EXPERIMENTAL STUDIES

A Novel Modified t-PA, E-6010, Induces Faster Recovery of Ventricular Function after Coronary Thrombolysis than Native t-PA in a Canine Thrombosis Model

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Using the centerline method in a canine model, we compared left ventricular function after coronary thrombolysis induced by a novel modified recombinant tissue plasminogen activator (rt-PA) (E6010: $^{13}$Cys$^{-14}$Ser) to that induced by rt-PA or urokinase. Thirty minutes after occlusion, a bolus injection of E6010 (0.2 mg/kg) or a continuous infusion of either rt-PA (0.6 mg/kg over 1 h) or urokinase (0.38 mg/kg over 1 h) was administered intravenously. Animals with sustained copper coil-occlusion served as non-reperfused controls. Left ventricular ejection fraction and regional wall motion (expressed as the infarction chord number; i.e., the number of chords < -2SD among chords 12 - 66) were 42 ± 5%** and 5 ± 3%* respectively, in the E6010 group, 31 ± 8% and 16 ± 12 in the rt-PA group, and 31 ± 2% and 32 ± 13 in the urokinase group 1 h after reperfusion, indicating earlier recovery of left ventricular function after thrombolysis in the E6010 group than in the rt-PA and urokinase groups (**p<0.01 vs control). Coronary reperfusion with E6010 induced earlier recovery of left ventricular function than reperfusion with rt-PA or urokinase. These results suggest that E6010 may be of clinical value in the treatment of coronary occlusion.

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Ischemia in myocardium due to coronary artery occlusion rapidly causes necrosis and reduces myocardial contractility. However, prompt reperfusion of the occluded coronary artery has been shown to limit infarct size1,2 and improve survival3 - 7. The benefits of early reperfusion, however, may be compromised by reperfusion injury, i.e., lethal damage to viable myocytes brought about by reperfusion itself, a phenomenon that is thought to be due to cytotoxic oxygen-derived free radicals produced at the time of reflow8 - 10. Percutaneous transluminal coronary angioplasty (PTCA) is the primary treatment for acute myocardial infarction. However, this can be performed in only a limited number of institutions and at a considerable time after the onset.

The purpose of thrombolytic therapy is to achieve early initiation of coronary artery reperfusion after the onset of symptoms to limit infarct size, preserve left ventricular function, and save lives11,12. In patients with acute myocardial infarction, a coronary reperfusion rate of 70% or higher was can be obtained with thrombolytic treatment using native recombinant tissue plasminogen activator (rt-PA)13 - 15. However, since rt-PA has a short half-life, it must be administered by continuous drip infusion, which requires

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complex maneuvers. Therefore, this treatment also can be applied in only a few institutions. To overcome this shortcoming of rt-PA, we developed a novel modified recombinant tissue plasminogen activator (E6010) that has a longer plasma half-life, by replacing the \(^{84}\)Cys of tissue plasminogen activator (t-PA) with \(^{84}\)Ser using recombinant DNA technology. We previously demonstrated that E6010 has a potent thrombolytic effect when given by bolus intravenous injection in a canine copper coil model of coronary thrombosis.

Reduced left ventricular function can reportedly be recovered by initiating reperfusion of the coronary artery soon after the onset of symptoms, and there may be a correlation between recovery of left ventricular function after thrombolytic therapy and survival rate in patients with acute myocardial infarction.

The centerline method first reported by Sheehan et al is now commonly used for the clinical evaluation of left ventricular function (in terms of left ventricular ejection fraction and regional wall motion). There have been few reports, however, on changes in left ventricular function associated with coronary occlusion and its recovery after thrombolysis in animal models primarily because the normal values and the control curve for regional wall motion that are required in the centerline method are not available in animal models. Therefore, before performing this study, in which we analyzed left ventricular function by the centerline method, we first created a control curve of regional wall motion from the left ventriculograms of 106 untreated anesthetized normal dogs.

