Tissue-Type Plasminogen Activator Improves Neurological Functions in a Rat Model of Thromboembolic Stroke

Teruo Sakurama, MSc; Ritsuko Kitamura, BSc; Munekiyo Kaneko, PhD

Background and Purpose The capacity of an intravenous infusion of double-stranded tissue-type plasminogen activator to salvage neurological functions in a rat model of thromboembolic stroke was studied.

Methods The model of thromboembolic stroke was induced by the intracarotid injection of 2-hour-old homologous blood clots to rats. Neurological functions were scored on a 5-point scale 48 hours after the injection of the clots. Infarction size was determined by triphenyltetrazolium chloride staining, and cerebral hemorrhage was examined macroscopically.

Results Intravenous infusion of tissue-type plasminogen activator (1 or 5 x 10⁵ IU/kg) within 3 hours after embolization significantly improved neurological functions (P<.01) and reduced infarction size (P<.05). Tissue-type plasminogen activator treatment 6 hours after embolization failed to attenuate the neurological status score. Treatment with tissue-type plasminogen activator did not increase the incidence of intracerebral hemorrhage and was not associated with a systemic fibrinolytic state. In comparison with tissue-type plasminogen activator treatment, although urokinase treatment (5 x 10⁵ IU/kg) improved neurological functions, it was associated with a systemic fibrinolytic state and a tendency to increase the incidence of intracerebral hemorrhage.

Conclusions These findings in this model suggest that tissue-type plasminogen activator should be given early after the onset of ischemic symptoms to effectively prevent or limit pathological infarction and improve neurological functions without an increase in the incidence of cerebral hemorrhage. (Stroke. 1994;25:451-456.)

Key Words • embolism • plasminogen activator, tissue-type • urokinase • rats

See Editorial Comment, page 456

The demonstration that acute stroke is typically an atherothrombotic or thromboembolic process provides a theoretical basis for the use of thrombolytic agents in the treatment of acute stroke. Therapeutic thrombosis can be achieved by using agents that activate endogenous plasminogen to plasmin. Available plasminogen activators, such as urokinase and streptokinase, activate plasminogen both at the thrombus surface and in the plasma, which causes the release of more plasmin that can be inhibited by available α2-plasmin inhibitor. The systemic release of plasmin is capable of paralyzing the clotting process by destroying many of its components and thereby causing a "lytic state." Therefore, treatment of a thrombotic cerebrovascular occlusion with such agents is a complex situation because of the possibility of intracerebral hemorrhage.²⁻⁶

Tissue-type plasminogen activator (TPA) has high affinity for fibrin and requires fibrin as a stimulator for plasminogen activation.¹² Thus, intravenous administration of TPA induces thrombosis with minimal systemic lytic effects. TPA has recently become available in beneficial effect on neurological functions, including mortality, because clinically stroke has a high lethal rate. Further, we examined the effects of the time lag between the beginning of TPA infusion and the onset of symptoms, and we monitored the effects of TPA on cerebral infarction size, incidence of intracerebral hemorrhage, and plasma fibrinogen levels. These effects of TPA were compared with those of urokinase, which has similar thrombolytic properties but which does not exhibit fibrin specificity.

Materials and Methods

Recombinant TPA consisting predominantly of two-chain molecules (96%) was produced from a cultured mammalian cell line, using recombinant DNA technology. The specific activity of TPA used was approximately 5 x 10⁵ IU/mg protein. Urokinase, derived from human urine (high molecular weight type, 1.2 x 10⁵ IU/mg protein), was purchased from Japan Chemical Research, Kobe, Japan. In this study the units of TPA and urokinase used were the aforementioned international units.

Five hundred fifty-five male Wistar rats were used. The animals were housed at 23 ± 2°C with a 12-hour/12-hour light/dark cycle with food and tap water provided ad libitum.
experiments reported herein were conducted according to the Guidelines of Experimental Animal Care issued by the Japanese Prime Minister's office.

