Establishment of evaluation model for thrombolytic activity in vivo

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Summary

Background and Purpose — The cardiovascular diseases is a high rank in the cause of death, and the thrombolytic therapy by rt-PA is a basic therapy as with a PTCA. However, the optimal assessment of an objective thrombolytic activity is not found in the field of medical technology. In the present study, our purpose is to establish the thrombolysis model to estimate thrombolytic activity in vivo.

Methods — Thrombolysis was evaluated by using a He-Ne-laser–induced thrombosis model in rat mesenteric microvessels. Changes in thrombus volume were analyzed with the image analysis software Image-Pro Plus (Media Cybernetics, USA).

There were two experimental groups (placebo, rt-PA 0.6 mg/kg). Sequential changes (0 to 60 min) in thrombus volume were compared by using a relative optical density method.

Results — In the placebo group, the thrombus volume at 60 min, reflecting the extent of thrombolysis, was 97.2% ± 5.7% of the initial value. In the rt-PA group, thrombus volume decreased to 70.7% ± 4.1% (P<0.01) after 20 min and 14.2% ± 6.6% after 60 min.

Conclusions — This model well reflects thrombolytic activity in vivo, and is available to basic investigation in the thrombolytic therapy.

Key words: Thrombolysis, Recombinant tissue plasminogen activator, He-Ne-laser, Cardiovascular disease

1. Introduction

Cardiovascular diseases are leading causes of morbidity and mortality in the developed world and in many developing countries. The recombinant tissue-type plasminogen activator (rt-PA) is the only medication approved for the treatment of acute ischemic diseases. This therapy was approved by the Food and Drug Administration in 1996 for selected patients who can be treated within 3 h of stroke onset. The approval was based on the results of a clinical trial. In recent years, rt-PA has been studied in several...
trials to assess its effectiveness in peripheral vascular thrombolysis\(^{19}\). The recombinant tissue-type plasminogen activator is increasingly becoming the thrombolytic agent of first choice for the treatment of peripheral arterial and venous thrombosis\(^{5,16}\). However, although many studies have shown the efficacy of thrombolytic therapy with rt-PA, many problems have remained, including hemorrhagic complications, neurologic injury induced by reperfusion, and potential neurotoxicity\(^{11,12}\). A lot of basic investigations in the thrombolytic therapy are not enough. Further basic investigations would be necessary in the future. However, the optimal assessment of an objective thrombolytic activity is not found. In the present study, we investigated the thrombolysis model in vivo, and the analysis method.

2. Materials and Methods

1. Experimental Animals

Male Wistar-ST rats weighing 250 to 330 g were obtained from Japan SLC, Inc. (Hamamatsu, Japan). All animals were maintained in air-conditioned rooms (temperature: 22.5 ± 0.5°C; humidity: 50% ± 5%) with a 12-h light-dark cycle. Animals had free access to food and drinking water. All procedures were conducted in compliance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan.

2. Recombinant tissue plasminogen activator (Alteplase)

Alteplase (GRTPA for injection) was kindly donated by Mitsubishi Pharma Corporation (Tokyo, Japan). Alteplase (6,000,000 IU/vial) was dissolved in distilled water and stored at -80°C until use. Alteplase was administered to rats by infusion into the femoral vein (10% bolus plus 90% perfusion over a 45-min additional period) in 1 ml saline solution. The dosage (0.12, 0.3, 0.6 mg/kg; n=8 animals in each group) was used to investigate dose-dependence. The dosage was tested and we found 0.6 mg/kg most appropriate and decided to use this.

3. Experimental Thrombolysis Model

Animals were anesthetized with sodium pentobarbital (60 mg/kg i.m.) and the femoral blood vessels exposed by a median incision. A cannula (PE 50, Becton Dickinson, USA) was introduced into the femoral vein for administration of the study agents, and another was introduced into the femoral artery to monitor blood pressure respectively. A loop of intestine was extracted through the midline abdominal incision and spread over a perspex plate. An O-ring was placed around the tissue to stop vessel movement during peristalsis. The perspex plate was attached to the stage of a microscope (Olympus BX51, Japan) and the preparation observed through a long-working-distance objective. The schema of this model was shown in Figure 1. Arterioles (30 to 35 μm in diameter) were selected for irradiation. The He-Ne laser beam was introduced into the microscope by using a dichroic mirror and focused on the center of each selected vessels. The diameter of the laser spot on the focal plane was 15 μm, and the power was 25 mW. Evans blue (1.6 mg/kg, E. Merck, Germany) was injected through the femoral vein cannula. Irradiation for 5 s was repeated every 30 s until a mural thrombus occluding 80% of the vessel lumen in monitor was formed. After stabilisation for 10 min, infusion of saline or alteplase (0.12, 0.3, 0.6 mg/kg) (10% bolus plus 90% perfusion over a 45-min additional period) in 1ml saline solution saline began through the femoral vein cannula.

4. Analysis of Thrombus Size

Thrombolysis was assessed by using a slight modification of our laser-induced thrombosis method\(^{11}\). Briefly, changes in thrombus volume were analyzed with the image analysis software Image-Pro Plus (Media Cybernetics, Rockville, MD). Two-dimensional outline images of thrombi were captured in situ on a computer at 5-min intervals. Subsequently, 3D images were constructed by establishing optical density values relative to that of an area of the blood vessel lumen not involved in thrombus formation (Fig. 2). Integrative optical density (IOD) values were computed corresponding to the thrombus size. Changes in thrombus size were calculated according to
the following formula. Thrombus size = IODn \div IODo (IODn = the integrative optical density at various times intervals during thrombolysis; IODo = the integrative optical density immediately after the stabilizing thrombus). The extent of thrombolysis was expressed as a percentage of the initial thrombus volume.

