Microplasmin Reduces Ischemic Brain Damage and Improves Neurological Function in a Rat Stroke Model Monitored With MRI

Yasuhiro Suzuki, PhD; Feng Chen, MD; Yicheng Ni, MD, PhD; Guy Marchal, MD, PhD; Desire Collen, MD, PhD; Nobuo Nagai, PhD

Background and Purpose—Microplasmin (μPl), a derivative of plasmin lacking the 5 “kringle” domains, was studied in a rat thrombotic stroke model with MRI monitoring.

Methods—Brain ischemia was induced by middle cerebral artery (MCA) occlusion with photochemically induced thrombosis. Brain tissue damage was assessed at 1 hour and 24 hours after MCA occlusion by MRI and 2,3,5-triphenyl tetrazolium chloride (TTC) staining. Neurological symptoms were scored at 24 hours. Animals with insufficient evidence of significant jeopardized brain tissue on perfusion-weighted imaging (PWI) at 1 hour were excluded before randomization. Included animals were randomized (blinded) to controls (solvent), 7.5 or 10 mg/kg μPl, administered as an intravenous bolus 90 minutes after MCA occlusion (n=8 per dose group).

Results—μPl treatment reduced cerebral damage measured by TTC staining at 24 hours, from 250±69 mm³ (mean±SD) in controls to 150±30 and 170±62 mm³ with 7.5 and 10 mg/kg μPl, respectively; it reduced the expansion of the PWI positive area between 1 and 24 hours, and it reduced neurological deficits from a Bederson score of 7 (6 to 9) in controls to 4.5 (3 to 8) and 4 (3 to 6), with 7.5 and 10 mg/kg μPl, respectively (median and range P<0.05 for each dose versus controls for all parameters).

Conclusions—Bolus intravenous μPl given 90 minutes after thrombotic MCA occlusion in rats reduces cerebral ischemic damage and improves neurological dysfunction, suggesting that μPl could be beneficial in ischemic stroke patients. (Stroke. 2004;35:2402-2406.)

Key Words: brain infarction ■ magnetic resonance imaging ■ models, animal

Presently, tissue plasminogen activator (tPA) is approved for treatment of thrombotic ischemic stroke. However, tPA may increase brain tissue damage in animal stroke models, suggesting that it might be neurotoxic and possibly harmful in a subgroup of patients. In the permanent middle cerebral artery (MCA) occlusion model, plasmin reduces brain tissue damage, suggesting that some of its effect might occur via neuroprotection. Microplasmin (μPl) is a recombinantly produced derivative of plasmin that lacks the 5 kringle domains and that can be produced as a pharmaceutical preparation. It reacts with α2-antiplasmin (α2-AP) and neutralizes it, although at a lower rate than intact plasmin. Previous studies have shown that μPl reduces brain tissue damage in a mouse thrombotic stroke model and in a rabbit small clot embolic stroke model. Furthermore, a lower bleeding tendency at equieffective doses of μPl than of tPA was demonstrated in a rabbit large clot embolic model. The present study evaluates the effect of μPl on thrombotic stroke in the rat, the species that is routinely used for ischemic stroke studies.

Most animal stroke models experience a wide interindividual variability in infarct size, which reduces the statistical power of pharmacological studies aimed at establishing significant differences between active and control groups. In the present study, the area of ischemic risk was noninvasively estimated at 1 hour after MCA occlusion by using perfusion-weighted imaging (PWI) on MRI analysis, as described and validated previously. Animals with insufficient brain area at risk for ischemic damage were excluded from randomization. The effect of 7.5 or 10 mg/kg μPl versus controls (solvent) on brain tissue damage and neurological function was then determined at 24 hours in a randomized blinded study design.

Materials and Methods

Animal Preparation
Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) weighing 220 to 350 g were used. Body temperature was maintained at 37.5±0.5°C with a heating pad (TR-100; FST) during surgery. All experiments were performed in compliance with the guidelines of the International Committee on...
Thrombosis and Hemostasis and the current institutional regulations for use and care of laboratory animals.

For physiological variable measurements, animals were anesthetized with 4% halothane and maintained with 2% halothane in a 20% oxygen–80% room air mixture. A plastic catheter was placed in the right femoral vein for injection of Rose Bengal and test agents.

**MCA Thrombotic Occlusion**

MCA was occluded by photochemically induced thrombosis, as described in detail previously.