In this study, to clarify the usefulness of E6010, we evaluated the improvements in left ventricular function after coronary thrombolysis induced by bolus intravenous injection of this agent, and compared these improvements to those observed in animals treated with either rt-PA or urokinase, animals in which balloon occlusion-reperfusion was performed (reperfused controls), and animals in which sustained copper coil-occlusion was performed (non-reperfused controls).

MATERIALS AND METHODS

Thrombolytic Agents
Recombinant t-PA and E6010 were prepared in Syrian hamster kidney cells by recombinant DNA technology, and purified by monoclonal anti-t-PA antibody-Sepharose affinity chromatography and gel chromatography. The specific activities, estimated by the fibrin clot lysis method, were \(50.0 \times 10^4\) and \(15.0 \times 10^4\) IU/mg, respectively.
Urokinase (specific activity $15.7 \times 10^4$ IU/mg) was purchased from Green Cross Corp (Osaka, Japan). Vials of the rt-PA or E6010 preparation, which contained 5 mg of rt-PA or E6010 dissolved in a vehicle (pH 5.0) containing 2.5 mL of 3% arginine-aspartic acid and 5% mannitol, were stored at $-20^\circ$C until use.

The entire predetermined dose (0.2 mg/kg) of E6010 was diluted to 5 mL with vehicle and administered intravenously as a bolus. For rt-PA, 10% of the predetermined dose (0.6 mg/kg) was diluted to 5 mL with vehicle and administered as a bolus, and the remaining 90% was diluted to 180 mL with 5% glucose solution and administered continuously over 60 min with an infusion pump (TFV-2100, Nihon Kohden Co, Ltd, Tokyo, Japan). For urokinase, the entire dose (0.38 mg/kg) was diluted to 180 mL with 5% glucose solution and administered continuously over 60 min.

**Experimental Protocols**

The experimental protocols are shown in Fig 1. All experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Thirty adult mongrel dogs of both sexes, weighing 13–25 kg, were randomly allocated to 5 groups of 6 animals each.

The animals were anesthetized with ketamine hydrochloride (Sankyo Co, Ltd, Tokyo, Japan), 50 mg/kg intramuscularly, followed by thiopental sodium (Tanabe Seiyaku Co., Ltd., Tokyo, Japan), 10 mg/kg intravenously. After endotracheal intubation, respiration was controlled with an artificial respirator (ARF-850E and EM-2, Acoma Co, Ltd, Tokyo, Japan) and anesthesia was maintained with a gas mixture of O$_2$, nitrous oxide (NO$_2$), and 0.8%–1.5% enflurane (Dainabot Co, Ltd, Osaka, Japan). All of the animals were ventilated with positive pressure at a stroke volume of 20 mL/kg and a frequency of 12 inflations/min. O$_2$ and NO$_2$ were mixed at a ratio of 1:2. Millar tip catheters (MPC-500, Millar Instruments Inc, Houston, TX, USA) were advanced from the bilateral femoral arteries; one was placed in the left ventricle to measure left ventricular pressure, and the other was placed in the thoracic aorta to measure aortic pressure. Polyethylene catheters (7-Fr: Atom Co, Ltd, Tokyo, Japan) were inserted in the left jugular vein and the left femoral vein for drug administration. Electrocardiograms (ECG) were recorded with limb leads I–III. Heart rate was measured with a tachometer (AT-601G: Nihon Kohden, Tokyo, Japan), using the left ventricular pressure waveform as the trigger. ECG (lead II), heart rate, phasic blood pressure, mean blood pressure, and LVP were continuously recorded with a polygraph (RM-6000: Nihon Kohden, Tokyo, Japan) during the experiment. The left carotid artery was secured for the insertion of either a Sones catheter (8-Fr: USCI-Bard Inc, Billerica, MA, USA) or a brachial guiding catheter (8.3-Fr, Baxter Corp, Irvine, CA, USA) for left ventriculography.