A modification of an experimental acute stroke model in rats, first described by Kudo et al., was used in this study. Blood was drawn from donor rats (10 to 12 weeks old) under ether anesthesia. The blood was allowed to clot for 2 hours at 37°C. The clot was suspended in calcium-free Dulbecco's phosphate-buffered saline (PBS) containing 1 mg/mL bovine serum albumin and was fragmented by use of a Physcissors (Nitto, Funabashi, Japan). The fragments were sized by sequential filtration through first a 250-μm screen and then a 104-μm screen. The particles were washed with PBS, and a clot suspension was prepared with PBS at a concentration of 30 or 60 mg clots per milliliter. Rats (8 to 10 weeks old) were randomly assigned into groups and then anesthetized with sodium pentobarbital (45 mg/kg IP). The left common carotid artery was surgically exposed. A silicone catheter was inserted into the common carotid artery and secured with ligatures. The clot suspension was then injected gently (0.15 mL per rat, 0.3 mL/min). After the clot injection system had been flushed with saline, the common carotid was ligated.

The mortality of each group and the neurological functions of each rat were evaluated carefully 48 hours after the injection of clot particles. Neurological function was scored on a 5-point scale, essentially according to Bederson et al.6: 0, normal activity; 1, forelimb flexion; 2, circling or flexion of whole body; 3, convulsion or coma; and 4, death.

Determination of infarction size was performed by the method of Bederson et al.21 At 48 hours after inducing embolization, the surviving rats were killed and the brains removed. Coronal slices were made at 7 mm from the frontal pole, and the slices were immersed in 1.5%, 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline for 30 minutes at 37°C. The sections were then fixed in 10% phosphate-buffered formalin to be photographed. The area of infarction was identified by the lack of staining of the tissue with TTC. Quantification of the infarcted area was carried out by cutting and weighing traced sections of TTC-stained and unstained areas.

In separate experiments in which the drugs were infused at 6 hours after embolization, the rats were killed 1, 3, or 6 hours after the termination of drug infusion, and the brains were removed. Hemorrhage was defined as the appearance of blood that was evident to the unaided eye on the dorsal and ventral surfaces of the brains. The brains were then cut into five coronal sections and examined for evidence of hemorrhage. Evaluation of neurological function, infarct size, and hemorrhage was performed by an observer who was blinded to the treatment the animal had received.

Blood samples were collected into plastic tubes containing trisodium citrate (3.8%, 9:1 vol/vol) before and after embolization, at the end of drug infusion, and 3 hours after embolization. The samples were placed on ice and centrifuged at 4°C. Plasma fibrinogen levels were determined by the thrombin time method immediately (within 1 hour) after the collection of plasma.

Infarction size was analyzed by Student's t test, and plasma fibrinogen levels were analyzed by paired t test. A Fisher's exact test was performed to compare the mortality and the frequency of hemorrhages in each group. Neurological grade was tested by the Wilcoxon rank sum test.

Results

On injection of emboli, facial twitching was observed in virtually all animals. All but some severely damaged animals took fluid and food spontaneously after recovering from anesthesia. Various clinical manifestations, such as pallor of the eyeball on the affected side, circling, head tilting, limping, or decreased spontaneous activity, appeared singly or in any combination.

To examine the effects of TPA and urokinase on mortality in this model, 60 mg/mL of clot suspension was injected into the animals. The mortality increased with time after clot injection, and in the control group mortality at 48 hours after embolization was 60% to 90%. Table 1 shows the effects of TPA and urokinase on mortality (48 hours after embolization). The intravenous administration of TPA (5×10^5 IU/kg) 5 minutes after embolization resulted in a significant reduction in mortality compared with the control group (20% in the TPA group versus 71.4% in controls). At lower doses, a small dose-related decrease in mortality was seen (53.8% in the 1×10^5 IU/kg TPA group and 45.5% in the 3×10^5 IU/kg TPA group), but this decrease was without statistical significance. The same experiment was done with urokinase (Table 1). The efficacy of urokinase (5×10^5 IU/kg) infusion was similar to that of TPA (40% in the urokinase group versus 40% in the TPA group).

The efficacy of TPA (5×10^5 IU/kg) depended on the time of administration after embolization. Thus, infusion within 5 minutes decreased mortality significantly, but infusion after 3 or 6 hours failed to cause a statistically significant reduction in mortality (Table 2). The lack of statistical significance at the 3-hour interval might represent a type II error because of the small sample size and the variability in mortality.

