Microplasmin: A Novel Thrombolytic That Improves Behavioral Outcome After Embolic Strokes in Rabbits

Paul A. Lapchak, PhD; Dalia M. Araujo, PhD; Steve Pakola, MD; Donghuan Song, MD; Jiandong Wei, MD; Justin A. Zivin, MD, PhD

Background and Purpose—It has been proposed that the novel thrombolytic microplasmin may be useful in the treatment of ischemic stroke. In the present study the effects and safety profile of microplasmin were evaluated in 2 rabbit embolic stroke models that have been used successfully to develop tissue plasminogen activator (tPA) as the only Food and Drug Administration–approved treatment for stroke. The rabbit small clot embolic stroke model (RSCEM) and rabbit large clot embolic stroke model (RLCEM) were used to determine the potential neuroprotective properties and safety profile of microplasmin, respectively, after an embolic stroke.

Methods—Rabbits were embolized by injecting small blood clots (RSCEM) or large blood clots (RLCEM) into the cerebral circulation. For the RSCEM, 126 rabbits were included, with behavioral analysis conducted 24 hours later, allowing for determination of the effective stroke dose (ES50) or clot amount (milligrams) that produces severe neurological deficits in 50% of rabbits. For RLCEM safety study analysis, 47 rabbits were included, with postmortem analyses consisting of assessment of hemorrhage and infarct rate and size. In test animals microplasmin was infused intravenously 60 minutes after embolization, whereas control rabbits were given infusions of the saline/Plasma-Lyte vehicle with all assessments performed in a blinded fashion.

Results—In the RSCEM, a drug is considered neuroprotective if it significantly increases the ES50 compared with the vehicle-treated control group. The ES50 of the vehicle-treated control group 24 hours after embolization was 1.36±0.42 mg (n=38). Microplasmin, infused starting 60 minutes after embolization, increased the ES50 to 2.32±0.57 (n=21), 1.89±0.48 (n=21), 2.81±0.55 (n=22), and 1.89±0.28 mg (n=24) for the 1-, 2-, 4-, and 8-mg/kg doses, respectively. There was a statistically significant behavioral improvement in the 4-mg/kg dose arm (P=0.040). The microplasmin dose of microplasmin that was statistically significant (4 mg/kg) was subsequently determined to be safe in the RLCEM because it did not increase the incidence of hemorrhages (56%) compared with vehicle-treated rabbits (63%), nor did it significantly alter hemorrhage volume, infarct rate, or infarct volume.

Conclusions—The present study shows that microplasmin improves behavioral rating scores in the RSCEM when administered 60 minutes after embolization, at a dose that does not increase hemorrhages in the RLCEM. This is in contrast to tPA, which significantly enhances the hemorrhage rate in the RLCEM. (Stroke. 2002;33:2279-2284.)

Key Words: ischemia □ neuroprotection □ reperfusion □ thrombolytic therapy □ tissue plasminogen activator □ rabbits

The direct and indirect costs of stroke currently exceed $51 billion in the United States.1 It is estimated that the overall incidence of stroke is approximately 2 to 2.5 per thousand population, resulting in 4.5 million deaths per year worldwide, with approximately 9 million stroke survivors.2 However, despite more than 50 years of efforts to develop effective treatments for stroke,3 the thrombolytic tissue plasminogen activator (tPA), which was shown to reduce neurological damage after cerebral embolization in rabbits,4 remains the only Food and Drug Administration–approved treatment for acute stroke. Subsequently, tPA was shown to improve clinical outcome in patients but only if administered within 3 hours of the onset of ischemic stroke.3,5 Furthermore, the increased incidence of symptomatic intracerebral hemorrhage6–10 has put constraints on the clinical use of tPA.