**MRI Measurements**

MRI measurement was performed twice in each animal, at 1 hour and 24 hours after MCA occlusion. It was performed with a 1.5-T Siemens Sonata scanner using a commercially available 4-channel phased array wrist coil (MRI Devices Corp). Rats were placed in the supine position into a plastic holder in the wrist coil and anesthetized with 2% isoflurane. Sagittal, coronal, and axial pilot scans were obtained for positioning for subsequent MRI acquisitions. Each imaging sequence, 12 coronal images were acquired with a slice thickness of 2 mm gapped at 0.2 mm. T2-weighted (T2WI) turbo-spin echo sequence, 12 coronal images were obtained in each measurement, as described in detail previously.

In the axial MRI, the entire brain and ischemic lesions were delineated manually on each slice by consensus of 2 observers. The image analysis was performed as described in detail previously.

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**Histological Determination of the Area of Brain Damage**

At 24 hours after MCA occlusion, animals were anesthetized with sodium pentobarbital. Brains were removed, and 6 coronal sections (2-mm-thick slices from anterior 3.5 mm to anterior 13.5 mm) were sliced using a brain matrix (Agar Scientific). Sections were scored for hemorrhage and stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC). For each animal, the following was calculated using a computerized image analysis system (NIH Image 1.61): (1) the infarcted area in the ipsilateral hemisphere, (2) the area of nondamaged tissue in the ipsilateral hemisphere, and (3) the area of the contralateral hemisphere. Each of the areas was determined by summing the calculated areas of the 6 slices. Brain damage was shown as a volume of infarct area containing edema (Figure 2). The cerebral damage corrected for cerebral edema was shown as ratio of net damaged area to the intact contralateral hemisphere calculated as (3−2)/3.1 (Table).

**Neurological Deficits Assessment**

Neurological deficits were evaluated just before euthanization for TTC staining at 24 hours after the MCA occlusion according to the method of Bederson et al. In the dysfunctional paw test, the contralateral forepaw or hindpaw was pulled toward the body, and the time to re-extend each paw was scored as: 0 (<1 second), 1 (<5 seconds), 2 (1–5 seconds), 3 (5–15 seconds), 4 (15–30 seconds), 5 (30–60 seconds), 6 (>60 seconds).

**Effect of Microplasmin on Cerebral Damage Corrected for Brain Edema**

<table>
<thead>
<tr>
<th>µPli</th>
<th>Cortex</th>
<th>Subcortical Region</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40±14%</td>
<td>24±6.9%</td>
<td>33±9.0%</td>
</tr>
<tr>
<td>7.5 mg/kg</td>
<td>21±6.6%*</td>
<td>17±7.2%</td>
<td>19±6.9%*</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>22±16%*</td>
<td>16±5.6%*</td>
<td>19±11%*</td>
</tr>
</tbody>
</table>

Data represent mean±SD of the damaged brain tissue in percentage of the ipsilateral hemisphere as determined by TTC staining after correction for brain edema, as defined in Methods (n=8). *P<0.05 vs control (Dunnett test).
seconds), or 2 (>5 seconds). In the postural reflex test, the animal was pushed in the contralateral direction and scored as: 0 (resistance to lateral push), 1 (initially reduced but progressive resistance), 2 (reduced resistance), or 3 (lateral down fall). In the circling test, movements were scored as: 0 (straight movement), 1 (movement to the right), 2 (circling movement), or 3 (no movement). In the dysfunction of paws, mild wrist flexion and full flexion of wrist and elbow were observed. In the postural reflex test and the circling test, hemiparesis and obtundation of animals were observed. Each score was summed and represented as a single overall neurological score (0 to 10).

Statistical Analysis
Data are presented as mean±SD or median and range. Comparison of groups was made by ANOVA followed by Dunnett multiple comparison test if statistically significant differences were observed by ANOVA. In the behavior test, comparison of groups was made by Mann–Whitney U test. A comparison of 1 hour and 24 hours on PWI were made by repeated-measurements ANOVA, which, when significant, was followed by Tukey–Kramer test. A P value <0.05 was considered statistically significant.

Results
Selection of Study Animals by PWI at 1 Hour After MCA Occlusion
Thirty-six rats were subjected to MCA occlusion; 27 rats were entered, and 9 rats were excluded before randomization. In the latter group, 8 rats were eliminated on the basis of insufficient PWI abnormalities at 1 hour: 4 rats with a small PWI-detected area at ischemic risk (Figure 1) and 4 rats with unclear PWI analysis. In addition, 1 animal did not recover from anesthesia during the MRI measurement. Twenty-seven rats were randomized to solvent, 7.5 or 10 mg/kg μPl, three randomized animals were excluded from analysis: 1 rat died shortly after randomization, and 2 rats did not reach MRI inclusion criteria on more detailed quantitative reanalysis, which was conducted before unblinding of the study. Finally, 8 animals were obtained in each group.

