Thrombolysis of Canine Femoral Artery Thrombus by a Novel Modified Tissue-Type Plasminogen Activator (E6010)

Noboru Suzuki, Suguru Suzuki, Naoko Nagaoka, Hitosi Mizuo, Teruaki Yuzuriha, Shinji Yoshitake and Katsuo Kanmatsu

1Tsukuba Research Laboratories, Eisai Co., Ltd., 1-3, Tokodai 5-chome, Tsukuba, Ibaraki 300-26, Japan
2The 2nd Department of Internal Medicine, Surugadai Nihon University Hospital, Kandasurugadai, Chiyoda-ku, Tokyo 101, Japan

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ABSTRACT—The thrombolytic activity of a novel modified tissue-type plasminogen activator (t-PA) (E6010) was examined in a canine model with copper coil-induced femoral artery thrombus. This model, in which thrombolytic activity can be easily and directly quantified by determining changes in thrombus weight, should be useful for comparing the activities of various thrombolytic agents. Using this model, the present study showed that the thrombolytic activity of bolus intravenous injection of E6010 was identical to that of continuous intravenous infusion of recombinant t-PA at the same dose. This thrombolytic activity can be explained by changes in blood concentrations of the administered thrombolytic agents. On the other hand, administration of the thrombolytic agents dose-dependently caused significant changes in the levels of hemostatic and fibrinolytic factors. These changes were not so marked with administration of E6010, and therefore we concluded that E6010 is unlikely to cause bleeding complications after administration.

Keywords: E6010 (novel modified t-PA), Plasminogen activator (tissue-type), Femoral artery thrombus model, Thrombolytic activity, Plasma concentration

Recently, coronary artery angiography has been commonly used and safely performed during early stages of acute myocardial infarction. As a result, coronary thrombi have been found in many patients with acute myocardial infarction, suggesting its involvement in the pathogenesis of myocardial infarction (1). In 1979, Rentrop et al. succeeded in lysing a coronary thrombus by direct intracoronary infusion of streptokinase (2). In Japan, percutaneous transluminal coronary recanalization (PTCR) with urokinase has been commonly performed (3). These drugs can lyse fibrin that is a component of thrombi by converting plasminogen into the active form, plasmin, in the circulating blood. The resulting plasmin lyses not only fibrin but also fibrinogen. The effect of plasmin is regulated by α2-plasmin inhibitor, which binds to plasmin to inactivate it. The increase in plasmin caused by these drugs results in the subsequent decrease in blood concentrations of fibrinogen and α2-plasmin inhibitor, frequently leading to bleeding complications. Therefore, it is necessary to develop drugs that have a high affinity with thrombi so that the production of plasmin will be localized near the thrombi.

Compared to intracoronary administration, intravenous administration has been found to produce more rapid recanalization of the coronary artery by thrombolytic therapy. A large dose of urokinase must be administered for thrombolysis, with a high risk of bleeding complications. In 1982, tissue-type plasminogen activator (t-PA), which has a high affinity to thrombi, was isolated and purified from human melanoma cells (4). The genetic recombinant technology has enabled the large-scale production of t-PA (5). T-PA has been used as an effective thrombolytic agent against acute myocardial infarction (6–8) and other diseases (9). However, pharmacokinetic studies revealed that t-PA is rapidly taken up by the liver after administration, with an elimination time of a few minutes (10, 11). Accordingly, continuous intravenous infusion has been adopted to maintain an adequate blood concentration of t-PA for efficient thrombolysis. Subsequently, various types of modified t-PA were constructed by recombinant DNA techniques (12–15), and these were reported to show a prolonged half-life in rats and rabbits (16–19).

We developed a modified t-PA (E6010) and demonstrated that it had a prolonged half-life in rats and rabbits (20, 21). Furthermore, we found that E6010 had more potent...
activity and was accompanied by fewer side effects than natural t-PA in a canine model with copper coil-induced coronary artery thrombus (22). In this model, animals showed symptoms similar to those of acute myocardial infarction because of thrombus formation in the coronary artery. However, quantification of thrombolytic activity was impossible because the time to reperfusion was determined as the thrombolytic activity.

In the present study, we attempted to determine the thrombus weight in a canine model with copper coil-induced femoral artery thrombus. We also evaluated the action of a thrombolytic agent by determining changes in thrombus weight.