Although the most recognized adverse effects of tPA are manifested as a general increase in intracerebral hemorrhage rate, recent studies suggest that tPA may also affect specific mechanisms in the fibrinolytic cascade that may lead to increased neuronal degeneration.7,8,11,12 For instance, individual components of the fibrinolytic system, or plasminogen activation system, have been proposed to contribute to...
damage after permanent middle cerebral artery (MCA) occlusion.\cite{9,13} Plasminogen activators like tPA are specific proteolytic enzymes that convert the inactive proenzyme plasminogen to plasmin, which is a potent, nonspecific protease that cleaves blood fibrin clots and several other extracellular proteins.\cite{14,15} In mice with specific targeted inactivation of genes encoding plasminogen, tPA, and α₂ antiplasmin (α₂AP), inactivation or neutralization of the latter significantly reduced infarct size after ischemia.\cite{13} In mice depleted of α₂AP by either intravenous plasmin or neutralizing antibodies, infarct size after permanent occlusion of the MCA was shown to be smaller, suggesting that α₂AP depletion might reduce infarct size in the absence of reperfusion.\cite{9} These results point toward the promising neuroprotective properties conferred by depleting circulating α₂AP. Moreover, the thrombolytic nature of plasmin and microplasmin\cite{15,16} (a truncated form of plasmin that allows for recombinant production) provides an additional positive property of these drugs. Given its thrombolytic and neuroprotective properties, microplasmin is an attractive candidate to develop for the treatment of stroke. However, thus far, the potential usefullness of microplasmin has not been investigated in animal models of stroke that reproduce many of the clinically observed symptoms of acute stroke. Therefore, the main objective of the present study was to test whether microplasmin is neuroprotective in a rabbit model of stroke and whether it presents a better safety profile than tPA.

**Materials and Methods**

Male New Zealand White rabbits were anesthetized with halothane (5% induction, 2% maintenance by face mask), the bifurcation of the right carotid artery was exposed, and the external carotid was ligated just distal to the bifurcation, where a catheter was inserted anteriorly into the common carotid artery and secured with ligatures. The incision was closed around the catheter with the distal ends left accessible outside the neck; the catheter was filled with heparinized saline and plugged with an injection cap. The rabbits were allowed to recover from anesthesia for a minimum of 3 hours until they were awake and behaving normally.

For the rabbit small clot embolic stroke model (RSCEM), small clots were prepared by allowing blood drawn from a donor rabbit to clot at 37°C, as described previously by Lapchak et al.\cite{17} Briefly, the clot was suspended in Dulbecco’s PBS solution containing 0.1% bovine serum albumin, fragmented with the use of a Polytron (setting 6, 3 seconds), and sized by sequential filtration through a 240-μm metal screen and a 100-μm nylon mesh. The “microclots” retained by the nylon mesh were resuspended in PBS, then washed and allowed to settle, followed by aspiration of the supernatant and spiking of the particles with tracer quantities of 15-μm radiolabeled microspheres. The specific activity of the particles was determined by removing an aliquot, after which appropriate volumes of PBS solution were added so that a predetermined weight of particles was suspended in 1 mL (approximately 4 to 7 mg of clots). At the time of intra-arterial injection, clot particles were rapidly injected through the catheter, and both the syringe and catheter were flushed with 5 mL of normal saline.

For the rabbit large clot embolic stroke model (RLCEM) experiments, blood clots were prepared as described previously by Lapchak et al.\cite{18,19,20} Briefly, tracer quantities of radiolabeled (57Co) 15-μm microspheres were mixed into blood collected from a donor rabbit, which was then allowed to clot (2 hours, 37°C) and cut into pieces weighing between 3.5 and 3.8 mg. Each clot was suspended in 0.5 mL of normal saline and transferred to the injection catheter hub with forceps, but only after the catheter was cleared of heparinized saline, and pushed into the cerebral arterial system by injecting 5 mL of saline. If the rabbit did not respond behaviorally to the first clot, a second clot was administered in the same way as described previously by Lapchak et al.\cite{18,19,20}

**Drug Administration**

Drug or vehicle was administered starting 60 minutes after embolization. Microplasmin was produced with the use of the Pichia Pastoris yeast expression system. Microplasmin, which contains the protease domain of plasminogen, has a molecular weight of 29 000 kDa, less affinity for fibrin, and reacts slowly with α₂AP, which results in a half-life in blood of approximately 4 seconds.