Our experiments were performed in a masked manner. The investigators performing the surgery gave each group of animals (n=8) a secret code that remained unknown to the experimenters in charge of assessing the effects of drugs on the thrombolysis

Fig. 1   The schema of bioimaging model for evaluation of thrombus size by using imaging software. Thrombus is formed by He-Ne laser irradiation at microvessels of mesentry in rat. Thrombus images are captured into PC and analyzed with image analyzing software (Image-Pro Plus)

Fig. 2   Evaluation of thrombus size by using imaging software. Thrombus size = IODn \div IODo; IODn = the IOD at various time intervals during thrombolysis and IODo = the IOD immediately after stabilization of the thrombus. The extent of thrombolysis was expressed as thrombus volume as a percentage of the initial thrombus volume.
outcome until the end of study.

5. Statistical Analysis

Results are expressed as means ± SEM. All data showed normal distributions. Comparisons among groups were made by using one-way factorial ANOVA followed by Fisher’s Protected Least Significant Difference test or Dunnett’s multiple comparison post-test. Differences between means were considered significant at $P < 0.05$.

3. Results

1. Spontaneous Thrombolysis

The stable thrombi induced by He-Ne laser irradiation demonstrated low levels of spontaneous thrombolysis, and at 60 min after irradiation, they retained 97.2% ± 5.7% of their original volume. Relative thrombus size did not differ significantly between times 0 and 60 min. Figure 3a shows typical images.

2. Thrombolysis induced by recombinant tissue plasminogen activator

Relative thrombus size decreased significantly after bolus infusion of rt-PA at doses of 0.12, 0.3, or 0.6 mg/kg, and the rate of thrombolysis was dependent on the dose of alteplase (Fig. 4). The volume of the thrombus after rt-PA (0.6 mg/kg) treatment was 70.7% ± 4.1% at 20 min and 14.2% ± 6.6% at 60 min (Fig. 4). Figure 3b shows typical images.

4. Discussion

Several experimental models have been reported to investigate thrombolytic agents in vivo\textsuperscript{14-17}. These models may be complex and can give rise to problems of reproducibility and technical artifacts. In addition, it can be very difficult to estimate the precise rate and extent of thrombus dissolution. We have developed in vivo thrombolysis models utilising laser technologies and have quantified the results using computerised image analysis. In particular, the models provided a means of assessing thrombolytic strategies in conditions, which reflected different levels of vascular damage. Using the He-Ne laser, the endothelial cell monolayer remained intact and injury to the vessel wall was minimal\textsuperscript{11}. The damage of endothelium by He-Ne laser will be minimal compared with other methods. Severe endothelial damage will be caused if the argon laser is used instead of He-Ne laser. The

Fig. 3 Changes in thrombus size.
Thrombus was induced by He-Ne laser irradiation until 80% occlusion. After a 10 min thrombus stabilization period, two-dimensional images of the thrombus were captured on a PC at 5 min intervals. a. Placebo group b. rt-PA group (0.6 mg/kg) (Typical images from each group are shown.)
thrombus formed by a severe endothelial damage contains a lot more leukocyte compared with the He-Ne laser. Leukocytic accumulation in the thrombus reflects the extent of the tissue damage, and might cause the expression of a lot of cell adhesion molecules. These reactions will have a significant influence on localized thrombolytic mechanism.

In the placebo group, the thrombi induced by laser irradiation demonstrated low levels of spontaneous thrombolysis for 60 min after laser irradiation. On the other hand, in rt-PA group, thrombus volume significantly decreased at 20 min (P<0.0001, Fig. 4).

In vivo, thrombin may be generated under conditions of high shear stress associated with vascular stenosis. Thrombin has numerous activities in haemostasis. It stimulates platelets to release plasminogen-activator inhibitor (PAI-1) 24 and alpha 2 plasmin inhibitor, promotes the function of several coagulation factors and activates a specific fibrinolytic inhibitor (thrombin activatable fibrinolytic inhibitor, TAFI). These mechanisms might limit thrombolysis. Furthermore, platelets also contain serotonin (5-HT2) and ADP, and vasoconstriction induced by the release of these agents from activated platelets could also restrict thrombolysis. For these reasons, thrombolytic activity should be invesigate in vivo.

In the present study, we have expanded a previous thrombosis model and have developed reproducible methods to investigate thrombolysis in vivo using different laser technologies and computerized image processing techniques. This model is available to basic investigations in the thrombolytic therapy.

Disclosure of Conflict of Interest

The authors state that the have no conflict of interest.

Reference


Fig. 4 Time course of thrombolysis.
a. Dose-dependent thrombolytic effect of Alteplase (○: placebo; ●: alteplase i.v. 0.12 mg/kg; △: alteplase i.v. 0.3 mg/kg; ■: alteplase i.v. 0.6 mg/kg) (n = 8 animals in each group; * P<0.005; ** P<0.001; *** P<0.0001, placebo vs each group)


