26</td>
<td>37.1±0.22</td>
<td>185.8±3.88</td>
<td>7.37±0.05</td>
<td>40.5±1.69</td>
<td>125.1±5.23</td>
</tr>
<tr>
<td>3 (tPA at 6 Hours)</td>
<td>285±8.20</td>
<td>37.0±0.15</td>
<td>183.5±6.25</td>
<td>7.37±0.02</td>
<td>38.9±5.98</td>
<td>128.2±4.05</td>
</tr>
<tr>
<td>4 (Minocycline at 4 Hours)</td>
<td>290±5.77</td>
<td>37.0±0.08</td>
<td>185±5.22</td>
<td>7.38±0.03</td>
<td>40.8±2.06</td>
<td>129.3±8.56</td>
</tr>
<tr>
<td>5 (Minocycline at 4 Hours, tPA at 6 Hours)</td>
<td>286±7.26</td>
<td>37.1±0.15</td>
<td>186.2±3.25</td>
<td>7.37±0.04</td>
<td>40.5±5.22</td>
<td>127.3±5.62</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MABP indicates mean arterial blood pressure.

stained with 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) to quantify infarct volumes. Cerebral hemorrhage was measured using a spectrophotometric assay to quantify hemoglobin in perfused brain. We have previously shown that hemoglobin measurements can be simultaneously performed on TTC-stained sections.5,10

MMP Zymography
Blood plasma samples were taken from the delayed 6-hour tPA only group and the combination minocycline plus tPA group. Samples were collected before ischemia, before tPA administration, 1 hour after tPA, and 24 hours after ischemia. Standard gel zymography was used to measure levels of MMP-2 and MMP-9.5 EDTA blood samples were immediately centrifuged at 4000 rpm for 15 minutes to obtain supernatants and total protein concentrations determined with the BCA assay (Pierce). Thirty micrograms of total protein were used to measure levels of MMP-2 and MMP-9.5 EDTA blood samples were immediately centrifuged at 4000 rpm for 15 minutes to obtain supernatants and total protein concentrations determined with the BCA assay (Pierce). Thirty micrograms of total protein were loaded in each gel to identify the bands and to standardize between gels. Band intensities of total MMP-9 and MMP-2 (including pro- and cleaved forms) were quantified via standard gel densitometry techniques as previously described.5,11 and expressed as a percentage of pro-MMP-9 standards in each gel. This calculation was used a common known protein amount as the baseline denominator a percentage of pro-MMP-9 standards in each gel. This calculation allowed quantitation and comparisons between all gels.

Statistical Analysis
Data were expressed as mean±SEM. Chi-square tests were used to evaluate differences in mortality. Plasma MMP levels, infarction, and hemorrhage volumes were assessed with ANOVA followed by Tukey-Kramer tests. Differences with P<0.05 were considered significant.

Results
Physiological parameters remained within normal range in all groups (Table 1). On clot injection, cerebral perfusion in the middle cerebral artery territory dropped below 20% of preischemic baselines (Figure 1). Early tPA treatment at 1 hour restored perfusion to almost 100%, but delayed tPA at 6 hours only reperfused to about 40% to 50% (Figure 1). Minocycline did not have detectable effects on cerebral perfusion values. As expected, early thrombolysis with tPA at 1 hour reduced 24-hour infarct volumes, but no efficacy was obtained with delayed 6-hour tPA administration (Figure 1). Early tPA at 1 hour almost fully restored perfusion and minimized infarctions, but delayed tPA at 6 hours did not have detectable effects on cerebral perfusion values.

Treatment with minocycline alone at 4 hours postischemia appeared to have measurable neuroprotective effects in our rat embolic stroke model; ie, infarction was reduced (Figure 2a and 2b) without an increase in cerebral hemorrhage and hemorrhagic conversion in the brain (Figure 2b and 2c). In this model, mortality rates in untreated stroke-only rats lie in the 30% range. Mortality rates with early (1 hour) and late (6 hour) tPA treatments were 10% and 45%, respectively. But with the limited numbers studied here, no statistically significant differences in mortality were detected between the various groups (Table 2).

