Thrombolytic Effects of a Novel Modified Tissue Plasminogen Activator, E6010, on Coronary Thrombosis in the Pig

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ABSTRACT — This study was conducted to compare the thrombolytic effect of a novel modified tissue plasminogen activator, E6010, with that of recombinant human tissue plasminogen activator (t-PA), administered by single intravenous bolus injection in pigs with occlusive coronary thrombosis. Thrombosis was induced by electrical stimulation of the intimal surface of the left circumflex coronary artery. Coronary blood flow velocity and hemodynamic parameters were observed for 1 hr after complete cessation of coronary flow. Ten minutes after heparin injection (300 U/kg), E6010, t-PA or placebo was intravenously administered as a bolus. E6010 at 0.2 and 0.4 mg/kg caused recanalization of the occluded coronary artery in 1 of 6 and 5 of 5 pigs, respectively. The time to recanalization after 0.4 mg/kg of E6010 was 22 ± 11 min (mean ± S.E.M.). t-PA (0.4 mg/kg) caused recanalization in only 1 of 5 pigs. Recanalization did not occur in any of the 6 animals administered placebo. Plasma clearance of E6010 was smaller than that of t-PA (4.9 ± 0.3 vs. 9.4 ± 3.8 ml/min/kg). There were no significant differences in plasma levels of fibrinogen, a2-plasmin inhibitor and plasminogen among the placebo, E6010 and t-PA groups. These results suggest that the slower clearance of E6010 from plasma contributes to the effective thrombolytic action of E6010 following single intravenous bolus injection.

A number of clinical studies have confirmed the effectiveness of recombinant tissue plasminogen activator (t-PA) as a thrombolytic agent in the treatment of acute myocardial infarction (1–3). One disadvantage associated with t-PA is its very rapid elimination from the systemic circulation by clearance in the liver, resulting in an initial plasma half-life in the order of a few minutes (4, 5). This rapid clearance of t-PA necessitates intravenous or intracoronary infusion of a relatively high dose in order to maintain therapeutic plasma levels. The rapid decrease in plasma level of t-PA after the termination of infusion may result in coronary reocclusion (6), and overdose of t-PA, which may occur with continuous infusion, may increase the risk of hemorrhagic complications (7).

t-PA derivatives with slower clearance from plasma would be effective by intravenous bolus injection and provide advantages over conventional t-PA in the treatment of acute myocardial infarction and other thrombotic diseases. E6010 is a novel modified human t-PA, in which cysteine 84 in the epidermal growth factor domain is replaced by serine (8). A slower rate of clearance of E6010 from plasma compared to that of t-PA has been dem-
onstrated in dogs (9) and rats (10, 11). In addition, E6010 has been shown to have a more potent and longer thrombolytic action than t-PA in a canine model with copper coil-induced coronary artery thrombosis (9) and in a rat arterio-venous shunt model (11).

The present study was conducted to compare the thrombolytic effects of E6010 with those of native t-PA, in an experimental model of occlusive coronary thrombosis in pigs. A single intravenous bolus injection was used to examine the pharmacological and pharmacokinetic properties of both drugs under the same experimental condition. A model of sustained occlusive coronary thrombosis that resembles the pathology of acute myocardial infarction was produced by applying an anodal current to the arterial lumen of the left circumflex coronary artery (LCX) (12).

MATERIALS AND METHODS

Surgical procedures and instrumentation

Thirty farm pigs of both sexes, weighing 16.1 ± 0.5 kg (mean ± S.E.M.), were used after an overnight fast. They were anesthetized with 15 mg/kg of sodium pentobarbital. Each animal was artificially ventilated, and anesthesia was maintained with inhalation of a mixture of O₂ and N₂O (1:2) to which 0.5–1% halothane was added. The monitoring apparatus was attached to the animals as described previously (12, 13). The external jugular vein was cannulated with a polyethylene catheter for infusion of saline and blood sampling. A microtip pressure catheter (MPC-500, Millar Instruments, Houston, TX, U.S.A.) was positioned in the aortic arch to monitor aortic pressure.

A left thoracotomy was performed and a microtip pressure catheter was inserted into the left ventricular cavity through the apex for measurement of left ventricular pressure (LVP). The first derivative of LVP (LV dP/dt) was obtained by differentiation of LVP. Heart rate was monitored with a cardiотachometer (AT-600G, Nihon Kohden).