Effect of μPl on Brain Damage
Both doses of μPl significantly reduced the size of brain tissue damage on TTC staining at 24 hours after the MCA occlusion (Figure 2). Results (mean±SD) of brain tissue damage in the cortex, subcortical area, and total brain were as follows: for controls 160±55, 91±20, and 250±69 mm³; for 7.5 mg/kg μPl 83±23, 71±15, and 150±30 mm³; and for 10 mg/kg μPl 93±51, 73±13, and 170±62 mm³ (n=8 in each group; P<0.05 for each dose versus controls). After correction of the size of cerebral damage for edema, the effect of μPl was still significant (Table). Intracranial hemorrhage was observed in 3, 1, and 0 of 8 animals in the control, 7.5 or 10 mg/kg group, respectively.

μPl significantly reduced volumes detected by DWI (from 260±25 mm³ in controls to 160±44 and 170±58 mm³ with 7.5 and 10 mg/kg μPl, respectively) and T₂WI (from 260±39 mm³ to 170±26 and 170±55 mm³, respectively) in the whole brain at 24 hours (n=6 in each group; P<0.05; for each dose versus controls; Figure 3). The dose of 7.5 mg/kg of μPl significantly reduced PWI (from 210±50 mm³ in controls to 140±34 mm³; n=6 in each group; P<0.05), but the difference with the 10 mg/kg dose (150±38 mm³; n=6) was not statistically significant.

Comparison of PWI, DWI, and T₂WI at 1 Hour and 24 Hours
Figure 4 shows the ratio of detected area to whole area at the coronal slice at 0.5 mm frontal from bregma in PWI at 1 hour and 24 hours after MCA occlusion. In the control group, PWI detected area increased from a value of 31±5.6% at 1 hour to a value of 40±4.7% at 24 hours (n=6; Figure 4G). With μPl, a significantly smaller increase was observed: from 27±3.6% to 31±4.2% with 7.5 mg/kg μPl; from 29±5.6% to 30±6.0% with 10 mg/kg μPl; n=6 in each group (P<0.05 for each dose versus controls; Figure 4G). The PWI positive area was larger at 24 hours than at 1 hour in all rats in the control group, whereas 2 of 6 rats in the 7.5 mg/kg μPl group and 3 of 6 rats in the 10 mg/kg μPl group had a smaller PWI positive area at 24 hours than at 1 hour (Figure 4H).

The area on DWI increased from 24±4.8% at 1 hour to 40±7.9% at 24 hours in controls (n=6) and from 22±8.5% to 32±9.5% and from 19±6.3% to 28±4.7% in the 7.5 and 10 mg/kg μPl groups, respectively (n=6 in each group). The increase in DWI positive area was significantly smaller in the 10 mg/kg group than in controls (P<0.05). Although the positive area in DWI at 1 hour was significantly smaller than the PWI positive area (P<0.05), the positive area in DWI at 24 hours was similar to the PWI positive area in controls.

T₂WI positive area changes were not observed at 1 hour. At 24 hours, the T₂WI positive area was 40±4.6% in the control group (n=6), similar to the area obtained from DWI or PWI (Figure 4B versus 4C and 4E versus 4F). Both doses of μPl decreased the T₂WI positive area at 24 hours significantly (31±2.3% and 31±8.8% for 7.5 mg/kg and 10 mg/kg μPl, respectively; n=6 in each group; P<0.05 for each dose versus controls).

Effect on Neurological Symptoms
All animals in the control group exhibited impairment of postural reflexes, with a median Bederson score of 7 (range 6-10). In the μPl group, three 7.5 mg/kg μPl, and the group with 10 mg/kg of μPl is expressed in cubic millimeters. The columns and vertical bars represent mean and SD. *P<0.05; **P<0.01 vs control (Dunnett test).
to 9; n=8). In the 7.5 and 10 mg/kg μPli groups, the median scores were 4.5 (range 3 to 8; n=8) and 4 (range 3 to 6; n=8), respectively, at 24 hours (P<0.05 for each dose versus controls; Figure 5).