Figure 1. Cerebral perfusion during embolic focal ischemia and reperfusion after tPA thrombolysis, as measured with laser Doppler flowmetry (mean ± SEM). Cerebral perfusion dropped rapidly below 20% after clot injection. Early tPA therapy at 1 hour almost fully restored perfusion. Delayed tPA therapy at 6 hours did not fully achieve reperfusion. No effects of minocycline on perfusion were detected.
Most importantly, combination therapy with minocycline further improved outcomes after delayed tPA at 6 hours. Infarct volumes at 24 hours were reduced to levels equivalent to those obtained with early 1 hour tPA treatments (Figure 2b). Correspondingly, hemorrhagic conversion associated with delayed tPA administration was significantly ameliorated as well (Figure 2c).

Because MMP-9 dysregulation has been previously implicated as a potential mechanism for tPA-associated hemorrhage, we compared plasma MMP-9 levels (total pro–MMP-9 and cleaved MMP-9 forms) in rats treated with delayed tPA alone at 6 hours versus rats treated with combined minocycline plus tPA at 6 hours. After ischemic onset, plasma MMP-9 was significantly elevated, and treatment with tPA at 6 hours further amplified total plasma MMP-9 levels over time (Figure 3a and 3b). However, in rats cotreated with minocycline, total levels of plasma MMP-9 post-tPA were significantly suppressed (Figure 3a and 3b). Within the

**Table 2. Mortality Rates**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 (Saline at 1 Hour)</th>
<th>2 (tPA at 1 Hour)</th>
<th>3 (tPA at 6 Hours)</th>
<th>4 (Minocycline at 4 Hours)</th>
<th>5 (Minocycline at 4 Hours, tPA at 6 Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=13</td>
<td>n=10</td>
<td>n=20</td>
<td>n=9</td>
<td>n=11</td>
</tr>
<tr>
<td>Dead (mortality rate)</td>
<td>4 (31%)</td>
<td>1 (10%)</td>
<td>9 (45%)</td>
<td>2 (22%)</td>
<td>2 (18%)</td>
</tr>
</tbody>
</table>
sensitivity limits of our model, no significant alterations were detected for MMP-2 (Figure 3c). Finally, average plasma levels of total MMP-9 at 1 hour after tPA administration were significantly correlated with both infarct volumes (Figure 4a) and the degree of hemorrhagic conversion (Figure 4b), consistent with the beneficial effects of minocycline administered in combination with tPA.

Discussion
Thrombolysis with tPA is the only FDA-approved treatment to dissolve clots and restore blood flow after ischemic stroke. However, use of tPA is limited by narrow time windows for thrombolysis, which in turn may be related to elevated risks of BBB injury, edema, and cerebral hemorrhage. Thus, it is important to identify new therapies that can prevent tPA-associated cerebral hemorrhage and also extend the time window for thrombolysis without reducing its benefits. Recent data suggest that MMPs might mediate some of these tPA-associated complications. Here, we show that combination therapy with minocycline can suppress plasma levels of MMP-9 after ischemic stroke, reduce tPA-associated hemorrhagic conversion, and extend the thrombolytic time window up to 6 hours after stroke onset in a rat model of embolic focal cerebral ischemia.

Minocycline has been shown to be beneficial in a wide range of acute neurological injuries such as focal cerebral ischemia, transient global brain ischemia, spinal cord injury, traumatic brain injury, and intracranial hemorrhage. Mechanisms of minocycline neuroprotection are broad and remain to be fully elucidated. Minocycline can downregulate apoptosis and protect against oxidative stress. Minocycline can also suppress cytokines and microglial activation in injured brain. In the present study, our data are only focused on the ability of minocycline to downregulate MMPs. But it is clear that these other mechanisms will surely contribute to the ischemic and hemorrhagic outcomes measured here as well.
Other studies have tested various MMP inhibitors in experimental cerebral ischemia. Our choice of minocycline was predicated on the fact that it is a relatively safe drug with a proven record of clinical use. We used a relatively low dose (3 mg/kg) of intravenous minocycline which results in serum levels close to that obtained with the standard 200-mg human dose. Although we did not directly measure minocycline levels in our study, others have demonstrated that systemic administration of minocycline can penetrate brain and downregulate MMPs. Many other labs have previously shown that minocycline can reduce infarction and improve neurological outcomes in experimental stroke models. In mechanical models of focal cerebral ischemia, minocycline effectively penetrates the parenchyma, downregulates brain levels of MMPs, and reduces infarction. Minocycline has also been tested as a combination therapy that augments hypothermic neuroprotection. In our study, minocycline alone effectively decreased infarct volumes by about 25%, whereas delayed tPA alone at 6 hours did not offer any protection. However, the combination of minocycline plus delayed tPA treatments reduced infarct volumes by about 50%. Thus adding minocycline to delayed 6-hour tPA therapy which by itself was not protective, produced further benefit. Our numbers are too small to truly support a conclusion of synergy. But these findings do suggest that minocycline may extend the therapeutic time window of tPA reperfusion therapy in stroke.