The animals were stabilized for 60 min, a catheter for left ventriculography (LVG) was inserted into the left ventricle from the left carotid artery in the 30° right anterior oblique (RAO) position, and LVGs were obtained with 5 mL of contrast medium (Urographin 76%, Nihon Schering Corp, Osaka, Japan), using a fluoroscopic system (Angioplus Series 9000 C-Arm, OEC-Diasonics Inc, Salt Lake City, UT, USA) equipped with a videographic apparatus (VA-5800, Sony Corp, Tokyo, Japan). These LVGs served as controls for the analysis of left ventricular function by the centerline method (LVG-1).

Hemodynamic parameters and ECG were used to evaluate the eligibility of the data, to confirm the absence of arrhythmias while LVGs were being recorded, and to determine end-diastole (ED) and end-systole (ES) in videotaped images of LVGs.

A Sones catheter was inserted from the left carotid artery under fluoroscopic monitoring. After coronary angiographic confirmation that the coronary artery was normal, coronary thrombosis was induced by advancing a copper coil (5-mm-long) attached to an intracoronary catheter through the carotid artery, distal to the first diagonal branch of the left anterior descending artery, according to the method of Kordenat et al$^{21,22}$ An occlusive thrombus formed within 5–10 min, and was confirmed by coronary angiography. The occlusive thrombus was then reconfirmed by coronary angiography.
30 min after induction (30 min thrombosis), and an LVG was obtained 30 min after occlusion (LVG-2).

Three hundred units per kg of heparin (Novo Nordisk Inc, Gentofte, Denmark) was then injected and the thrombolytic agents were administered 1 min later. E6010 (0.2 mg/kg) was administered intravenously as a bolus (Group I), rt-PA (0.6 mg/kg/h) was administered by continuous intravenous infusion (Group II), and urokinase (60,000 IU/kg/h; 0.38 mg/kg/h) was administered by continuous intravenous infusion (Group III). Reperfusion, as assessed by angiography, was performed at about 10 min intervals after bolus injection or after the initiation of the infusion. Complete reperfusion was defined as TIMI grade 3. The drugs were given in doses of 0.2 mg/kg for E6010, 0.6 mg/kg/h for native t-PA, and 0.38 mg/kg/h for urokinase, since the time to complete reperfusion was about 20 min after the administration of the drugs at these doses in a previous study.\textsuperscript{17,23}

In the balloon occlusion-reperfusion group (Group IV), a 3-Fr balloon catheter (CV-1040: Baxter Corp, Irvine, CA, USA) filled with a 10% dilution of Urographin with physiologic saline was advanced along the guiding catheter (RF-GA35153, Terumo Corp, Tokyo), and the coronary artery was occluded distal to the first diagonal branch of the left anterior descending artery by applying a fixed pressure (150 μl) to the balloon. The vessel was reperfused by deflating and withdrawing the balloon 50 min after occlusion. In the sustained copper coil-occlusion group (Group V), the coronary artery was occluded for 4 h and 50 min with a copper coil. Group IV served as the reperfused control group, and Group V served as the non-reperfused control group. An LVG in the 30° RAO projection was obtained immediately after reperfusion in Groups I–III, immediately after reperfusion (withdrawal of the balloon) in Group IV, and 50 min after occlusion in Group V (LVG-3). LVG-4, 5, 6, and 7 were obtained in the 30° RAO projection 1, 2, 3, and 4 h, respectively, after reperfusion (Fig. 1). At each left ventriculography, maintenance of patency in all animals in Groups I–IV was confirmed by coronary angiography. LVG-4, 5, 6, and 7 in Group V were obtained 110, 170, 230, and 290 min, respectively, after occlusion (Fig. 1). Coronary angiography showed that there was no spontaneous reperfusion in Group V during the experiment. All data were expressed as means ± standard deviations for each group.