Discussion

In an ischemic stroke model in rats, μPli significantly reduced brain tissue damage versus controls as measured by TTC staining and MRI (including PWI, DWI, and T2WI) at 24 hours after thrombotic MCA occlusion. μPli also reduced the expansion of PWI area between 1 and 24 hours. Furthermore, μPli-treated animals displayed less neurological deficit at 24 hours than controls.

Brain damage measured with TTC staining at 24 hours was 37% and 32% smaller with 7.5 and 10 mg/kg of μPli, respectively, than in controls. This resulted mainly from reduction of cortical damage, in agreement with the fact that the subcortical region is more sensitive to ischemic damage. Noninvasive MRI measurements (PWI, DWI, and T2WI) revealed the similar reduction by μPli compared with TTC (Figures 2 and 3). A previous report has shown that infarction at 24 hours after MCA occlusion is fully established and that the neuronal deficit starts to improve from the second day after ischemia. Therefore, the reduction of brain damage by μPli is most likely attributable not to a delayed infarct expansion, but to reduction of initial brain tissue damage. μPli was found not to increase bleeding tendency, in line with previous observations.

PWI, DWI, and T2WI showed different alteration between 1 and 24 hours. A positive area of PWI, which is sensitive to impaired perfusion and reveals the area at risk for the progression of ischemic stroke, was ~30% at 1 hour in control. However, a positive area of 20% was detected in DWI that is sensitive to the restriction of water diffusion by cell swelling, which is thought to result in cell injury when no abnormalities were detected yet in T2WI, which is sensitive to the presence of vasogenic edema resulting from blood–brain barrier breakdown. The expansion of PWI between 1 and 24 hours in controls is in line with previous observations. This expansion was significantly reduced by μPli; in some animals given μPli, the PWI positive area at 24 hours was smaller than at 1 hour, supporting the conclusion that μPli reduces ischemic damage at least in part by restoring vascular patency.

In the rat photochemically induced thrombotic MCA occlusion model, photoillumination induced not only MCA occlusion but also injury in the directly illuminated region. This area that is located in the ventral half of in the section through the MCA region at 0.5 mm frontal from bregma.
cannot be affected by treatment. Thus, animals with a PWI positive area limited to the ventral half at the MRI slice would not be expected to be responsive to treatment and would only reduce the statistical power of a randomized blinded study design. Therefore, in the present study, 4 animals with smaller PWI-detected area than the ventral half in the coronal slice at 0.5 mm frontal from bregma and 4 animals with unclear images in PWI were not randomized into the study. This selection to reduce interindividual variability optimizes the experimental design in a randomized blind design. Therefore, in the present study, 4 animals with high yield in the present study, which are the most widely used animals for ischemic stroke studies.

Improvement of neurological deficits is the main clinical goal of antistroke treatment to improve the quality of life in affected patients. The significant improvement of neurological deficits by μPli was apparently attributable to reduction of brain tissue damage and edematous volume in the cortex.

Rationale for the use of μPli as antistroke agent is based on previous observations that gene inactivation of α₂-AP by plasmin or anti-α₂-AP antibody reduced ischemic brain damage in mice. These observations are extended to rats with a thrombotic MCA occlusion in the present study, which are the most widely used animals for ischemic stroke studies.

The mechanism of brain tissue damage reduction by μPli requires further elucidation. Probable mechanisms include partial recanalization of the occluded vessel or thrombolysis in the microvasculature, consistent with the reduction of PWI positivity at 24 hours in some animals given μPli. Alternatively, μPli may reduce damage via pathways independent of thrombolysis, as supported by the observation that μPli also reduced brain damage in a permanent MCA occlusion model in mice. The doses of 7.5 and 10 mg/kg were selected as the minimal doses to reduce circulating α₂-AP levels to near zero while staying as far as possible below the doses that affected blood pressure (15 to 20 mg/kg in rats; unpublished toxicity study data, on file 2003).

In view of the similar pharmacodynamics effects of doses and their absence of hemodynamic side effects, their similar efficacy, in retrospect, is not surprising. The advantage of μPli over plasmin is that it can be produced recombinantly and expressed with high yield in Pichia pastoris. Furthermore, μPli may be associated with less hemorrhage risk than tPA.

In conclusion, μPli prevents progression of cerebral ischemic damage in animals with cortical ischemia after thrombotic MCA occlusion. μPli also significantly improved neurological dysfunction, suggesting that it may be beneficial for treatment of ischemic stroke in humans.

References

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