To examine the effects of TPA and urokinase on neurological functions, 30 mg/mL of clot suspension was injected into the animals. In the control group mortality was 20% to 30% at 48 hours after embolization, and all animals that evaded death were grossly abnormal neurologically. Thrombolytic treatment 5 minutes after embolization attenuated the neurological status score in a dose-dependent manner (Fig 1). Neurological func-

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**Table 1. Effects of Tissue-Type Plasminogen Activator and Urokinase on Mortality in Rats With Acute Thromboembolic Stroke**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals, Dead/Total</th>
<th>% Mortality</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA</td>
<td>10/14</td>
<td>71.4</td>
<td>.</td>
</tr>
<tr>
<td>TPA 1×10^4 IU/kg</td>
<td>7/12</td>
<td>58.3</td>
<td>NS</td>
</tr>
<tr>
<td>TPA 3×10^4 IU/kg</td>
<td>5/11</td>
<td>45.5</td>
<td>NS</td>
</tr>
<tr>
<td>TPA 1×10^5 IU/kg</td>
<td>7/13</td>
<td>53.8</td>
<td>NS</td>
</tr>
<tr>
<td>TPA 5×10^5 IU/kg</td>
<td>2/10</td>
<td>20</td>
<td>.05</td>
</tr>
<tr>
<td>Urokinase</td>
<td>10/11</td>
<td>90.9</td>
<td>.</td>
</tr>
<tr>
<td>Urokinase 3×10^4 IU/kg</td>
<td>8/10</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>Urokinase 1×10^5 IU/kg</td>
<td>8/11</td>
<td>72.7</td>
<td>NS</td>
</tr>
<tr>
<td>Urokinase 5×10^5 IU/kg</td>
<td>4/10</td>
<td>40</td>
<td>.05</td>
</tr>
<tr>
<td>TPA 5×10^5 IU/kg</td>
<td>4/10</td>
<td>40</td>
<td>.05</td>
</tr>
</tbody>
</table>

Drugs were administered at 5 minutes after embolization. TPA indicates tissue-type plasminogen activator.

*Fisher's exact test.
TABLE 2. Effects of Time of Tissue-Type Plasminogen Activator Infusion After Embolization on Mortality

<table>
<thead>
<tr>
<th>Time*</th>
<th>Group</th>
<th>No. of Animals, Dead/Total</th>
<th>Mortality, %</th>
<th>Pt†</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>Control</td>
<td>7/9</td>
<td>77.8</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>TPA 5x10⁵ IU/kg</td>
<td>3/9</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>3 h</td>
<td>Control</td>
<td>5/9</td>
<td>55.6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TPA 5x10⁶ IU/kg</td>
<td>4/9</td>
<td>44.4</td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>Control</td>
<td>6/8</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPA 5x10⁶ IU/kg</td>
<td>6/9</td>
<td>66.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are given as mortality at 48 hours after embolization. TPA indicates tissue-type plasminogen activator.

*Time of administration after embolization.
†Fisher's exact test.

Injections were significantly improved in the groups treated with TPA (1x10⁵ and 5x10⁵ IU/kg) and in the group treated with urokinase (5x10⁶ IU/kg) in comparison with the control group. However, 3x10⁶ IU/kg TPA treatment did not lead to a significant improvement.

Fig 2 shows the effects of the time from the induction of stroke to the time of treatment of TPA on recovery from neurological deficits in this model. It was demonstrated that TPA treatment received within 3 hours after embolization improved neurological functions; however, TPA treatment at 6 hours after embolization did not attenuate the neurological status score.

