Evaluation of the Combination of a Tissue-Type Plasminogen Activator, SUN9216, and a Thromboxane A₂ Receptor Antagonist, Vapiprost, in a Rat Middle Cerebral Artery Thrombosis Model

K. Umemura, MD; K. Wada; T. Uematsu; M. Nakashima

Background and Purpose: We aimed to evaluate a modified tissue-type plasminogen activator, SUN9216, and the combination of SUN9216 and a thromboxane A₂ receptor antagonist, vapiprost, in a rat middle cerebral artery thrombosis model.

Methods: Under anesthesia, the left middle cerebral artery was observed under an operation microscope without cutting the dura mater via a subtemporal craniotomy. Photoillumination (wave length, 540 nm) was applied to the middle cerebral artery, and then rose bengal (20 mg/kg) was administered intravenously. The reopening of the middle cerebral artery by SUN9216, injected 30 minutes after middle cerebral artery occlusion, was observed under an operation microscope for a 60-minute observation period. Twenty-four hours after the operation, sections of the cerebrum were stained with triphenyltetrazolium chloride, and the area of cerebral infarction was analyzed by a computer.

Results: The combination of SUN9216 and vapiprost caused reopening of the middle cerebral artery in 58.8% of the rats, which was a greater percentage than that achieved with SUN9216 alone (31.6%). In contrast, saline did not cause reopening of the middle cerebral artery during the 60-minute observation period. The area of cerebral infarction in rats reperfused with SUN9216 was significantly reduced compared with that in the control group. The infarction area in rats treated with the combination of SUN9216 and vapiprost was reduced compared with that in rats treated with SUN9216 alone; this was the case whether or not the occlusion was reperfused. There was a significant correlation between the time of reopening of the middle cerebral artery and area of cerebral infarction.

Conclusions: A single injection of SUN9216 was effective in recanalizing the vessel and reducing the area of cerebral infarction. (Stroke 1993;24:1077-1082)

KEY WORDS • plasminogen activator, tissue type • thrombosis • thromboxane antagonists • rats

The potential for treatment of acute cerebral artery thrombosis with fibrinolytic agents has generated much interest. However, the use of thrombolytic agents in acute thrombotic stroke may predispose to hemorrhage. It has been reported that intracerebral hemorrhage is a severe but infrequent complication associated with the use of tissue plasminogen activator (t-PA) in acute myocardial infarction. However, the risk of intracerebral hemorrhage after the use of t-PA in patients with acute thrombotic stroke is unknown, and therefore animal models of acute thrombosis in the cerebrum would be useful to evaluate this risk. Several animal models of stroke caused by middle cerebral artery (MCA) occlusion have been reported, which have used ligation, cauterization, or coagulation.

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cutting, or embolization in the MCA. We aimed to produce a model of MCA occlusion in rats as a result of thrombosis, which was induced by a photochemical reaction between green light and the intravenous injection of rose bengal, which has been reported previously, and to evaluate the effect of t-PA in cerebral infarction using this model of rat MCA occlusion due to thrombosis.

In this study, the effect of a modified t-PA, SUN9216, was evaluated. SUN9216 is constructed by modifying a single amino acid (Asn117-Gln117), which yields a t-PA finger and growth, and by deleting the factor domains with a longer half-life in the blood than native t-PA. It was reported that SUN9216 is cleared approximately 20-fold more slowly than native t-PA and is 8.6-fold more potent as a fibrinolytic agent than native t-PA when compared in a rabbit after bolus injection.

It has been suggested that thromboxane A₂ (TXA₂) and prostacyclin play an important role in regulating local blood flow in ischemic cerebral microcirculation.
The reperfusion of the MCA by thrombolytic agents may increase the production of TXA₂ in the ischemic area, because the activity of cyclooxygenase depends on oxygen. It is postulated that this contributes to postischemic hypoperfusion. From these findings we hypothesize that blocking TXA₂ action, such as contraction of vessels and aggregation of platelets, may enhance the efficacy of the thrombolytic agents.