MATERIALS AND METHODS

Recombinant t-PA (rt-PA) and E6010

The c-DNA of t-PA was isolated from the melanoma cell line as previous reported (4). E6010 was prepared by modifying only a single amino acid in the epidermal growth factor domain of t-PA (Cys84-Ser84) by site-directed mutagenesis. Using genetic recombinant technology, t-PA and E6010 were expressed in baby Syrian hamster kidney cells (BHK cells) as the host cell and then purified from conditioned medium by affinity chromatography through monoclonal anti-t-PA antibody-coupled gel followed by gel filtration. The molecular weights of these recombinant products (rt-PA and E6010) were shown to be about 70 kilodaltons (kDa) by SDS-PAGE. The specific activities of rt-PA and E6010 were 500,000 IU/mg (lot K702301) and 150,000 IU/mg (lot K810800), respectively (22). The test substances were dissolved in solutions containing 3% arginine aspartate salt and 5% mannitol, pH 5.0.

E6010 shows CNBr-digested fibrinogen-dependent plasminogen activation and fibrin binding ability that is slightly weaker than those of rt-PA (23). In rats, the half-lives of E6010 were t1/2(α)=2.3 and t1/2(β)=153 min, whereas the half-lives of rt-PA were t1/2(α)=1.6 and t1/2(β)=32 min (24).

Induction of thrombus

The canine model with a copper coil-induced femoral artery thrombus that was developed by Bush et al. (25) and Cercek et al. (26) was modified and used to examine the efficacy of the thrombolytic agents. Mongrel dogs (body weight: 9–15 kg) were anesthetized with sodium pentobarbital and artificially ventilated. The right carotid artery was carefully exposed, and cannulation was performed with a Tygon tube to monitor the blood pressure. On the left brachial vein and right femoral veins, cannulation was also performed to facilitate administration of rt-PA or E6010 (left branchial vein) and as sampling routes for blood (right femoral vein). The right and left femoral arteries were carefully exposed and covered with gauze moistened with saline to prevent dryness. All branches belonging to the femoral artery were ligated, but one main artery was left for blood flow. Copper coils for the induction of thrombus were made by coiling a 24-gauge wire to fit into the inside diameter of the main femoral artery (about 1 cm in length). The coils were weighed (A) and then inserted into both femoral arteries from the distal end. Blood flow ceased a few minutes after insertion, indicating the occlusion of the artery. Bush et al. (25) reported the time to occlusion after the insertion of the coil to be 12±1 min. Therefore, we left the coils for 1 hr to allow sufficient thrombus formation. The copper coils were removed from both femoral arteries and weighed (B). The difference between the coil weights before and after insertion (B−A) represents the weight of the first thrombus formed. The coils retaining the thrombus were carefully reinserted into their identical original sites.

Administration of rt-PA and E6010

Immediately after insertion of the coil, 300 U/kg of heparin was injected via the left brachial vein. Then, continuous intravenous infusion of rt-PA over 30 min or bolus intravenous injection of rt-PA or E6010 was performed from the same site as heparin injection. Blood samples were collected serially before and after administration of rt-PA or E6010 from the right femoral vein, and their blood concentrations and the effect on hemostatic and fibrinolytic factors were evaluated. Figure 1 shows the protocol of this experiment. Thirty minutes after the start of thrombolytic agent administration, the copper coils were removed and weighed (C) to determine the weight of the remaining thrombus. The activities of the thrombolytic agents were calculated as the thrombolytic rate according to the following formula:

\[
\text{Thrombolytic rate (\%)} = \frac{(B-C)}{(B-A)} \times 100
\]

Determination

Blood was collected by a syringe with 1/10 volume of 3.8% sodium citrate and then centrifuged. The plasma was preserved at −80°C until use. The blood concentrations of rt-PA or E6010 and plasma levels of plasminogen, fibrinogen and α2-plasmin inhibitor were determined.

The blood concentrations of rt-PA and E6010 were measured by ELISA with a polyclonal antibody specific for t-PA (26, 27). The plasma fibrinogen level was determined by measuring thrombin time with the Fibrinogen B-test (Wako Pure Chemical Co., Ltd., Osaka). Plasma plasminogen and α2-plasmin inhibitor levels were determined with a Testzyme PLG kit and Testzyme APL kit (Daiichi Pure Chemical Co., Ltd., Tokyo). In these
experiments, we used mongrel dogs, so the plasma levels in these parameters differed among individual animals. Therefore, pre-collected plasma from each mongrel dog that was sampled before the administration of heparin served as the control. The results are shown as percentages of each control value.