Cerebral infarction size was determined by staining of live tissue with TTC, and the results are shown in Fig 3. Fifteen rats for each group were used to examine the effects on infarction size of TPA and urokinase administered 5 minutes after embolization. Three hours after embolization, 17 rats for the control group and 18 rats for the TPA-treated group were used. Six hours after embolization, 15 rats for the control group and 16 rats for the TPA-treated group were used. Excess mortality in the control groups caused an imbalance in the treatment group sizes. The injection of a thrombus (30 mg/mL of clot suspension) resulted in large infarctions (approximately 20% of the whole section) in all rats in the control groups. In the groups treated with TPA and...
urokinase (5 x 10^5 IU/kg) 5 minutes after embolization, the percentage of infarction size was significantly lower than that in the controls. Moreover, no infarcted area was seen in 7 of 11 rats treated with TPA or in 5 of 12 rats treated with urokinase. When animals were treated with TPA (5 x 10^5 IU/kg) 3 or 6 hours after embolization, the infarction size decreased to 7.2% and 12%, respectively. No infarcted area was detected in 7 of 16 rats and in 4 of 13 rats treated with TPA 3 and 6 hours after embolization, respectively.

Table 3 shows the effects of TPA and urokinase on intracerebral hemorrhage at various times after thrombolytic treatment. Vascular damage may be a prerequisite for hemorrhage, and this damage is dependent on the duration of ischemia. Therefore, drug infusion was begun 6 hours after embolization (30 mg/mL of clot suspension). This was the longest duration of ischemia before TPA treatment in this study. No hemorrhage was present in the control group. Of a total of 19 rats treated with TPA at 5 x 10^5 IU/kg, only 1 exhibited gross hemorrhage at 6 hours after the termination of infusion. In the group treated with urokinase (5 x 10^5 IU/kg), hemorrhage was present in 33.3% (2 of 6), 20% (1 of 5), and 28.6% (2 of 7) of the animals at 1, 3, and 6 hours, respectively, after the termination of infusion. By macroscopic examination of coronal sections, the observed hemorrhages in 3 of 5 rats treated with urokinase were intraparenchymal, and the other hemorrhages in TPA- and urokinase-treated rats were subarachnoid. There was no statistically significant difference in the incidence of hemorrhage among the groups. However, because 5 of the 18 rats treated with urokinase exhibited hemorrhage, there was an obvious tendency for urokinase treatment (P = 0.078 versus TPA, by Fisher's exact test) to increase the incidence of hemorrhage in comparison with TPA treatment.

The effects of TPA and urokinase on plasma fibrinogen levels are shown in Fig 4. A slight but significant decrease in fibrinogen concentration was observed at the end of infusion and 3 hours after embolization in the urokinase-treated but not in the TPA-treated group.

**Table 3. Intracerebral Hemorrhage at Various Times After Thrombolytic Treatment**

<table>
<thead>
<tr>
<th>Time After Drug Infusion</th>
<th>Group</th>
<th>n</th>
<th>No.</th>
<th>Incidence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>Control</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Urokinase</td>
<td>8</td>
<td>2</td>
<td>33.3</td>
</tr>
<tr>
<td>3 h</td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Urokinase</td>
<td>5</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>6 h</td>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>7</td>
<td>1</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>Urokinase</td>
<td>7</td>
<td>2</td>
<td>28.6</td>
</tr>
</tbody>
</table>

Doses of tissue-type plasminogen activator (TPA) and urokinase were both 5 x 10^5 IU/kg. Drug infusion was begun 6 hours after embolization, and the rats were killed 1, 3, and 6 hours after drug infusion.

**Discussion**

In this study we demonstrated that TPA significantly improved the neurological status score when treatment began up to 3 hours after embolization. The dose-response relation of neurological score for TPA given at 5 minutes after embolization was statistically significant (P < 0.01 by two-way cumulative x^2 test). Although TPA treatment administered 6 hours after embolization failed to attenuate the neurological status score, it reduced the infarction size. Since the 5-point scale that we used to assess neurological function was not sensitive enough to detect slight improvements of neurological function, it is possible that such neurological improvements could be detected in the TPA-treated group 6 hours after embolization if a more detailed grading scale was used. Since this study focused on the effects of TPA in this model, we did not investigate urokinase at later intervals.

