Experimental Model of Carotid Artery Thrombosis in Rats and the Thrombolytic Activity of YM866, a Novel Modified Tissue-Type Plasminogen Activator†

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ABSTRACT—We compared the thrombolytic activity of a novel modified t-PA, YM866, with that of t-PA in a rat model of electrically-induced thrombosis. Histological examination revealed the thrombus to be composed mainly of platelet clumps. Measurement of the decrease in carotid blood flow showed that complete occlusion occurred within 14 min. At 10 min after the induction of thrombus, a test drug (YM866, t-PA, or saline) was administered by i.v. bolus injection under heparinization (300 IU/kg, i.v.). Both YM866 and t-PA exhibited dose-dependent thrombolytic activity; the reperfusion rate of YM866 was twice that of t-PA. There was no significant difference in time to reperfusion between the agents, but YM866 showed a greater improvement in patency status after successful thrombolysis than t-PA. Plasma fibrinogen fell slightly but significantly (14% of baseline value) in animals given 1 mg/kg of YM866. All groups of rats showed a significant decrease in carotid artery blood flow at 1 hr after successful reperfusion or injection of the drug, but this decrease showed significant recovery in animals given 1 mg/kg of YM866. These results suggest that YM866 by single bolus injection is a superior thrombolytic agent to t-PA, and that YM866 can improve the patency status after successful thrombolysis. Furthermore, this platelet-rich thrombosis model permits continuous observation of the process of thrombus formation and subsequent thrombolysis and provides a useful tool for the screening and evaluation of efficacy of new antithrombotic agents.

Keywords: Tissue-type plasminogen activator (t-PA) (modified), Thrombolysis, Thrombosis (electrically-induced), Patency status, Histological examination

A number of experimental models of arterial thrombosis suitable for evaluating the efficacy of antithrombotic agents have been described (1, 2). Because of its simplicity and reproducibility, electrical stimulation has long been used to induce thrombus formation (3). In 1972, Hladovec (4) reported a rat model of thrombosis electrically induced in the carotid artery in which the process of thrombus formation was monitored in real time by a continuous registration thermometer. In the present study, we prepared a rat model of occlusive thrombosis according to the method of Hladovec in which thrombus formation and subsequent thrombolysis following injection of a thrombolytic agent were continuously monitored with an electromagnetic blood flowmeter.

Tissue-type plasminogen activator (t-PA) has a high affinity for fibrin and is used clinically as a thrombolytic agent specifically able to lyse thrombi. However, due to the extremely short half-life of this agent, administration must be by high-dosage infusion. This increases the risk of systemic bleeding and results in a high incidence of acute reocclusion (5). To eliminate these shortcomings, attempts have been made to modify t-PA by recombinant DNA technology, and several mutants of t-PA with prolonged plasma half-life that can be administered by i.v. bolus injection have been reported (6, 7).

YM866 is a novel modified t-PA with the deletion of the kringel-1 domain and with a point mutation at the site of the kringel-2 domain linkage to the light chain (8). It has

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been shown in vitro to possess a pronounced affinity for fibrin and to retain the same specific activity as t-PA (9). This study was performed to establish a method to induce acute thrombosis in rats, and to then use this method to evaluate the thrombolytic activity of YM866 and t-PA. We also investigated this experimental model for its suitability for the evaluation or screening of anti-thrombotic agents in routine studies.

MATERIALS AND METHODS

**Thrombolytic agents**

YM866 is a recombinant tissue-plasminogen activator analogue that contains a finger domain, growth factor domain, kringle-2 domain, and serine protease domain, as well as a point mutation at the kringle-2-serine protease linkage site (del 92-173, 275Arg → Glu) (8). The preparations of YM866 used in this study contained more than 98% of the single chain form. Lyophilized preparations of YM866 and t-PA (ACTIVASE®, 50 mg/vial; Genentech, Inc., South San Francisco, CA, USA) were dissolved in distilled water for injection and then diluted with saline. The specific activities of YM866 and t-PA as determined by fibrin clot lysis assay calibrated with the international standard (83/157) were 570,000 and 600,000 IU/mg, respectively (9).