Left ventricular function was analyzed by the centerline method.\textsuperscript{18} The endocardial outlines at ED and ES in videotaped LVGs were traced on sheets in an overhead projector and analyzed with a computer, using CAMAC-300 LVG analysis software (Goodman Corp., Nagoya, Japan), which is based on the centerline method, and an image analyzer (KD4030A, Graphtec Co, Ltd, Tokyo, Japan).

The control curve for the centerline method was obtained from the LVGs of 106 untreated anesthetized dogs. Cardiac output was calculated either from the measured values from the tip of the Millar tip catheter used to measure left ventricular pressure or from the measured values of the copper coil in the videotaped images in LVGs.

Regional wall motion was expressed as the infarction chord number, ie, the number of
chords showing less than −2 standard deviations in among chords No. 12–66 located in the regions dominated by the left anterior descending artery.\textsuperscript{19,20,24}

Statistics
Statistical analysis was performed by Tukey’s multiple comparison test. Results were considered significant at $p<0.05$.

RESULTS

Reperfusion Time
The time to complete reperfusion was $18.3 \pm 2.6 \text{ min}$ in Group I, $19.2 \pm 2.0 \text{ min}$ in Group II, $20.0 \pm 6.3 \text{ min}$ in Group III, and $20.0 \text{ min}$ in Group IV, with no significant differences among the groups. No reocclusion was observed for 4 h after complete reperfusion in these 4 groups. No spontaneous reperfusion was observed in Group V.

Left Ventricular Ejection Fraction
Fig. 2 shows the left ventricular ejection fraction in each group before occlusion, 30 min after occlusion, and after reperfusion. In Group V, left ventricular ejection fraction decreased after occlusion and remained reduced until the end of the experiment. In Group I, left ventricular ejection fraction, which was reduced after occlusion, recovered significantly, beginning immediately after reperfusion, compared to that in Group V. The recovery was also significantly greater than that in Group IV 1 h or more after reperfusion. In Group II, left ventricular ejection fraction recovered significantly 2 h or more after reperfusion compared to that in Group V, but this recovery was not significantly greater than that in Group IV. In Group III, left ventricular ejection fraction recovered significantly to that in Group V 4 h after reperfusion. In Group IV, left ventricular ejection fraction recovered slightly after reperfusion, but this recovery, compared to that in Group V, was not significant, even at 4 h after reperfusion.

Regional Left Ventricular Wall Motion
Fig. 3 shows changes in the regional left ventricular wall motion expressed as infarction chord number (Inf No.; number of chords showing less than −2SD) before occlusion, 30 min after occlusion, and after reperfusion. As with left ventricular ejection fraction, regional left ventricular wall motion was decreased, ie Inf No. was increased, after occlusion. In Group V, regional left ventricular wall motion decreased after occlusion and remained reduced until the end of the experiment. In Group I, regional wall motion, which was reduced after occlusion, recovered significantly, beginning immediately after reperfusion, compared to that in Group V. Regional wall motion in Group I also recovered significantly compared to that in Group IV 1 h or more after reperfusion.

In Group II, regional left ventricular wall motion recovered significantly, beginning immediately after reperfusion, compared to that in Group V, but this recovery was not significant compared to that in Group IV. In Group III, wall motion recovered significantly 3 h after reperfusion compared to that in Group V and 4 h after reperfusion compared to that in Group IV. In Group IV, regional wall motion was further reduced, though not significantly, immediately after reperfusion.
compared to that in Group V, but recovered slightly thereafter and was improved, compared to that in Group V, 2 h or more after reperfusion. These results indicate that left ventricular function, as reflected by left ventricular ejection fraction and regional left ventricular wall motion, was decreased after occlusion but recovered significantly, beginning immediately after the reperfusion induced either by the bolus intravenous administration of E6010 or by the continuous infusion of rt-PA or urokinase. Left ventricular function recovered more rapidly after reperfusion with E6010 than after reperfusion with rt-PA or urokinase. Left ventricular function also recovered slightly after reperfusion in Group IV However, this recovery, compared to that in Group V, was not significant.