For neuroprotective studies, microplasmin was administered intravenously at a dose of 8 mg/kg, with 50% of the dose given in a bolus injection and the rest infused over 60 minutes. This treatment schedule was used because microplasmin has a half-life (approximately 4 seconds) in blood. The control group was given a vehicle consisting of a 1:1 mix of normal saline and Plasma-Lyte (Baxter Pharmaceuticals). Rabbits were observed continuously for a minimum of 2 hours after embolization and treatment, at which time neurological function was scored, and again at 24 hours after embolization. For the latter, an observer who was naive to the treatments did the end point analysis.

**RSCEM Analyses**

For the RSCEM, a quantal dose-response data analysis technique was used, as described previously.\cite{17,21} A wide range of clot doses was tested to create both normal and abnormal animals, with the result that small numbers of microclots caused no grossly apparent neurological dysfunction, whereas large numbers of microclots invariably caused encephalopathy or death. With the use of a simple rating system, each animal was rated by a naive observer as either behaviorally normal or abnormal, with a reproducible composite result for a group of animals, and interrater variability was very low (<5%). The effective stroke dose (ES50) was then calculated as the amount of microclots that produce neurological dysfunction (impairment) in 50% of the rabbits within a specific treatment group.

**RLCEM Analyses**

For the RLCEM, emboli were prepared, and the rabbits were embolized according to previous procedures.\cite{6,18,20} If an animal did not react behaviorally (nystagmus, head lean/wave, struggling, hemiparesis) to the embolization, a second blood clot was injected in the same way 3 minutes later. Animals that had no behavioral reaction after administration of 2 clots were treated in the same manner as animals that responded to emboli. After the embolization process was completed, the catheter was ligated close to the neck, and the rest of the catheter and injection port was cut off. Animals that died during the procedure were excluded from the study, and their brains were fixed and sectioned as described below. Surviving animals were euthanized 48 hours after embolization, and their brains were removed, immersion-fixed in 4% paraformaldehyde for at least 5 days, and then examined by an observer blinded to the treatment groups. The right MCA of each brain was examined for the presence of emboli, surface blood vessels were stripped from the cerebral hemispheres, and the cerebellum was removed from the brain stem. Hemispheres and brain stem were cut into seven 5-mm-thick coronal slices, each having 2 faces, for a total of 14. The presence, location, size, and type of each hemorrhage and infarct were noted. Hemorrhage size was recorded as the number of section faces showing hemorrhage, while infarction was grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the brain sections. Four major types of hemorrhage were identified with the use of a previously documented grading system.\cite{6,18,20,22} Subarachnoid hemorrhage was identified as grossly visible accumulation of blood under the pial layer on the surface of the brain; hemorrhagic infarction necrotic tissue consisted of red speckling of an area, usually surrounded by soft infarcted tissue; punctate hemorrhage was isolated small red marks within the tissue that did not extend through the tissue as a blood vessel would; and parenchymatous intracerebral hemorrhage was a large homogeneous
mass of blood within the tissue. Finally, total radioactivity in the brain slices, cerebellum, brain stem, and the surface vessels from the right hemisphere was measured with the use of a gamma counter. The amount of radiolabel counted in the brains (including the right hemisphere vessels) was compared with that present in the labeled blood clot at embolization. If \(10\%\) of the counts were found in the brain and vessels, it was assumed that the labeled blood clot had not reached the brain, and data from these animals were excluded from further analyses.

For all experiments in this study, rabbits were randomly allocated into treatment groups before the embolization procedure, with concealment of the randomization guaranteed by using an independent third party. The randomization sequence was not revealed until all postmortem analyses were complete.

Statistical Analysis

For the RSCEM, a separate curve was generated for each treatment tested. The \(t\) test was used for comparison between groups, with the Bonferroni correction for multiple comparisons where appropriate. The KLCEM data were analyzed with the \(\chi^2\) test for hemorrhage/infarct rate and ANOVA where relevant.

