Enhanced Thrombolysis Induced by Argatroban or Activated Protein C in the Presence or Absence of Staphylokinase, Measured in an in vivo Animal Model Using Mesenteric Arterioles

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Key Words

Endogenous thrombolysis · Thrombin inhibitors · Activated protein C

Abstract

Successful administration of thrombolytic agents is associated with vessel reocclusion in a high proportion of cases. We have previously established an animal model to investigate platelet-rich thrombolytic mechanisms in vivo and demonstrated that recombinant staphylokinase (rSAK)-induced thrombolysis was enhanced by the concomitant administration of the direct thrombin inhibitor argatroban. The present study expanded the use of this model by comparing

arterial and venous thrombolysis using advanced image analysis software. Mural thrombi were formed by Helium-Neon laser irradiation in mesenteric arterioles and were shown to be lysed, dose-dependently, by smaller amounts of rSAK than those required in venules. Activated protein C (APC), as well as argatroban, enhanced the rSAK-induced thrombolysis. APC or argatroban also induced thrombolysis in the absence of rSAK, and the effect was inhibited by tranexamic acid. The enhanced thrombolysis induced by APC or argatroban in the presence or absence of rSAK may be due to increased endogenous thrombolysis mediated by the inhibition of thrombin activity or delayed thrombin generation.

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Introduction

Plasminogen activator (PA) provides an effective means of treatment for patients with arterial thrombosis such as occurs in acute myocardial and cerebrovascular infarction. PA alone dissolves thrombi, but induces transient thrombin activity and is associated with reocclusion in a high proportion of cases [1, 2]. In recent multicentre trials of thrombolysis therapy, antithrombotic agents have therefore been administered together with PA [3, 4].

We have previously established a simple, accurate and reproducible animal model of thrombolysis in vivo in which platelet-rich thrombi were initially formed by Helium-Neon (He-Ne) laser irradiation of rat mesenteric venules. The controlled administration of therapeutic agents enabled a detailed study of thrombolytic mechanisms and provided novel information on the efficiency of potential therapeutic procedures [5, 6]. We have also shown, however, that differences exist between arterioles and venules with regard to leucocyte behaviour and the effects of aspirin on thrombus formation [7]. In the present study, we have therefore expanded our model to quantitate thrombolytic mechanisms in arterioles in vivo using advanced image analysis software. The main aim of the study was to assess the simultaneous effects of the specific thrombin inhibitor argatroban and of activated protein C (APC) on PA-induced thrombolysis.

Materials and Methods

Animals

Male Wistar ST rats aged 8 weeks and weighing 230–270 g were obtained from SLC Co. Ltd. (Hama-matsu, Japan). The animals were allowed to drink ad libitum and were fasted overnight prior to the thrombolytic experiments to suppress the peristaltic movement of the intestines and to minimise the effect of

diet. All procedures were conducted in compliance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, published by the Physiological Society of Japan.

Agents

Recombinant staphylokinase (rSAK, 1.0 mg/ml) was donated by Yakult Co. Ltd. (Tokyo, Japan) and was stored at -80°C . It was dissolved and diluted in 0.05% Tween80/phosphate-buffered saline (PBS, pH 7.4) immediately before use. Argatroban (Novastan, 0.5 mg/ml) was purchased from Tokyo Tanabe Pharmaceutical Co. Ltd. (Tokyo, Japan) and diluted in physiological saline. APC 3.5 mg/ml was donated by Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) and was stored at -80°C . It was dissolved and diluted in 20 mM citrate/0.7% NaCl/0.5% glycine (pH 7.0) immediately before use. Tranexamic acid (AMCA) was provided by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) and was dissolved and diluted in physiological saline.

He-Ne Laser-Induced Thrombosis and Thrombolysis

Microvascular thrombi were produced in rat mesenteric arterioles by a slight modification of the method described previously [8, 9]. Briefly, animals were anaesthetised with Nembutal (70 mg/kg, i.m.), and cannulae (PE50: inner diameter 0.58 mm, outer diameter 0.97 mm) were inserted into both femoral veins for the administration of test substances and Evans blue. The mesentery was secured flat in Tyrode solution at 37°C on the stage of a microscope. Arterioles (inner diameter 35–45 μm) were irradiated with a He-Ne laser beam at the midpoint between the internal vessel wall and the centre line of the lumen after the injection of Evans blue (14.2 mg/kg). The power and diameter of the laser spot at the focal plane were 15 mW and 15 μm , respectively, and irradiation was repeated for 2 s at 15-second intervals until the extent of the thrombus reached 90% of the lumen diameter. The thrombus was then allowed to stabilise for 10 min before rSAK and/or test substances were given through the femoral vein [5]. rSAK (0.39–3.9 mg/7.7 ml/kg/h), argatroban (0.67 or 2.0 mg/4.0 ml/kg/h) and/or AMCA (121 mg/7.7 ml/kg/h) were infused for 60 min through the femoral veins. APC (0.54 or 1.62 mg/2.31 ml/kg) was given as a bolus injection. Experiments were performed in as short a time as possible to minimise possible inter-batch variations in the animal model.