DISCUSSION

Left ventricular function deteriorates rapidly after occlusion of the coronary artery, but recovers with rapid reperfusion of the occluded vessel. However, reperfusion of the occluded coronary artery is has been reported to cause reperfusion injury of the myocardium, which leads to inferior recovery of ventricular function. Murdock et al.\textsuperscript{25} reported that the rapid release of coronary occlusion induced serious arrhythmias. The rapid washout of extracellular electrolytes during reperfusion may also account for the electrophysiological changes related to arrhythmogenesis.\textsuperscript{26} Yamazaki et al.\textsuperscript{27} reported that the incidence of serious arrhythmias in a staged-reperfusion group was much lower than that in a sudden-reperfusion group.

In previous studies in which we used a canine copper coil-thrombosis model, we often observed that, in the E6010 group, partial reperfusion of TIMI grade 1 or 2 was obtained before complete reperfusion was achieved.\textsuperscript{17} In addition, we found that reperfusion arrhythmias occurred less frequently, and that the mortality rate was lower, after the bolus intravenous injection of E6010 than after the continuous intravenous infusion of rt-PA or urokinase.\textsuperscript{23} We have also reported differences in the increase in coronary blood flow in an open-chest canine copper coil-thrombosis model during reperfusion with various thrombolytic agents. In that study, we showed that the gradual increase in coronary blood flow induced by E6010 during reperfusion, which began early after administration, as opposed to sudden thrombolysis, led to a lower incidence of reperfusion arrhythmias, and to a lower mortality rate.\textsuperscript{28}

In the present study, we investigated whether recovery of left ventricular function after reperfusion varied with the treatment employed to induce reperfusion; i.e., bolus intravenous injection of E6010, continuous intravenous infusion of either rt-PA or urokinase, and withdrawal of a balloon. We found that left ventricular function recovered significantly more rapidly after the bolus injection of E6010 than after the continuous infusion of rt-PA or urokinase or after the withdrawal of the balloon. The rates of recovery being were E6010 > rt-PA > urokinase. In Group IV, left ventricular function recovered after withdrawal of the balloon, but this recovery was not significant compared to that in Group V, in which occlusion was sustained with a copper coil.

Since there were no differences among the groups with regard to time to complete reperfusion, it would appear that differences among the thrombolytic agents with regard to time to recovery of left ventricular function, as well as the absence of significant recovery after abrupt reperfusion following withdrawal of the balloon, were due to differences in how the thrombus was lysed in the occluded vessel. The results of this study appear to agree with our previous findings that E6010 reduced the frequency of reperfusion arrhythmias and the mortality rate.\textsuperscript{23,28}

These results suggest that the gradual increase of coronary blood flow induced by E6010 during reperfusion, beginning early after administration, as opposed to sudden thrombolysis, leads to the rapid recovery of left ventricular function after complete reperfusion.

Recently, Purvis et al.\textsuperscript{29} reported that a double-bolus administration of alteplase in patients with acute myocardial infarction had therapeutic effects superior to those observed with an infusion of alteplase. This finding indicates that the complete reperfusion of occluded coronary arteries induced by bolus injections of plasminogen activators may lead to the preservation of viable
myocytes and to rapid recovery of left ventricular function after reperfusion.

At present, however, we have no biochemical or pharmacological evidence for the protective effect of E6010 on myocytes. This aspect of E6010 must be examined in further studies.

The findings of this study suggest that the clinical value of thrombolytic therapy with E6010, given as a bolus intravenous injection for the treatment of coronary occlusion, is superior to those of therapies which use other thrombolytic agents, since it allows for a more rapid recovery of left ventricular function after lysis of the intracoronary thrombi. A multicenter trial of E6010, given by intravenous bolus injection to patients with acute myocardial infarction, is now being conducted in Japan.

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REFERENCES