**Statistical methods**

The thrombolytic data were compared by Tukey's multiple comparison test, and the percentage data of plasma levels of hemostatic and fibrinolytic factors were compared by Fisher's test. The weights of thrombi were compared by the Kruskal-Wallis test.

**RESULTS**

In a previous study on the efficacy of E6010 in a canine model with copper coil-induced coronary artery thrombus, the thrombus, formed with the coil for 1 hr, showed network-like fibrin growth, which included erythrocytes and other blood components (22). The thrombus in the present study was considered to have a similar constitution.

Table 1 shows the average weights of thrombi formed after 1 hr in the femoral arteries of two legs. The weights of thrombi in the inserted coils ranged from 22.5 to 38.8 mg.

<table>
<thead>
<tr>
<th>Sample</th>
<th>dose (mg/kg)</th>
<th>n</th>
<th>Thrombus weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA infusion</td>
<td>0.1</td>
<td>6</td>
<td>22.5±2.5</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>8</td>
<td>30.7±11.8</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10</td>
<td>38.8±7.7</td>
</tr>
<tr>
<td>rt-PA bolus</td>
<td>0.2</td>
<td>8</td>
<td>23.4±4.6</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>7</td>
<td>33.7±10.3</td>
</tr>
<tr>
<td>E6010 bolus</td>
<td>0.1</td>
<td>10</td>
<td>31.7±9.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>12</td>
<td>29.2±8.8</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>12</td>
<td>30.4±32.7</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>11</td>
<td>32.9±13.2</td>
</tr>
</tbody>
</table>

Values are mean±S.D. n represents the number of coils.

![Fig. 1. Protocol of the canine model.](image)

![Fig. 2. Thrombolytic activities of E6010 and rt-PA. Each point represents the mean±S.E.M. ■: placebo, n=11; △: rt-PA (continuous infusion), n=6–10; ○: rt-PA (bolus injection), n=7–8; ●: E6010 (bolus injection), n=10–12.](image)
mg and were not significantly different among the groups.

Figure 2 shows the thrombolytic activities of E6010 and rt-PA. In the control group, treated with a placebo, the thrombolytic rate was 6.7%. After 30 min of continuous intravenous infusion of rt-PA, the thrombolytic rate was 21.5% in the 0.1 mg/kg group, 48.1% in the 0.2 mg/kg group and 54.9% in the 0.4 mg/kg group. However, after bolus intravenous injection, the thrombolytic rate was only 24% in both the 0.2 mg/kg and 0.4 mg/kg groups, which was not significantly different from that in the control group.

After bolus intravenous injection of E6010, the thrombolytic rate was 14.1% in the 0.1 mg/kg group, 52.1% in the 0.2 mg/kg group and 65.4% in the 0.4 mg/kg group. The 0.2 mg/kg group of E6010 showed the same thrombolytic effects as the 0.2 mg/kg infusion group of rt-PA. On the other hand, the 0.4 mg/kg group of E6010 exhibited a higher effect than the 0.4 mg/kg infusion group of rt-PA.

Next, we compared the plasma concentrations of E6010 and rt-PA in this model (Fig. 3). E6010 had a much longer half-life than rt-PA, which explained the observed differences between their activities caused by differences in respective plasma concentrations. The prolonged half-life of E6010 in plasma agreed well with that reported previously (20–22, 24).

In general, plasmin activators that are non-specific for thrombi, such as urokinase, reduce the plasma concentrations of hemostatic and fibrinolytic factors, resulting in
the development of bleeding complications. To evaluate the safety of E6010, we examined the changes in the concentrations of hemostatic and fibrinolytic factors (fibrinogen, plasminogen and α2-plasmin inhibitor) after administration of E6010 and rt-PA. In the group given rt-PA by bolus injection and the groups given rt-PA by continuous infusion, no significant changes were observed in the plasma fibrinogen levels (Fig. 4). On the other hand, at 40 and 60 min, the levels were significantly lower in the 0.4 mg/kg E6010 group than in the 0.2 mg/kg E6010 group. However, at 120 min, there were no significant differences among the three dose groups. As shown in Fig. 5, significant decreases in the plasma plasminogen levels were observed after continuous infusion of t-PA for 120 min in the 0.4 mg/kg dose group and at 30, 40 and 120 min in the 0.4 mg/kg E6010 groups.