**Induction of thrombosis**

Thrombi were produced in male SD rats weighing 290–395 g (Clea Japan Inc., Tokyo) by an electric method modified from that of Hladovec, as follows: Rats were anesthetized by an intraperitoneal injection of 1.3 g of urethane/kg of body weight and then fixed on an operating table. The left carotid artery was carefully freed from the surrounding tissue along a minimum length of 15 mm. An electromagnetic flow probe (1 mm-diameter) was placed in contact with the exposed artery proximal to the electrodes and connected to an electromagnetic blood flowmeter (MVF-2100; Nihon Kohden Co., Tokyo) for recording on a polygraph. A stainless steel bipolar hook electrode was attached to the carotid artery at the proximal portion of the probe site, with a thin gum-rubber sheet (5 mm × 30 mm) inserted underneath, to deliver electrical stimulation (3 mA, 1 min) via an ammeter connected to the electrode. Immediately after electrical stimulation, the electrode was removed from the artery, and the exposed surface of the artery was covered with wet gauze moistened with saline, followed by continuous blood flow measurement. The femoral vein and artery were cannulated for injection of test drug and collection of blood samples, respectively.

**Measurement of thrombolytic activity**

The experimental protocol is shown in Fig. 1. Carotid blood flow began to fall from several minutes after the end of electrical stimulation and stabilized at the zero level within 14 min. The time from the end of electrical stimulation to a 50% decrease in baseline blood flow level was designated the thrombosis time. Heparin (Novo Heparin®; Novo BioLabs, Copenhagen, Denmark) was given to all animals by i.v. bolus injection at 300 IU/kg (1 ml/kg) immediately before the administration of the thrombolytic agent. Ten minutes after the induction of thrombus, test drug (YM866, t-PA, or saline) was administered by i.v. bolus injection. Reperfusion as assessed by blood flow was observed for 1 hr after bolus injection. Animals showing no evidence of successful reperfusion at 1 hr were considered to have failed to attain reperfusion. If reperfusion occurred, blood flow was monitored for a further 1-hr period to assess the patency status after thrombolysis. Reperfusion and subsequent reocclusion were defined here as a recovery of blood flow to ≥50% and a decrease to ≤25% of the baseline level, respectively. To schematically represent the patency status after successful reperfusion, blood flow for the 1-hr period after reperfusion was classified under categories of ≥50%, >25% to <50%, and ≤25% of the baseline level in each animal. Percent duration of these blood flow patency statuses during this 1-hr period were thereby calculated for each animal. Similar calculations were made for the 1-hr period after drug injection in animals failing to attain reperfusion. To give a parametric score, carotid patency

![Fig. 1. Experimental protocol. Ten minutes after the induction of thrombus, the test drug was administered by i.v. bolus injection. Carotid patency status was monitored continuously for 1 hr after drug injection. Animals attaining reperfusion were monitored for a further 1 hr to assess the patency status after thrombolysis. Blood samples were collected at baseline and 1 hr after drug injection.](image-url)
status was expressed according to the following classification scheme: 1) persistent occlusion (PO): no reflow, 2) cyclic reflow (CR): alternating reocclusion and reperfusion after initial reflow, 3) persistent patency (PP): persistent flow without reocclusion after initial reflow.

Measurement of fibrinolytic system parameters
Citrated blood samples (0.5 ml) were collected in 1 µM PPACK (D-Phe-Pro-Arg-chloromethylketone; Calbiochem, San Diego, CA, USA) (10) for measurement of fibrinogen and α2-plasmin inhibitor. Plasma samples were stored frozen at -70°C until assay. Fibrinogen was determined by a thrombin time method (Fibrinogen Test "BMY®", Boehringer-Mannheim, Mannheim, Germany) (11) and α2-plasmin inhibitor by a synthetic substrate method (Testzym APL kit®, Daiichi Pure Chemicals Co., Tokyo).

Histological examination
The histological characteristics of the thrombi were observed in 13 male, 8-week-old SD rats. Ten minutes after the induction of thrombus, the animals were killed with an overdose of pentobarbital, and then the thrombus segment of the carotid artery was immediately removed and fixed with 10% phosphate-buffered neutral formalin solution or phosphate-buffered, 2.5%-glutaraldehyde and 2%-paraformaldehyde solution at 4°C. For light microscopy, paraffin sections were stained with hematoxylin and phosphotungstic acid-hematoxylin (PTAH). For transmission electron microscopy (TEM), the fixed materials were processed as described elsewhere (12).

Statistical analyses
The experiments were performed on groups of 8-9 rats each. Data were expressed as percentages or as the mean ± standard deviation of the mean. Changes in plasma fibrinogen and α2-plasmin inhibitor were analyzed by the paired t-test, with one-way ANOVA for intergroup comparisons. A P value of less than 0.05 was considered significant.