Fig. 1. Evaluation of thrombus size using Image Analyst software. **a** The thrombus within the lumen of the arteriole is boxed and a threshold level defining the thrombus area is set on a grey scale. The extent of thrombolysis was calculated from the following formula: $A_n \times G_n / A_0 \times G_0$, where A_0 is the thrombus area immediately before infusion of the test agents (time 0) and A_n is the area of the thrombus at time intervals during thrombolysis (time n). G_0 is the grey reading at time 0 and G_n is the equivalent reading at time n. **b** Three-dimensional thrombus image obtained by multiplication of area and grey scale. T = Thrombus; V = vessel wall; L = lumen.

Computerised Image Analysis of Thrombolysis

The process of thrombolysis was continuously recorded on a videotape recorder. Subsequently, images at fixed time intervals were transferred to a personal computer and were analysed with Image Analyst software (Automatix, USA). The method used to calculate the dimensions of a thrombus is shown in figure 1. The consolidated thrombus within the vessel lumen was enclosed by a box. The grey scale threshold level was set to delineate the thrombus and the thrombus area was measured. The thrombus size was calculated by multiplication of area and grey scale value. This calcu-

lation provided an estimate of the thrombus volume. Thrombus size during thrombolysis was expressed relative to that of the thrombus immediately before agent administration.

Transmission Electron Microscopy

Sections of mesenteric arterioles with or without thrombi were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 postfixed with 1% osmium tetroxide, dehydrated through a graded series of acetone and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and

Fig. 2. Representative transmission electron micrographs of thrombi in arterioles. **a** Magnification $\times 3,400$, bar = 1.0 μm . **b** $\times 17,600$, bar = 0.5 μm . E = Endothelial cell.

were examined under a JEM 2000EX electron microscope.

Statistical Analysis

The results were analysed by multiple repeated ANOVA followed by Duncan's post hoc test and were expressed as mean $\pm$ SEM.

Results

Transmission Electron Microscopy Observation of He-Ne Laser-Induced Thrombi

Representative electron micrographs are shown in figure 2. The thrombi formed in arterioles were shown to be composed of densely packed platelets. Within the thrombi, the platelets were degranulated. Erythrocytes

and fibrin were not observed. Moreover, fibrin fibres were not detected even at high magnification. Endothelial cells under the thrombi were morphologically intact and denudation was not evident.

Thrombolysis Induced by rSAK

rSAK was infused after stabilising the thrombi for 10 min. The process of thrombolysis is shown in figure 3. Thrombus size decreased over time in the absence of rSAK. Thrombolysis was significantly enhanced by rSAK above a dose of 0.39 mg/7.7 ml/kg/h in a dose-dependent manner. The minimum effective dose of rSAK was 0.77 mg/7.7 ml/kg/h and was evident for at least 25 min after infusion.

Fig. 3. Thrombolysis induced by various doses of rSAK in rat mesenteric arterioles. rSAK or PBS was infused at a rate of 7.7 ml/kg/h (0–60 min). □ = 3.9 mg/7.7 ml/kg/h; △ = 1.9 mg/7.7 ml/kg/h; ◇ = 0.77 mg/7.7 ml/kg/h; ▽ = 0.39 mg/7.7 ml/kg/h; ○ = PBS (control). n = 5–6 rats in each group. * $p < 0.05$; ** $p < 0.01$ versus control at each time interval.

Effect of Concomitant Administration of Coagulation Inhibitors on rSAK-Induced Thrombolysis

Effect of APC. The thrombi were stabilised for 10 min and the minimum effective dose of rSAK (0.77 mg/7.7 ml/kg/h) was infused immediately after a bolus injection of APC (0.54 or 1.62 mg/2.31 ml/kg). The results are shown in figure 4. Significantly enhanced thrombolysis was observed at an APC dose of 1.62 mg/2.31 ml/kg. The effects were observed 10 min after APC administration and lasted at least 60 min. 0.54 mg/2.31 ml/kg of APC did not enhance thrombolysis.