On the other hand, the α2-plasmin inhibitor levels in the blood showed greater changes than fibrinogen and plasminogen (Fig. 6). Especially, in groups receiving rt-PA (continuous infusion) and E6010 (bolus injection) at a dose of 0.4 mg/kg, blood α2-plasmin inhibitor levels were significantly reduced compared with those in the 0.1 and 0.2 mg/kg dose groups.

However, these three plasma parameters were all significantly reduced in the highest dose groups of rt-PA and E6010. Therefore, the risk of bleeding complications was expected to be low with the administration of either E6010 or rt-PA.

![Fig. 5](image1.png) **Fig. 5.** Effects of rt-PA (A: bolus injection and B: continuous infusion) and E6010 (C: bolus injection) on the plasma plasminogen level. Each point represents the mean ± S.E.M. (⁎P < 0.05), n = 3–6. □: 0.1 mg/kg, ○●: 0.2 mg/kg, △▲: 0.4 mg/kg.

![Fig. 6](image2.png) **Fig. 6.** Effects of rt-PA (A: bolus injection and B: continuous infusion) and E6010 (C: bolus injection) on the plasma α2-plasmin inhibitor level. Each point represents the mean ± S.E.M. (⁎P < 0.05), n = 3–6. □: 0.1 mg/kg, ○●: 0.2 mg/kg, △▲: 0.4 mg/kg.
DISCUSSION

Bush et al. studied the efficacy of thrombolytic agents in a canine model with copper coil-induced femoral artery thrombus (25). They inserted a copper coil by passing a guiding wire from the carotid artery through the femoral artery, to which a blood flow meter was attached. After blood flow ceased, thrombolytic agents were administered, and time to reperfusion was determined to evaluate the efficacy of the thrombolytic agents. This technique requires special skill and angiography to insert the coil. In addition, direct quantification of thrombolytic rate is not possible with the blood flow meter.

Badylak et al. developed a method using canine femoral artery for evaluating thrombolytic agents (28). In this method, the femoral artery was ligated and injured with hot saline, where a thrombus was formed by injection of labeled fibrinogen and thrombin. After treatment with thrombolytic agents, the increase in blood radioactivity was determined as thrombolytic activity.

In recent years, new methods using animal models with copper coil-induced coronary artery thrombi have been developed to evaluate the thrombolytic efficacy of various agents (12, 23, 29). These methods include the insertion of a coil into the coronary artery and confirmation of thrombolysis by coronary angiography after thrombus formation. Activity was presented as time to reperfusion. Since acute myocardial infarction develops in the coronary artery, the model with the thrombus formed in the coronary artery is close to the clinical condition of acute myocardial infarction and is thus useful for the evaluation of thrombolytic agents.

We reported previously the thrombolytic efficacy of E6010 in this model (22). The time to reperfusion (TR) of E6010 with a single bolus intravenous injection at a dose of 0.2 mg/kg was 30 ± 15.3 min, which was the same as the TR with continuous infusion of rt-PA at a dose of 0.3 – 0.6 mg/kg. These findings agreed with the thrombolytic activity of E6010 and rt-PA in the femoral model in the present study. However, it is difficult to quantify thrombolytic activity with this model. In the present study, we quantified the thrombolytic activity of rt-PA and E6010 by determining changes in the weight of the thrombus formed with a copper coil inserted into the femoral artery. The rt-PA in this experimental model showed high thrombolytic activity after continuous intravenous infusion, but low activity after bolus intravenous injection. This implied that this model is useful for evaluating the efficacies of thrombolytic agents. We found that a bolus intravenous injection of E6010 has the same thrombolytic activity as continuous intravenous infusion of rt-PA. This can be explained by changes in their blood concentrations. Because rt-PA has a short half-life, only its continuous intravenous infusion is employed for the treatment of acute myocardial infarction in clinical practice. E6010 showed a sufficient effect after a bolus intravenous injection, which is a very useful property for emergency use.

Neither rt-PA nor E6010 substantially affected hemostatic or fibrinolytic factors. Especially, bolus intravenous injection of E6010 caused no abnormal decrease in the levels of hemostatic or fibrinolytic factors, although the initial dose of E6010 was higher than that of rt-PA, suggesting that E6010 is highly safe and does not cause bleeding complications. Thus, E6010 appears to be a novel thrombolytic agent that had sufficient therapeutic effects even after bolus injection in the treatment of acute myocardial infarction.

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