RESULTS
Thrombolytic activity of YM866 and t-PA
Figure 2 shows representative tracings of carotid blood flow. Occlusive thrombus formation and subsequent thrombolysis are clearly seen. In all animals, carotid blood flow decreased gradually after electrical stimulation and reached a stable zero level within 14 min. Thrombosis time ranged from 2 min 30 sec to 13 min 5 sec. Group mean values were 6 min 8 sec to 7 min 59 sec, showing no significant intergroup difference in thrombosis time (one-way ANOVA, data not shown). Thrombolytic activities of YM866 and t-PA are shown in Fig. 3. No reperfusion occurred in animals given saline. Both YM866 and t-PA exhibited dose-dependent thrombolysis. Reperfusion rates of YM866 were twice as high as those of t-PA. Time to reperfusion was decreased dose-dependently in animals given YM866, while no distinct dose-dependency was observed with t-PA. Earlier reperfusion was achieved among animals receiving YM866 at 0.5 and 1.0 mg/kg than those given t-PA. Both the YM866- and t-PA-treated groups exhibited high reocclusion rates.

Fig. 2. Typical patterns of flow records obtained after i.v. bolus injection of saline (A), YM866 (B) and t-PA (C). Persistent patency characterized by stable blood flow after successful reperfusion was seen with YM866 at 1 mg/kg (B), whereas t-PA at 2 mg/kg (C) frequently showed cyclic reflow with alternating reocclusion and reperfusion.
Fig. 3. Thrombolytic activity of YM866 and t-PA in rats with carotid artery thrombosis. Open circles represent the time to reperfusion in each rat. Bars indicate the mean value of the time to reperfusion of animals that achieved successful reperfusion.

Carotid patency status

The patency status of individual animals is schematically represented in Fig. 4. Animals attaining reperfusion were observed for 1 hr after successful reperfusion, while animals without reperfusion were observed for 1 hr after drug administration. Only 2 of the 9 animals given saline showed any restoration in blood flow, although only to less than 50% of the baseline value. Both the YM866- and t-PA-treated groups showed a dose-dependent recovery in blood flow. When compared between dosage groups exhibiting comparable reperfusion rates (upper versus lower graphs), patency status showed a markedly greater improvement with YM866 than with t-PA. Table 1 shows carotid patency status scored for each group. Persistent patency, characterized by a stable course of blood flow after successful reperfusion (Fig. 2B), was frequently seen with YM866 at 1 mg/kg, whereas t-PA at 2 mg/kg frequently showed cyclic reflow with alternating reocclusion and reperfusion (Fig. 2C).

Changes in fibrinolytic system parameters

Changes in plasma levels of fibrinogen and α2-plasmin inhibitor before and after drug injection are shown in Fig. 5. Data are plotted as percentages of the baseline.

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### Table 1: Carotid Patency Status

| Group          | 0 mg/kg | 0.25 mg/kg | 0.5 mg/kg | 1 mg/kg |
|----------------|---------|------------|-----------|---------|
| Reperfusion rate (%) | 0       | 25         | 63        | 89      |
| Reocclusion rate (%)   | -       | 50         | 80        | 50      |
| Saline               | 100     | 100        | 100       | 100     |
| YM866 0.25 mg/kg     | 90      | 50         | 100       |          |
| YM866 0.5 mg/kg      | 50      | 100        | 100       |          |
| YM866 1 mg/kg        | 50      | 100        | 100       |          |
| t-PA 0.25 mg/kg      | 50      | 100        | 100       |          |
| t-PA 0.5 mg/kg       | 0       | 50         | 100       |          |
| t-PA 1 mg/kg         | 0       | 50         | 100       |          |
| t-PA 2 mg/kg         | 0       | 50         | 100       |          |

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Fig. 4. Schematic representation of the carotid patency status at 1 hr after successful thrombolysis or after i.v. bolus injection of saline, YM866, or t-PA. Open, hatched and closed bars represent percentage durations of blood flow at ≥50%, >25% to <50%, and ≤25% of the baseline level in each animal, respectively. __ >1/2 flow, __ 1/4 to 1/2 flow, __ <1/4 flow.
These parameters showed no change after the injection of saline (data not shown). Fibrinogen significantly decreased to 86±8.2% of the baseline value with YM866 at 1 mg/kg (P<0.01, paired t-test), whereas the other groups showed no significant change. The α2-plasmin inhibitor level with YM866 at 1 mg/kg and t-PA at 2 mg/kg showed a dose-dependent and significant decrease to 43±16% and 71±17% of the baseline values, respectively, but there was no significant difference between these two values.