Effect of Argatroban. The thrombi were stabilised for 10 min and different doses of argatroban (0.67 or 2.0 mg/4.0 ml/kg/h) were infused together with the minimum effective dose of rSAK (0.77 mg/7.7 ml/kg/h). The results are shown in figure 5. Argatroban sig-

nificantly enhanced thrombolysis at a dose of 2.0 mg/4.0 ml/kg/h. At a dose of 0.67 mg/4.0 ml/kg/h, argatroban did not enhance rSAK-induced thrombolysis.

Effect of Coagulation Inhibitors on Spontaneous Thrombolysis in the Absence of rSAK

Effect of AMCA and APC Alone on Spontaneous Thrombolysis. AMCA alone at a dose of 121 mg/7.7 ml/kg/h and APC alone at a dose of 1.62 mg/2.31 ml/kg were given as bolus injections after stabilisation of the thrombus. The results are shown in figure 6. AMCA alone inhibited spontaneous thrombolysis compared to the control group (PBS + vehicle). APC at a dose of 1.62 mg/2.31 ml/kg significantly enhanced spontaneous thrombolysis. AMCA (121 mg/7.7 ml/kg/h) infused together with APC at a dose of 1.62 mg/

Fig. 4. Effect of APC administration on rSAK-induced thrombolysis in rat mesenteric arterioles. rSAK at a dose of 0.77 mg/kg/h was infused through a femoral vein. APC at a dose of 0.54 or 1.62 mg/2.31 ml/kg or vehicle was administered as a bolus injection (0 min) immediately before rSAK infusion (0–60 min). □ = 1.62 mg/2.31 ml/kg + rSAK; △ = 0.54 mg/2.31 ml/kg + rSAK; ○ = vehicle + rSAK (control). n = 6–8 rats in each group. * $p < 0.05$; ** $p < 0.01$ versus vehicle + rSAK at each time interval.

Fig. 5. The effect of concomitant argatroban administration on rSAK-induced thrombolysis in rat mesenteric arterioles. rSAK and argatroban were infused through separate femoral veins at a dose of 0.77 mg/7.7 ml/kg/h and 0.6 or 2.0 mg/4.0 ml/kg/h, respectively (0–60 min). □ = Argatroban (2.0 mg/4.0 ml/kg/h) + rSAK; △ = argatroban (0.67 mg/4.0 ml/kg/h) + rSAK; ○ = vehicle + rSAK (control). n = 6–8 rats in each group. * $p < 0.05$; ** $p < 0.01$ versus vehicle + rSAK at each time interval.

Fig. 6. Endogenous thrombolysis induced by APC alone in rat mesenteric arterioles and its inhibition by AMCA. APC at a dose of 1.62 mg/2.0 ml/kg or vehicle was administered as a bolus injection into a femoral vein (0 min). AMCA at a dose of 121 mg/7.7 ml/kg/h or PBS (7.7 ml/kg/h) was infused through the alternative femoral vein (0–60 min). $\square$ = PBS + APC; $\circ$ = PBS + vehicle; $\diamond$ = AMCA + APC; $\triangle$ = AMCA + vehicle. $n = 6-8$ in each group. ^a $p < 0.05$ versus PBS + vehicle; ^b $p < 0.05$ versus PBS + APC at each time interval.

Fig. 7. Endogenous thrombolysis induced by argatroban alone in rat mesenteric arterioles and its inhibition by AMCA. Argatroban at a dose of 2.0 mg/4.0 ml/kg/h or vehicle was infused through a femoral vein, and AMCA at a dose of 121 mg/7.7 ml/kg/h or PBS was infused through the alternative femoral vein (0–60 min). $\diamond$ = PBS + argatroban; $\circ$ = PBS + vehicle; $\square$ = AMCA + argatroban; $\triangle$ = AMCA + vehicle. $n = 6-8$ in each group. ^a $p < 0.05$; ^b $p < 0.01$ versus PBS + vehicle; ^c $p < 0.05$ versus PBS + argatroban at each time interval.

2.31 ml/kg not only prevented the acceleration of thrombolysis induced by APC, but also inhibited thrombolysis to the level observed in the presence of AMCA alone (AMCA + vehicle).