A Doppler flow probe was placed around the LCX (approximately 1 cm distal to the origin of the obtuse marginal branch) to measure coronary flow velocity (CFV). A 25 gauge silver wire electrode for induction of coronary thrombosis was inserted into the arterial lumen proximal to the flow probe (12). The tip (2–3 mm) of this electrode was in contact with the vessel intima. This electrode served as the anode and the cathodal electrode was sutured to the chest wall. The surface electrocardiogram (ECG) was recorded with a wire electrode sutured to the epicardium in the area supplied by the LCX.

Experimental protocol

The pigs were allowed to stabilize for 60 min, and hemodynamic parameters were continuously recorded thereafter. An anodal current of 300 μA from a battery (Model LS-500B, Physio-Tech, Tokyo, Japan) was then delivered to the intimal surface of the LCX through the anodal electrode. The electrical current was stopped 30 min after the mean CFV decreased to zero. One hour after the complete cessation of coronary flow, 300 U/kg of heparin sodium (Green Cross, Osaka, Japan) was injected into the dorsal ear vein. Ten minutes after the administration of heparin, E6010 (0.2 or 0.4 mg/kg), t-PA (0.4 mg/kg) or vehicle as the placebo (0.9% saline solution containing 3% arginine-aspartic acid and 5% mannitol) was intravenously injected as a bolus.

The animals in which sustained total coronary occlusion occurred were divided into four groups as follows: the control group (n = 6) which received a placebo, a group administered 0.2 mg/kg of E6010 (n = 6), a group administered 0.4 mg/kg of E6010 (n = 5), and a group that received 0.4 mg/kg of t-PA (n = 5). CFV and hemodynamic parameters were measured for 2 hr after the administration of the drugs. Recanalization was defined as the recovery of CFV equal to or greater than the baseline value prior to electrical stimulation.

Plasma levels of E6010, t-PA and hemostatic parameters
To determine the plasma levels of E6010, t-PA, fibrinogen, $\alpha_2$-plasmin inhibitor and plasminogen, blood samples were collected using a syringe containing sodium citrate solution before and at 5, 10, 30, 60, 90 and 120 min after the administration of E6010, t-PA or placebo. The blood samples were chilled immediately on ice and centrifuged at 4°C to separate the plasma which was stored at -20°C until use. Special care was taken to avoid prolonged blood processing and repeated freezing and thawing so that no artifactual changes in hemostatic parameters occurred (14). The plasma levels of E6010 and t-PA were determined by a plasminogen activator-specific enzyme-linked immunosorbent assay (9, 15). The plasma level of fibrinogen was assessed by measuring the thrombin time using a Fibrinogen B-Test® kit (Wako Pure Chemical Industries, Osaka, Japan). The plasma levels of $\alpha_2$-plasmin inhibitor and plasminogen were determined using a Testzym APL® kit (Daiichi Pure Chemicals, Tokyo, Japan) and a Testzym PLGC® kit (Daiichi Pure Chemicals), respectively. Pharmacokinetic parameters were calculated by fitting data to a two-compartment model using the method of residuals (16) and the method of statistical moments analysis (17).

LCX microscopy

At the end of the experiment, pigs in the control group with complete coronary occlusion were killed by intravenous administration of a saturated solution of potassium chloride, and the LCX was examined by light microscopy. The heart was excised and the LCX at the site of the electrode insertion was dissected free from the surrounding connective tissue. The tissue samples were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin.

Drugs

E6010 is a mutant of t-PA with modification of the epidermal growth factor (Cys84→Ser84) by site-directed mutagenesis (8). E6010 and native type recombinant t-PA were expressed in Syrian hamster kidney cells and purified from a conditioned medium by affinity chromatography on monoclonal anti-t-PA antibody-coupled Sepharose and then by gel chromatography. Purified E6010 was treated with plasmin-Sepharose according to the method of Wallén et al (18). E6010 consisted of a two-chain form, and its specific activity was 150,000 IU/mg protein (purity 100%). t-PA consisted of a 2:3 mixture of one-chain and two-chain peptides with a specific activity of 500,000 IU/mg protein (purity 100%). E6010 has the ability to activate CNBr-digested fibrinogen-dependent plasminogen. Its binding property to fibrin is slightly weaker than that of t-PA. Both drugs were dissolved in vehicle to form solutions of 2 mg/ml. The solutions of E6010 and t-PA were prepared freshly before each experiment. Drugs were injected over a 15-sec period.