The aims of the present study were to investigate the effect of t-PA in this MCA thrombosis model and to assess the risk of intracerebral hemorrhage after the use of thrombolytic agents. Furthermore, we wished to investigate the effect of coadministration of a TXA₂ receptor blocking drug on the efficacy of the thrombolytic agent.

Materials and Methods

Animal Preparation

Wistar male rats weighing 240 to 260 g were used. Body temperature of the animals was maintained at 37.5°C with a heating pad (K-module model K-20, American Pharmaseal Company). Under pentobarbital sodium anesthesia, a catheter for the administration of rose bengal or agents was placed in the femoral vein. The scalp and temporalis muscle were reflected, and a subtemporal craniotomy was performed using a dental drill under an operation microscope. A window (2 mm in diameter) just anterior to the foramen of the mandibular nerve in the skull base bone was opened. The main trunk of the left MCA was observed under an operation microscope without cutting dura mater. Photoillumination of green light (wave length, 540 nm) was achieved by using a xenon lamp (model L-4887, Hamamatsu Photonics, Hamamatsu, Japan) with a heat-absorbing filter and a green filter. The irradiation was directed by a 3-mm-diameter optic fiber mounted on a micromanipulator. The head of the optic fiber was placed on the window in the skull base, and rose bengal (20 mg/kg) was injected intravenously. Photoillumination was performed for 10 minutes. The incisions were closed after a 90-minute observation period. Twenty-four hours after the completion of the irradiation, the cerebrum was blindly removed under pentobarbital anesthesia by another investigator for later analysis. The cerebrum was coronally sectioned in 1-mm thicknesses from the frontal lobe with a microslicer, and then six consecutive slices were stained with triphenyltetrazolium chloride (TTC; Katayama, Japan). Photographs of the slices were then taken. For each slice, the relation of the area of infarction to the whole area of the corresponding cerebrum was calculated using a computerized image analysis system.

Experimental Procedure

t-PA (SUN9216, 1 mg/kg) was administered intravenously by a single bolus injection at a volume of 0.5 mL through the femoral vein 30 minutes after occlusion of the MCA. The thrombus in the MCA was observed by an operation microscope for 60 minutes after the injection of SUN9216. The time to reperfusion was determined when blood flow in the MCA could be observed by an operation microscope. Twenty-four hours after the administration of SUN9216, the infarcted area of the cerebrum was analyzed with TTC. In another experiment, the effect of the combination of SUN9216 and a TXA₂ receptor antagonist, vapiprost (1 mg/kg), which was injected at a volume of 0.25 mL through the femoral vein, was evaluated. Vapiprost was injected just before the administration of SUN9216, which was administered as before. Control animals were injected with saline at the same volume of SUN9216 or vapiprost. Saline, SUN9216 alone, or a combination of SUN9216 and vapiprost was administered at random.

In a preliminary experiment, blood pressure, heart rate, and arterial blood gas were not affected by the combination of rose bengal and green light, the operation, and the injection of drugs.

Histopathological Observation of Thrombus in the Middle Cerebral Artery

To evaluate thrombus in the MCA in the morphological study, rats were anesthetized with pentobarbital sodium, killed by exsanguination, and perfused transcardially for 1 minute with saline followed by 10% formaldehyde solution for 5 minutes, at 10 minutes after the occlusion of the MCA. The brain was immediately removed and fixed in 10% formaldehyde. Each section was stained by hematoxylin and eosin for light microscopic study.

Statistical Analysis

Data are expressed as mean±SE. Statistical analysis was performed with unpaired Student's t test for comparisons between groups, and the comparisons of more than three groups were analyzed by variance. In regard to incidence, the comparisons of groups were analyzed by Fisher's Exact Test. A value of P<.05 was considered significant.

Results

By operation microscope, we observed that the MCA was completely occluded by thrombus approximately 6
minutes after the administration of rose bengal. Histologically, the thrombus was massive and was firmly anchored to the damaged arterial inner wall (Fig 1). Transluminal thrombus consists of numerous red blood cells and amorphous materials, such as fibrin nets and platelets.