Effect of Argatroban Alone on Spontaneous Thrombolysis. Argatroban alone at a dose of 2.0 mg/4.0 ml/kg/h was infused after stabilisation of the thrombus. The results are shown in figure 7. Argatroban alone (PBS + argatroban) significantly accelerated spontaneous thrombolysis compared to the control (PBS + vehicle). AMCA prevented the accelerated thrombolysis induced by argatroban to the level observed in the control animals (PBS + vehicle), but not to the level observed in those given AMCA alone (AMCA + vehicle).

Discussion

In the presence of Evans blue, He-Ne laser irradiation induces thrombogenesis in vessels. Evans blue converts laser energy to heat resulting in disturbances of endothelial integrity and in thrombus formation. We [10] and others [11] have shown that the thrombi formed in this way are primarily platelet-rich and that platelets adhere and aggregate on morphologically uninjured endothelial cells [10, 11]. The absence of overt endothelial damage was confirmed in the present study (fig. 2). This experimental procedure differs, therefore, from other models, which depend on endothelial denudation and fibrin-rich thrombus formation [12–17]. The minimum dose of rSAK required to significantly enhance thrombolysis in arterioles in the present study was 0.77 mg/kg/h. In contrast, we have previously demonstrated that the corresponding minimum dose of rSAK in venules was 5.8 mg/kg/h [5]. This difference in thrombolytic sensitivity might be related to the higher rate of blood flow in the arterial circulation.

The thrombi observed in the current investigation were densely packed with platelets and fibrin was not detected by high-magnification, transmission electron microscopy. Nevertheless, the thrombi were lysed in a dose-dependent manner by rSAK, which has a high specificity for fibrin [18–20]. Moreover, the dimensions of the He-Ne laser-induced thrombi were decreased by 29–36% in 60 min in the absence of any exogenous fibrinolytic agents, and these decreases were partially suppressed by AMCA (fig. 6, 7). Some spontaneous decrease in thrombus size was apparent, even in the presence of AMCA; the role that other cellular products, such as leukocyte elastase, play in these thrombin-related reactions remains to be evaluated. Moreover, AMCA not only inhibited spontaneous or endogenous thrombolysis but also prevented the APC- or argatroban-induced enhancement of thrombolysis. In the presence of argatroban, however, the level of inhibition did not reach that observed with AMCA alone. The reasons for these discrepancies remain to be fully explored, but it may be that the continuous inactivation of thrombin by argatroban contributed to the thrombolytic mechanisms. AMCA is not an active site-directed inhibitor of plasmin but specifically inhibits the binding of plasminogen or plasmin to fibrin resulting in depressed fibrinolysis [21]. It seems likely, therefore, that although we failed to detect fibrin morphologically in our He-Ne laser-induced thrombi, fibrin-related reactions were involved in the thrombogenic mechanisms.

The rSAK-induced thrombolysis observed in the present in vivo experiments was further enhanced by the inhibition of thrombin and its generation by argatroban and APC, respectively. These results were consistent with other in vitro investigations which demonstrated that PA-induced thrombolysis was accelerated by APC [22]. APC is known to inhibit the

coagulation cascade, resulting in decreased thrombin generation. Thus, fibrin formation and platelet aggregation may be suppressed leading to more rapid thrombus dissolution. This seems to be true also for argatroban. Our original hypothesis was that the enhancement of rSAK-induced thrombolysis by argatroban and APC may be due to the inhibition of thrombus growth. The present study demonstrated, however, that spontaneous thrombolysis occurred in the absence of any agents, and that APC and argatroban enhanced these endogenous mechanisms. The precise modes of action of APC and argatroban remain to be clarified, but the present findings were in keeping with the suggestion that APC and argatroban moderate endogenous thrombolysis or fibrinolysis by attenuating the activity of thrombin-activatable fibrinolysis inhibitor [23–27]. Furthermore, it has been shown that rSAK per se can increase endogenous APC [28], and it may be that the inactivation of thrombin-activatable fibrinolysis inhibitor

contributes to the enhancement of rSAK-induced thrombolysis mediated by APC and argatroban.

In conclusion, we have established a simple and reproducible model of arterial thrombolysis using rat mesenteric microvessels. The present findings demonstrated that enhanced thrombolysis induced by argatroban or APC in the presence or absence of rSAK is mediated by accelerated endogenous thrombolytic activities. The present model offers a useful protocol for assessing the relationship between thrombin-activatable fibrinolysis inhibitor, APC, argatroban, and thrombolysis in vivo.

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