Statistical analysis

Data were expressed as means ± S.E.M. Comparison of hemodynamic, hemostatic and pharmacokinetic parameters among groups or before and after drug administration was performed using one-way analysis of variance. When a significant difference was detected by analysis of variance, the data were further analyzed by Duncan's multiple range test. Fisher's exact test was used to compare the recanalization rate in the four groups. Differences with P values less than 0.05 were considered to be statistically significant.

RESULTS

Formation of coronary thrombus

Sustained coronary occlusion was observed in 22 of 30 pigs subjected to electrical stimulation of the intimal surface of the LCX. The fall in CFV to zero occurred at 26 ± 4 min after the start of the electrical stimulation. Among the remaining 8 pigs, sudden death occurred in 4 animals, and unsustained occlusion with either fluctuating coronary flow or no change in CFV occurred in the other 4 animals. These animals were excluded from
the experiment.

In each control pig with complete cessation of CFV, the LCX was found to contain an occlusive thrombus. Light microscopy of the region of the LCX where the electrode had been inserted showed severe endothelial damage. The thrombus was a mixture of aggregated platelets, erythrocytes, leukocytes and fibrin (Fig. 1A and B).

**Effects of E6010 and t-PA**

The 22 pigs with sustained coronary occlusion were divided into four groups. There were no significant differences among the groups with respect to time from the start of electrical stimulation to the complete cessation of coronary flow (Table 1).

A representative tracing of the fall in CFV to zero after electrical stimulation and the effect of an intravenous bolus injection of 0.4 mg/kg of E6010 is shown in Fig. 2. A gradual decline in CFV was accompanied by a marked elevation of the ST segment in the epicardial ECG, indicating the presence of ischemia in the myocardium supplied by the LCX. A progressive increase in coronary flow was observed approximately 10 min after injection of E6010, and recovery to the level prior to electrical stimulation was observed within 1 hr after the injection. There was no change in aortic pressure, LV dP/dt\textsubscript{max} or heart rate. The elevation of the ST segment in the surface ECG disappeared after the recovery of CFV.

The effects of E6010 and t-PA on coronary thrombosis are summarized in Table 1. Placebo administration did not result in recanalization in any case. Administration of both E6010 and t-PA resulted in recanalization of the occluded LCX. The recanalization rates in the groups administered E6010 at 0.2 and 0.4 mg/kg and t-PA at 0.4 mg/kg were 17%, 100% and 20%, respectively. The recanalization rate after the administration of E6010 at 0.4 mg/kg was statistically significantly greater than the placebo value (P < 0.01). The thrombolytic effect of E6010 was greater than that of t-PA. The mean time from the injection of 0.4 mg/kg of E6010 to recanalization was 22 ± 11 min (n = 5). Reoclusion occurred in only 1 of the 5 pigs.

**Hemodynamic parameters**

Mean aortic pressure and LV dP/dt\textsubscript{max} did not change significantly throughout the experiment in any of the groups (Fig. 3). Heart rate tended to increase in all groups after electrical stimulation. There were no significant differences in heart rate among the E6010, t-PA and placebo groups after administration.

**Table 1.** Coronary thrombolysis by E6010 and t-PA

| Drug (mg/kg) | Time to occlusion (min) | Recanalization incidence | Recanalization time (min) |
|--------------|-------------------------|--------------------------|---------------------------|
| Placebo      | 39 ± 7                  | 0/6 (0%)                 |                           |
| E6010 0.2    | 20 ± 5                  | 1/6 (17%)                | 10                        |
| E6010 0.4    | 21 ± 7                  | 5/5 (100%)*              | 22 ± 11                   |
| t-PA 0.4     | 24 ± 9                  | 1/5 (20%)                | 8                         |

Values are expressed as means ± S.E.M. *P < 0.01, significant difference from placebo (Fisher's exact test).