In Fig 2, a typical photograph of a cerebral infarction stained with TTC 24 hours after occlusion of the MCA in rats treated with saline is shown. The left dorsolateral frontoparietal cortex and the left dorsolateral portion of the striatum were consistently infarcted.

SUN9216 restored blood flow in the MCA occlusion in 6 of 19 rats (31.6%) compared with 0 of 10 rats treated with saline (Table 1). Furthermore, it significantly reduced the area of cerebral infarction compared with rats in which reopening of the MCA was not successful and those treated with saline, as shown in Fig 3.

The combination of SUN9216 and a TXA2 receptor antagonist, vapiprost, also caused reopening and reduced the area of cerebral infarction. Moreover, this treatment significantly reduced the time to reperfusion, increased the incidence of reopening, and reduced the area of cerebral infarction compared with rats treated with SUN9216 alone (Table 1, Fig 3).

There was a significant correlation between the time of reopening of MCA occlusion and the area of cerebral infarction, as shown in Fig 4. The correlation curve between the time of reopening of MCA occlusion and the area of cerebral infarction in rats treated with SUN9216 alone was almost equivalent to that in those treated with the combination of SUN9216 and vapiprost.

No bleeding in the cerebrum in rats treated with the combination of SUN9216 and vapiprost was observed macroscopically.

**Discussion**

We have demonstrated that a single bolus injection of modified t-PA, SUN9216, caused reopening of MCA occlusion by thrombus and reduced the area of cerebral infarction 24 hours after the completion of photoillumination.

The method applied to this study, which has been reported previously,11,12 causes the formation of active oxygen species that damage the endothelium. Consequently, platelets adhere and aggregate on the damaged vessel, which results in occlusion. Dietrich et al12 reported that this photochemically induced endothelial alteration may stimulate platelet activation and implant abnormal endothelial function as a primary event. Our model is also characterized by platelet-rich thrombosis in a scanning electron microscopic study.19 The size and location of cerebral infarction induced by our method were very similar to those obtained with MCA cauterization in the rat.19 The combination of green light

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**FIG 2. Typical photograph of cerebral infarction stained with triphenyltetrazolium chloride 24 hours after occlusion of middle cerebral artery in control animals. Each section was cut coronally in 1-mm thickness from frontal lobe. Left dorsolateral frontoparietal cortex and left dorsolateral portion of striatum were consistently infarcted. Bar, 10 mm. Order of numbers represents position from frontal lobe.**

**TABLE 1. Effect of Combination of SUN9216 and Vapiprost in Incidence of Reopening or Time of Reopening of Middle Cerebral Artery**

<table>
<thead>
<tr>
<th>No.</th>
<th>Incidence of reopening</th>
<th>Time of reopening (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline 10</td>
<td>0/10 (0%)</td>
<td>...</td>
</tr>
<tr>
<td>SUN9216 (1.0 mg/kg) 19</td>
<td>6/19 (31.6%)</td>
<td>38.3±6.0</td>
</tr>
<tr>
<td>SUN9216 (1.0 mg/kg) and vapiprost (1.0 mg/kg) 17</td>
<td>10/17 (58.8%)</td>
<td>21.0±2.9*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SE. Time indicates time of reopening of middle cerebral artery after injection of SUN9216. 
P<.05 vs SUN9216 alone.
and rose bengal did not damage smooth muscle in the tunica media and lamina elastica interna of rat femoral arteries in a transmission electron microscopic study.\textsuperscript{10} It is unclear whether this combination affects polymorphonuclear leukocyte function.

Recently, thrombolytic therapy has been performed in patients within several hours of acute cerebral thrombosis and has been demonstrated to be effective without severe intracerebral hemorrhage. In this study, no cases of intracerebral hemorrhage were found macroscopically in rats treated with SUN9216 when it was injected 30 minutes after MCA occlusion.