**Table 1. Incidence of carotid patency at 1 hr after successful thrombolysis followed by i.v. bolus injection of saline, YM866 and t-PA**

| Drug   | Dose (mg/kg) | N | Classification of patency | PO | CR | PP |
|--------|--------------|---|---------------------------|----|----|----|
| Saline | 1 ml/kg      | 9 | 9                         | 0  | 0  | 0  |
| YM866  | 0.25         | 8 | 6                         | 1  | 1  | 1  |
| YM866  | 0.50         | 8 | 3                         | 4  | 1  | 1  |
| YM866  | 1.00         | 9 | 1                         | 4  | 4  | 4  |
| t-PA   | 0.50         | 8 | 7                         | 1  | 0  | 0  |
| t-PA   | 1.00         | 9 | 5                         | 3  | 1  | 1  |
| t-PA   | 2.00         | 9 | 2                         | 5  | 2  | 2  |

PO: persistent occlusion, CR: cyclic reflow, PP: persistent patency. Details of the classification scheme are given in the Methods section.

**Histological characteristics of thrombi**

Longitudinal sections of thrombi were examined by light microscopy (Fig. 6, A and B). The lumen was nearly completely occluded by thrombus, in which some erythrocytes and a few leukocytes were seen. At the site of electrical stimulation, endothelial cells were detached from the basal lamina (Fig. 6A). The thrombus was composed of thick fibrin nets, and fibrin-rich portions were seen at the surface of the thrombus and at the thrombus-basal lamina contact site (Fig. 6B). Latitudinal sections of thrombi were examined by TEM (Fig. 7). Numerous platelets (P), with extending pseudopods tightly adherent to the disrupted luminal surface, and some fibrin and erythrocytes (E) were seen in the thrombus.

**DISCUSSION**

Blood flow was decreased to zero by electrical stimulation at 3 mA for 1 min, suggesting the formation of an occlusive thrombus. The average thrombosis time in our experiments agreed with that reported by Hladovec under conditions of 2 mA for 5 min. Occasionally, blood flow that had once diminished after electrical stimulation was seen to recover, implying dissolution of the thrombus. We therefore used only those animals whose carotid flow remained stable at the zero level without recovery after initial thrombus formation. Heparin was given to all animals immediately before injection of the test drug, the need for which has been previously established (13). The doses of thrombolytic agents used in this study were essentially the same as those reported in several papers with t-PA or modified t-PA in rat models of thrombosis, although considerably lower doses have been used in experiments with other species of animals (14, 15). Extrapolation of animal experimental data to humans thus appears to require caution. Histological examination by light microscopy and TEM showed that the endothelial injuries were caused by electrical stimulation, and that the injury was followed by platelet activation and eventually by the formation of a platelet-rich thrombus.

Both YM866 and t-PA showed dose-dependent thrombolytic activity; YM866 achieved reperfusion rates twice as high as those with t-PA. There was no difference between the agents in time to reperfusion. This might be attributed to the difficulty in delivering a thrombolytic...
agent into the occluded vessel. Several clinical trials have used i.v bolus injection of t-PA with the aim of inducing early recanalization of the occluded coronary artery. Reperfusion rates and time to reperfusion were comparable to those by i.v infusion of this agent, but higher acute reocclusion rates were seen (16, 17). Although the reocclusion rate in the present study was generally high for both agents, with the definition of reocclusion being set at 25% of the baseline blood flow, measurement of the blood flow indicated that complete reocclusion was infrequent. Figure 4 shows that YM866 produced greater improvement in the blood flow when a comparison was made between groups showing comparable reperfusion rates. In particular, persistent patency was more frequent with YM866 at the dose of 1 mg/kg (Table 1). Pharmacokinetically, YM866 is characterized by its 7-fold slower clear-
Fig. 7. Transmission electron micrograph of the latitudinal section of an occlusive thrombus: the thrombus is packed with numerous platelets (P) with extending pseudopods, and fibrin and erythrocytes (E) are seen. Ti: tunica interna, Tm: tunica media, × 8,000.

ance from the plasma than t-PA (9). This higher concentration may contribute to the better patency status with YM866 than t-PA after successful thrombolysis. Further improvement of vascular patency may be expected with adjunctive use of antiplatelet agents such as anti-GPIIb/IIIa monoclonal antibody (18) and GPIIb/IIIa-receptor antagonist (19), or anticoagulants such as thrombin inhibitors (20) and Xa inhibitors (21). A slight but significant decrease (14%) in plasma fibrinogen occurred only with YM866 at 1 mg/kg. Enhancement of systemic fibrinolytic activity was minimal in this study as compared with streptokinase (22) and urokinase (23), which lack affinity for fibrin, thus suggesting the high affinity of YM866 and t-PA for fibrin.

The present data suggest the potential usefulness of YM866 as a novel thrombolytic agent which can be administered by i.v. bolus injection. The results also show that this rat model of thrombosis permits continuous observation of the process of thrombus formation and subsequent thrombolysis, and that it can serve as a useful tool for the screening and evaluation of the efficacy of newly developed antithrombotic agents.

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