Results

RSCEM Analyses

Neuroprotection

Quantal dose-response analysis was used to determine whether microplasmin was neuroprotective after an embolic stroke and whether there was a significant behavioral difference between vehicle-treated and microplasmin-treated rabbits. Figure 1 shows the quantal dose-response curve for the 4-mg/kg dose of microplasmin and for the vehicle-treated group constructed from the raw data presented in Table 1. In the RSCEM, neuroprotection occurs when a drug significantly increases the \(ES_{50}\) compared with the vehicle-treated control group. The \(ES_{50}\) of the vehicle-treated control group 24 hours after embolization was 1.36±0.42 mg \((n=38)\). When infused starting 60 minutes after embolization, microplasmin (4 mg/kg) significantly \((P=0.040)\) increased the \(ES_{50}\) by 107% compared with vehicle control to a value of 2.81±0.55 mg \((n=22)\), indicating a neuroprotective effect of the drug at this dose (Figure 1, Table 2). When the complete dose-response curves for microplasmin were analyzed, no significant differences between the vehicle-treated group and the 1-, 2-, or 8-mg/kg doses of microplasmin were observed (Figure 2, Table 2), with \(ES_{50}\) values of 2.32±0.57 \((n=21)\).
1.89±0.48 mg (n=21), and 1.89±0.28 mg (n=24), respectively.

**RLCEM Analyses**

**Hemorrhage Incidence, Rate, and Volume**
The microplasmin dose of 4 mg/kg, which in the RSCEM experiments resulted in statistically significant improvement compared with vehicle control, was subsequently evaluated in the RLCEM. In this model, this dose was also determined to be safe because it did not increase the hemorrhage rate, which was defined as the proportion of rabbits within a group that had at least 1 intracerebral hemorrhage (Table 3). Hemorrhage rate was 14 of 22 (63%) and 14 of 25 (56%) for the vehicle- and microplasmin-treated groups, respectively. The total number of intracerebral hemorrhages counted in each group was 20, with some of the rabbits in each group exhibiting >1 hemorrhage (Table 4). In addition, there were fewer incidences of punctate hemorrhage in microplasmin-treated rabbits compared with the vehicle control group. Microplasmin also did not significantly alter hemorrhage volume (Table 3).

**Infarct Rate and Volume**
There was no statistically significant effect (P>0.05) of microplasmin administration on infarct rate or volume. The proportion of rabbits with at least 1 grossly visible ischemic infarct (rate) was 14 of 22 (63%) and 21 of 25 (84%) in the vehicle-treated and microplasmin-treated groups, respectively. Microplasmin also did not significantly (P>0.05) affect infarct volume (Table 3).

**Discussion**
Recent studies have provided some insight into possible approaches for the treatment of stroke.1,3,8 Currently, there is a consensus that thrombolytics are valuable agents because recanalization is necessary to allow for reperfusion of ischemic tissue when stroke is the result of a thromboembolus.3,8 In the present study the pharmacological effects of microplasmin, a novel thrombolytic, which may in addition possess non–lytic-dependent neuroprotective properties,9 were investigated to determine not only its safety profile but also whether it is a potential neuroprotective agent. Our results indicate that microplasmin improved behavioral ratings scores after an embolic stroke; furthermore, microplasmin presented a better safety profile than previous observations with tPA in this model.8,18–20 This is consistent with earlier findings showing that a single injection of plasmin, which significantly reduces α1-AP levels, decreased infarct volume in the absence of reperfusion in the mouse MCA occlusion model.9 However, in our dose-response analysis, microplasmin, when administered at 4 mg/kg, resulted in an unequivocal behavioral improvement represented by an ES$_{50}$ value that was statistically different (improved) compared with the vehicle control group. All other doses were not statistically different from control.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Vehicle (n=22)</th>
<th>Microplasmin (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhage rate</td>
<td>14/22 (63.6%)</td>
<td>14/25 (56.0%)</td>
<td>0.558</td>
</tr>
<tr>
<td>Hemorrhage volume</td>
<td>2.45±0.57</td>
<td>3.20±0.92</td>
<td>0.509</td>
</tr>
<tr>
<td>Infarct rate</td>
<td>14/22 (63.6%)</td>
<td>21/25 (84.0%)</td>
<td>0.795</td>
</tr>
<tr>
<td>Infarct volume</td>
<td>4.36±0.98</td>
<td>7.00±0.973</td>
<td>0.063</td>
</tr>
</tbody>
</table>