A recent hypothesis regarding hemorrhagic transformations of brain infarcts suggests that they are due to ischemic damage of the blood vessel wall, which leads to vessel disruption on subsequent exposure to systemic arterial blood pressure after thrombolysis. According to this hypothesis, the duration of the obstruction would seem to be a critical factor. Therefore, we observed gross hemorrhage in the group that had the longest duration of ischemia in this study. We did not detect an increase in cerebral hemorrhage rate or a systemic fibrinolytic state after TPA treatment. The study by del Zoppo et al, in which TPA did not increase the incidence or severity of hemorrhage when given early (less than 3.5 hours) after the onset of focal cerebral ischemia, supports the view that TPA does not increase the incidence of hemorrhage. However, species differences in the extent of thrombus lysis by TPA in in vitro assays suggest that those results should be extrapolated with caution to clinical use of TPA in humans. Further, the animals used here were young and free of preexisting atherosclerotic vascular disease, and many stroke patients suffer some degree of arterial hypertension, which is associated with an increased risk of cerebral hemorrhage. Compared with TPA treatment, we found that urokinase treatment was associated both
with a tendency toward a higher incidence of hemorrhage and with a systemic fibrinolytic state. Our data at least suggest that TPA, at an effective dose, might be a safer thrombolytic agent than urokinase.

Zivin et al13 demonstrated that TPA infusion caused a significant reduction in neurological damage in a rabbit embolic stroke model. However, we chose a rat model for this study because compared with other species, the cerebral circulation of the rat more closely resembles that of humans.20 It is also an inexpensive and readily available animal to use for this reliable model of embolic cerebral ischemia.

The dose of 5 x 10^5 IU/kg of TPA was significantly effective in reducing the neurological status score in rats. In vitro studies have shown that the fibrinolytic effect of human TPA in rat plasma is approximately less than one fifth of that in the human plasma system.28 Therefore, we can expect that the effective doses of TPA for the treatment of human stroke would be similar or less than those used in this study. Recently Mori et al27 reported the outcome of a clinical trial of TPA on acute thrombotic stroke. Patients treated with 3 x 10^7 IU of TPA per body showed a significantly earlier improvement of neurological scale than did those treated with placebo. The average body weight of the patients treated with TPA in this trial was 51.2 kg, and therefore the dose of 5 x 10^5 IU/kg we used in rats very closely corresponds to that used in the clinical trial. The dosage is also similar to that used in reported clinical trials for the treatment of acute myocardial infarction.28,29 The dose of urokinase (6 x 10^5 IU for 7 days) has been widely administered in humans and is the dosage used in reported clinical trials for the treatment of embolic stroke. In Japan, a very low dosage of urokinase (6 x 10^5 IU/kg) was used for acute cerebral thrombosis.30-33 Clinical improvement was evident after this treatment, and there was no increase of hemorrhage. In a clinical trial of TPA for acute cerebral thrombosis, an infusion of 2 x 10^4 IU was used for 7 days.31,32 This thrombolytic therapy showed a higher clinical efficacy and safety than urokinase.

In conclusion, TPA should be given early after the onset of ischemic symptoms to effectively prevent or limit pathological infarction and to improve neurological functions.

References

Thrombolytic therapy for acute ischemic stroke, while of unproven benefit in humans, continues to draw considerable interest as a potentially promising treatment for stroke. In this series of experiments by Sakurama and colleagues, a rat model of thromboembolic stroke is used to explore a number of issues including dose response, efficacy, and safety of double-stranded human recombinant tissue-type plasminogen activator (TPA) (duteplase) as therapy compared with urokinase. The lack of a urokinase-treated group in some of the experiments is distracting, but the remaining observations do confirm the reports of others that intravenous recombinant TPA administered soon after the onset of thromboembolic occlusion of cerebral arteries results in reduced infarction size and improved neurological outcome. Delay in treatment administration results in progressive reduction in both benefit and safety. Moreover, with duteplase in this model there appears to be a dose-response relation with clinical outcome that may be difficult to demonstrate in the human condition because of inability to control for clot burden in the latter situation.

Whether recombinant TPA should be preferred over urokinase is not adequately established by the results of these experiments. A dose escalation was not performed with urokinase, as it was with recombinant TPA, and the number of animals studied was too small to demonstrate a convincing difference in the hemorrhage rates. Because of the cost differences, this issue deserves further study.

E. Clarke Haley, Jr, MD, Guest Editor
Department of Neurology
University of Virginia Health Sciences Center
Charlottesville
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T Sakurama, R Kitamura and M Kaneko

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