In the present study, there appeared to be a significant correlation between the time of reopening and the area of infarction 24 hours after MCA occlusion. From this correlation it would appear that the area of infarction in animals reperfusing later than 90 minutes after occlusion would be equivalent to that seen in saline-treated animals (approximately 17%). Similar infarction areas were also observed in animals treated with SUN9216 with or without vapiprost that did not reperfuse. This suggests that the area of infarction can only be reduced with thrombolytic therapy if this therapy achieves reperfusion within 90 minutes of occlusion in this model. Studies by many investigators, using various animal models, have demonstrated a critical period of MCA occlusion ranging from 2 to 7 hours that may be tolerated without, or with limited, infarction.\textsuperscript{20-24}

The combination of SUN9216 and vapiprost significantly reduced the time for reopening of MCA occlusion compared with SUN9216 alone. It is believed that the combination of thrombolytic and antiplatelet agents is effective in cerebral thrombosis, because the area of cerebral infarction primarily depended on the time of reopening of the MCA in this study.

Although the biochemistry of free fatty acids and their metabolites in cerebral ischemic insult has been investigated,\textsuperscript{25-29} the role of these substances in cerebral ischemia is still under discussion. The reperfusion of the MCA by t-PA might increase the production of TXA\textsubscript{2}, because arachidonic acid may be metabolized to TXA\textsubscript{2} in the presence of the oxygen “burst” accompanying reperfusion. TXA\textsubscript{2} is derived primarily from platelets, but it has been shown that cerebral arteries\textsuperscript{30} and glial cells\textsuperscript{31} are capable of producing TXA\textsubscript{2}. In this study, TXA\textsubscript{2} may be derived from these sources. The combination of SUN9216 and vapiprost tended to reduce the area of cerebral infarction compared with SUN9216 alone. However, the combination of drugs did not affect the correlation between the time of reopening and the area of cerebral infarction. These findings suggest that reperfusion of the MCA might cause an increase in the production of TXA\textsubscript{2}; however, the area of cerebral infarction might primarily depend on the time of the reopening of the MCA and not on the TXA\textsubscript{2} level in the cerebrum in acute cerebral infarction. It was reported that no correlation was found between local cerebral blood flow and regional concentration of TXA\textsubscript{2} after reperfusion of the MCA occlusion in cats.\textsuperscript{26}

In conclusion, a single bolus intravenous injection of a modified t-PA, SUN9216, caused reopening of the MCA occlusion caused by thrombosis in our model. In addition, the combination of SUN9216 and vapiprost tended to reduce the area of cerebral infarction and the time for reopening and caused an intracerebral hemorrhage macroscopically. However, the combination of drugs did not affect the correlation between the time of reopening and the area of cerebral infarction. These results may indicate that TXA\textsubscript{2} does not play a major role in the establishment of cerebral infarction caused by acute thrombosis.

Acknowledgments
A modified human tissue-type plasminogen activator, SUN9216, was a gift from Suntory Ltd, Japan. A TXA\textsubscript{2} receptor antagonist, vapiprost, was a gift from Glaxo Group Research Ltd, England.

References
In their work reported above, Umemura and colleagues present evidence that inhibition of thromboxane A2 (TXA2)-mediated activities can, in combination with a long t1/2 mutant of tissue plasminogen activator (t-PA), improve reperfusion in experimental thrombosis of the middle cerebral artery (MCA). The clinical implications of this work are obvious, but this experimental model presents other interesting issues for discussion.

The use of laser-assisted photoocoagulation has found a niche in experimental cerebral ischemia. Dietrich and coworkers1,2 have studied the ramifications and limitations of 540-nm photolllumination of cerebral tissue after the infusion of rose bengal, the technique used by Umemura et al. A somewhat different approach using the substance Photofrin II with an incident wavelength of 632 nm has also been used.3,4 Curiously, tissue injury induced by porphyrins and their precursors in the presence of light have a basis in clinical disease, in the rare congenital erythropoietic protoporphyria.5-7 Here, incident light at 400 nm produces singlet oxygen, which promotes lipid peroxidation, amino acid oxidation, nucleic acid changes, protein cross-linking, and the cutaneous manifestations of the disease. A third technique using direct incident laser light (486 nm) has been used to generate “thombi” in vital preparations without the use of a chemical intermediary (W. Eisert, personal communication). The latter process has been used in many settings but has the disadvantage of generating significant heat denaturation of perivascular and vascu-
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