Results are expressed as either hemorrhage/infarct rate (percentage of rabbits in each group) or hemorrhage/infarct volume mean±SEM, where n is the number of rabbits in each group. There were no statistically significant differences between the microplasmin- and vehicle-treated groups.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Vehicle (n=22)</th>
<th>Microplasmin (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subarachnoid hemorrhage incidence</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Parenchymatous ICH incidence</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Punctate hemorrhage incidence</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic infarction necrotic tissue incidence</td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Total hemorrhage incidence</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

ICH indicates intracerebral hemorrhage. There were no significant differences in total hemorrhage incidence between the 2 groups.
A dose-response curve similar to that of microplasmin also has been reported for tPA in the RSCEM, where a comparable dose was neuroprotective and increased the ES$_{50}$ by 153% over vehicle control. The ES$_{50}$ measured in the vehicle control group is comparable to that previously described in the RSCEM. However, very high doses of tPA were ineffective as a thrombolytic. The reason for the lack of effect of these thrombolytics at higher doses is not clear; however, at least for tPA, the hypothesis is that the drop-off in activity may be related to the “plasminogen-steal” effect, or depletion of available plasminogen, a phenomenon that appears to be associated with blood flow rates at the clot site and the concentration of plasminogen, the precursor to plasmin, on or near the surface of the clot. Another possibility is that higher doses of tPA may inhibit the fibrinolytic action of plasmin. However, because microplasmin appears to have a low affinity for fibrin and acts directly to lyse a clot, it is unlikely that the classic plasminogen-steal effect applies to its lack of effect at high doses. Nevertheless, our observation that high doses of microplasmin were ineffective suggests that there may be inhibition of the fibrinolytic action or that microplasmin may have affected other components involved in clot lysis that ultimately translated into reduced neuroprotective activity in the RSCEM.

When microplasmin was tested for activity in the RLCEM at the dose of 4 mg/kg, which improved behavior in the RSCEM, it resulted in a fewer hemorrhages than in the vehicle-treated group ($P<0.05$). However, microplasmin did not reduce the infarct volume in the model. In fact, there was a slight insignificant increase in infarct volume. It is not surprising that microplasmin did not reduce infarct rate in the large clot model because the model is based on injection of a 3- to 4-mg clot directed toward the MCA. Previous studies with tPA have also shown that infarct rate is not attenuated even though reperfusion is increased. The observation that microplasmin did not increase hemorrhage rate is noteworthy, particularly in view of the fact that tissue plasminogen activators such as tPA and tenecteplase increase hemorrhage rate by 104% to 220% in the same model. It is important to point out that because of the higher basal hemorrhage rate, the elevated rates of hemorrhage may have artifactually masked deleterious effects of microplasmin. It is possible that the specific vehicle required to stabilize and administer microplasmin increased baseline hemorrhage rates above that previously observed with this model. Nevertheless, because we used a fully randomized design in the present study, we could directly compare the hemorrhage rate in microplasmin-treated rabbits with that in vehicle-treated control rabbits. Additional preclinical safety studies with microplasmin are warranted before the initiation of a clinical trial.

In a separate rabbit model tPA has been shown to induce rebleeding in 90% of puncture sites, whereas full-length plasmin does not produce any rebleeding.

Conclusions

Microplasmin is neuroprotective in a thromboembolic model of stroke since it significantly improved behavioral rating scores in embolized rabbits. Moreover, since microplasmin did not increase hemorrhage rate, it may be a safer alternative to tPA for stroke therapy. This potential safety advantage, combined with the dual mechanism of action (thrombolytic plus non-lytic-dependent neuroprotection), indicates that microplasmin is a promising candidate for development as a treatment for acute stroke.

Acknowledgments

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References


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