**Fig. 1.** Photomicrographs of a section of coronary artery with complete occlusive thrombus. A: This coronary artery was taken from a pig that received the vehicle. The intact internal elastic lamina is indicated by arrows. B: Higher magnification of occlusive thrombus. The arterial wall shows multiple foci of endothelial denudation (arrows). The thrombus is composed of aggregated platelets, erythrocytes, leukocytes and fibrin. Adv, adventitia; Med, media; Thr, thrombus. Hematoxylin and eosin staining.
Fig. 2. A representative tracing showing cessation of coronary flow following thrombus formation and its recovery following intravenous bolus injection of E6010. Prior to electrical stimulation, the electrocardiogram (ECG), coronary blood flow velocity (CFV), aortic pressure (AoP), left ventricular pressure (LVP), the first derivative of LVP (LV dP/dt), and heart rate (HR) were stable. Following electrical stimulation, elevation of the ST segment and a decrease in CFV were observed. E6010 at 0.4 mg/kg was intravenously injected (solid circle) 70 min after CFV had decreased to zero. CFV began to steadily increase from approximately 10 min after the injection of E6010 and recovered to the pre-stimulation level within 1 hr after the injection. No reocclusion of the coronary artery was observed during the observation period. The time scale represents 1 min or 1 sec.

Pharmacokinetic parameters
The plasma levels of E6010 and t-PA following single intravenous bolus administration are shown in Fig. 4. The clearance rate of both drugs from plasma could be described by the sum of two exponential terms by a graphical curve. Pharmacokinetic parameters related to the clearance of E6010 and t-PA are summarized in Table 2. The plasma half-lives and plasma clearances of E6010 and t-PA indicate that the clearance rate of E6010 is approximately half that of t-PA.

Hemostatic parameters
Hemostatic parameters before and after administration of E6010 at 0.2 and 0.4 mg/kg, t-PA at 0.4 mg/kg, and placebo are summarized in Table 3. There were no significant differences in plasma levels of fibrinogen, $\alpha_2$-plasmin inhibitor and plasminogen among the four groups. No significant changes could be detected in any of the hemostatic parameters before and after administration of drugs.

DISCUSSION
Various in vivo models for coronary artery thrombosis in dogs have been reported: insertion of a copper coil into the artery (19, 20), stenosis by a plastic constrictor (21) and thrombin infusion into the coronary artery (22). Romson et al. (23) first reported a mod-
el of coronary artery thrombosis in conscious dogs induced by continuous electrical stimulation via an electrode placed in the lumen of a coronary artery. Benedict et al. (24) demonstrated that an occlusive coronary thrombus could occur spontaneously after the discontinuation of electrical stimulation in anesthetized open-chest dogs. Although canine models of coronary thrombosis have been widely used for the evaluation of thrombolytic agents (25–27), there have been few reports in pigs. We have recently reported a pig model of coronary thrombosis with spontaneous cyclic coronary flow variations induced by electrical stimulation of the intima of the coronary artery (12). In the present study, we employed a higher level of electrical stimulation (300 μA instead of 150 μA as used in the previous study), which resulted in formation of a sustained occlusive thrombus. The electrical current was discontinued 30 min after CFV fell to zero, in order to eliminate the influence of

**Fig. 3.** Time course of changes in mean aortic pressure (mAoP), maximal rate of rise in left ventricular pressure (LV dp/dt\textsuperscript{max}) and heart rate (HR) after single intravenous bolus injection of placebo (solid circles, n = 6), E6010 at 0.4 mg/kg (solid triangles, n = 5) or t-PA at 0.4 mg/kg (open circles, n = 5). Data are expressed as means ± S.E.M. Hemodynamics were measured before the start of electrical stimulation (Control) and before heparin administration (Hep). Placebo and drugs were injected intravenously at 0 time. There were no significant differences among the treatment groups with regard to any of these hemodynamic variables.