Besides its effects on infarction, our data also suggest that minocycline may ameliorate hemorrhage. Thrombolysis with tPA can elevate the risks of intracranial hemorrhage after ischemic stroke. Hemorrhagic transformation occurs more frequently in patients receiving treatment at least 6 hours after symptom onset. Our data here may be consistent with these clinical findings. Delayed treatment with tPA at 6 hours significantly increased hemorrhage volume compared with saline and 1-hour tPA. However, the hemorrhage volume was significantly decreased by the coadministration of minocycline even if delayed tPA treatment was performed. Of course, our study is limited to only 2 thrombolysis time points (1 hour and 6 hours), so further studies are required to determine whether minocycline can benefit intermediate treatment times. Combination therapy with minocycline may have also ameliorated the high rates of mortality associated with delayed tPA reperfusion. But with the small numbers tested here, no statistically significant effects were detected. Mortality in rat models of embolic stroke are typically related to vascular causes such as hemorrhage, edema, and elevations in intracranial pressure. It is tempting to speculate that in addition to neuroprotection, minocycline might in fact have vasculoprotective actions as well. However, our present study cannot dissect mechanisms, and it is important to acknowledge that interactions between parenchyma and vascular compartments in response to stroke and treatments will likely make it very difficult to separate these closely-related effects.

Overall, our data suggest that minocycline can suppress tPA-induced MMP dysregulation and prevent tPA-associated hemorrhagic conversion in ischemic stroke. However, there might be a few caveats. First, we only focus MMP-9. Previous studies in knockout mice suggest that MMP-9 plays a dominant role in mediating BBB injury during cerebral ischemia, so this initial focus may be reasonable. But other proteases in the MMP family and the endogenous inhibitors TIMPs will be involved as well. How minocycline affects these other systems should be examined further. Second, our data on plasma MMP-9 levels are only correlated with infarction and hemorrhage outcomes, so we cannot unequivocally prove causality (true and true but not related). We measured “biomarker” MMP-9 in plasma but not brain. Nevertheless, we and others have shown that MMP-9 is clearly upregulated in ischemic brain, and there is good correlation between plasma MMP-9 “biomarkers” and patient outcomes. These findings may also support the notion that plasma MMP-9 levels can potentially be used as a biomarker in future combination stroke therapies that target these neurovascular protease mechanisms. What remains to be determined, however, is the proximal source of these MMP responses. Cerebral endothelium, astrocytes, and neurons can upregulate MMPs in stroke, but our data are also consistent with the idea that neutrophils comprise an important source as well. A third caveat relates to mechanism. Even if it is true that MMPs comprise the target here, what are the functional substrates of these phenomenon? Elevation of MMP-9 level degrades neurovascular matrix, and tPA amplifies levels of MMP-9 after ischemia. The loss of vascular matrix is related to hemorrhage in primate and rat models of stroke. In this broader context of inflammation, MMP-2 and MMP-9 may rapidly degrade the blood brain barrier. Whether these substrates are affected by minocycline remains to be determined. Finally, our primary end points in this study are centered on tissue outcomes, i.e., infarction and hemorrhage. But how these changes in brain tissue integrity influence long-term functional profiles warrants further investigation. Recent data suggest that whereas MMPs mediate neurovascular damage during acute stroke, these same proteases contribute to neurovascular remodeling during stroke recovery. The use of minocycline (or other MMP inhibitors) will have to be carefully titrated to optimize its efficacy without compromising MMP-mediated remodeling during the recovery phase. The precise timing and duration of short-term treatments to augment tPA thrombolysis may need to be carefully assessed to avoid potential detrimental effects of prolonged MMP inhibition. A phase 2 clinical trial for Parkinson disease suggested that minocycline may have clinically relevant neuroprotective effects in humans as well. However, the recent finding that long term use of minocycline worsened outcomes in amyotrophic lateral sclerosis serves a cautionary note.

Overcoming the hemorrhagic complications of thrombolysis may extend its therapeutic time window, and allow tPA to be more widely used. Our data suggest that combining minocycline with tPA may be a clinically feasible way toward future attempts at combination stroke therapy.

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Disclosures
None.

References


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