**Fig. 4.** Time course of plasma levels after single intravenous bolus injection of E6010 at 0.4 mg/kg (solid circles, n = 5) and 0.2 mg/kg (solid triangles, n = 4), and t-PA at 0.4 mg/kg (open circles, n = 5). Data are expressed as means ± S.E.M.
Table 2. Pharmacokinetic parameters for E6010 and t-PA

| Drug  | N  | A      | B      | t1/2α  | t1/2β  | AUC    | Clp    | Vc     |
|-------|----|--------|--------|--------|--------|--------|--------|--------|
|       | (mg/kg) | (μg/ml) | (μg/ml) | (min)  | (min)  | (μg·min/ml) | (ml/min/kg) | (ml/kg) |
| E6010 | 0.2 | 4      | 1.39 ± 0.09 | 0.50 ± 0.12 | 5.2 ± 1.0 | 56.2 ± 9.6* | 46.4 ± 2.5 | 4.3 ± 0.2 | 108 ± 9 |
|       | 0.4 | 5      | 6.45 ± 2.78 | 1.28 ± 0.32 | 3.4 ± 1.6 | 45.8 ± 11.6 | 83.5 ± 4.1 | 4.9 ± 0.3 | 84 ± 26 |
| t-PA  | 0.4 | 5      | 91.6 ± 57.5 | 0.55 ± 0.21 | 2.2 ± 0.8 | 19.1 ± 3.7 | 109.0 ± 49.5 | 9.4 ± 3.8 | 54 ± 32 |

Values are expressed as means ± S.E.M. A, B, α, and β were calculated by nonlinear least squares fitting of the plasma clearance of t-PA-related antigen to biexponential elimination models: C(t) = A exp (-αt) + B exp (-βt). The half-lives α and β were calculated by dividing ln 2 by α or β. N, number of experiments; AUC, area under the plasma concentration-time curve; Clp, clearance of t-PA-related antigen from plasma; Vc, volume of central compartment; C, plasma levels of drugs. *P < 0.01, significant difference from t-PA.

Table 3. Hemostatic parameters before and after intravenous administrations of E6010 and t-PA

| Parameter       | Drug     | N  | Time after administration (min) |
|-----------------|----------|----|---------------------------------|
|                 |          | (mg/kg) | 0 | 5 | 10 | 30 | 60 | 90 | 120 |
| Fibrinogen      | Placebo  | 4  | 100 | 103 ± 2 | 100 ± 3 | 115 ± 13 | 111 ± 3 | 112 ± 4 | 122 ± 4 |
|                 | E6010    | 0.2| 4  | 100 | 100 ± 6 | 100 ± 4 | 105 ± 6 | 109 ± 7 | 112 ± 7 | 118 ± 9 |
|                 |          | 0.4| 5  | 100 | 99 ± 3  | 119 ± 11| 109 ± 7 | 112 ± 8 | 114 ± 8 | 122 ± 14|
|                 | t-PA     | 0.4| 5  | 100 | 102 ± 1 | 106 ± 4 | 101 ± 3 | 103 ± 4 | 110 ± 4 | 122 ± 6 |
| α2-Plasmin inhibitor | Placebo  | 4  | 100 | 104 ± 2 | 100 ± 3 | 103 ± 3 | 102 ± 2 | 103 ± 2 | 106 ± 3 |
|                 | E6010    | 0.2| 4  | 100 | 83 ± 4  | 88 ± 3  | 89 ± 4  | 94 ± 2  | 94 ± 3  | 98 ± 3  |
|                 |          | 0.4| 5  | 100 | 84 ± 9  | 94 ± 5  | 96 ± 6  | 95 ± 5  | 90 ± 8  | 91 ± 3  |
|                 | t-PA     | 0.4| 5  | 100 | 90 ± 2  | 95 ± 2  | 99 ± 2  | 97 ± 3  | 98 ± 5  | 101 ± 2 |
| Plasminogen     | Placebo  | 4  | 100 | 97 ± 2 | 104 ± 6 | 106 ± 5 | 114 ± 16| 113 ± 13| 109 ± 11|
|                 | E6010    | 0.2| 4  | 100 | 95 ± 2  | 93 ± 4  | 90 ± 6  | 99 ± 4  | 98 ± 4  | 104 ± 7  |
|                 |          | 0.4| 5  | 100 | 97 ± 3  | 101 ± 4 | 101 ± 3 | 97 ± 4  | 102 ± 13| 106 ± 15|
|                 | t-PA     | 0.4| 5  | 100 | 95 ± 5  | 100 ± 4 | 96 ± 2  | 98 ± 4  | 113 ± 11| 125 ± 14|

Levels prior to administration are defined as 100%. Values are expressed as means ± S.E.M. N, number of experiments. There were no significant differences among the treatment groups with regard to any of these hemostatic parameters.
continuous electrical stimulation. The formation of an occlusive mixed thrombus consisting of aggregated platelets, erythrocytes, leukocytes and fibrin was confirmed at the postmortem examination (Fig. 1).

Thrombus is generally considered to form by continued platelet activation and fibrin accretion at a site of endothelium-injured blood vessels. Clot lysis and recanalization of the occluded coronary artery induced in vivo by t-PA can be accelerated by adjunctive therapy with antiplatelet agents such as aspirin or antithrombin agents such as heparin and hirudin (28, 29). It has been reported that heparin enhances thrombolysis and maintains coronary artery-patency after the administration of t-PA (6, 30). As the balance between fibrin deposition and lysis is a key factor for thrombosis, we administered heparin to prevent the formation of fibrin and its incorporation into the thrombus during thrombolysis by plasminogen activators.

Recently, several t-PA variants have been developed to overcome potential limitations of native t-PA such as: (I) a short biologic half-life, (II) relative lack of fibrin specificity when used at higher doses, and (III) inactivation due to interaction with plasma inhibitors (31). Two t-PA variants lacking the finger-like and growth factor-like domains (26) and LY210825 (27), which possess longer plasma half lives than native t-PA, were shown to produce rapid recanalization of a coronary thrombus and prevent reocclusion in a canine model of coronary artery thrombosis.

E6010 is a new modified t-PA with longer half-life of clearance from plasma than conventional recombinant human t-PA. The binding property of E6010 to fibrin is slightly weaker than that of t-PA (8) and the binding properties of E6010 to endogenous inhibitors of t-PA and other plasma inhibitors are almost the same as those of t-PA (9). E6010 does not inhibit human platelet aggregation induced by ADP or collagen in vitro (M. Kogushi et al., unpublished data). In this study, we have demonstrated that an intravenous bolus injection of E6010 exhibits an effective thrombolytic action in pigs with occlusive coronary thrombosis. The thrombolytic effect of E6010 was not associated with any hemodynamic changes or systemic activation of the fibrinolytic system, as demonstrated by the constant plasma levels of fibrinogen, $\alpha_2$-plasmin inhibitor and plasminogen. These results indicate that E6010 can induce thrombolysis without affecting systemic circulation or systemic consumption of coagulation factors which leads to hypofibrinogenemia and a hemorrhagic tendency.

In this study, the thrombolytic activity of E6010 was more potent than that of native t-PA, although the specific activity of E6010 is less than that of t-PA (150,000 vs. 500,000 IU/mg protein) (9). The rate of elimination of E6010 from plasma, which is approximately half of that of t-PA, is considered to be a factor which contributes to its higher thrombolytic activity. Suzuki et al. (9) reported on the thrombolytic properties of E6010 in a canine model with copper coil-induced coronary artery thrombosis. In their study, an intravenous bolus injection of E6010 at 0.2 mg/kg was more effective than that of intravenous infusion of t-PA at 0.6 mg/kg/hr, with respect to the time to recanalization, recanalization rate and reocclusion rate for thrombi aged 1, 3 and 6 hr. The measurements of antigen levels and functional fibrinolytic activities of both drugs showed that E6010 has a slower clearance rate than t-PA, and the relatively long half-life calculated from the antigen level of E6010 is parallel to the half-life calculated from its activity. Plasma clearances of E6010 and t-PA obtained from the antigen level in the study of Suzuki et al. were 6.8 and 16 ml/min/kg, respectively, and the clearance rate of E6010 from plasma was 2.4 times slower than t-PA. Our results were consistent with these findings on the effectiveness and pharmacokinetics of E6010 compared with those of t-PA.

The slower elimination of E6010 from plasma has been demonstrated in dogs (9), rats (10, 11) and rabbits (H. Mizuo et al., unpublished data). These pharmacokinetic findings and evidence for the effective thrombolytic
activity of E6010 on coronary thrombosis in dogs (9) and pigs (present study) indicate that the action of E6010 is not species-specific, but will also be present in humans. Therefore, E6010 appears to be of potential benefit for the treatment of acute myocardial infarction by intravenous bolus injection.

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