Introduction

Thrombin is a key enzyme converting fibrinogen to fibrin and a physiologically important agonist of platelet activation. Clinical trials and animal experiments have shown that thrombin inhibition prevents thrombosis, accelerates exogenous plasminogen activator-induced thrombolysis and prevents reocclusion after successful thrombolytic therapy (1-9).

Recent studies have indicated that thrombin makes fibrin lysis-resistant via activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (10-16). TAFI is activated by thrombin. Activated TAFI (TAFIa) cleaves the plasminogen binding site on fibrin or fibrinogen, resulting in lysis-resistant fibrin. Hashimoto et al. have shown that thrombin inhibition induces endogenous thrombolysis and that this lysis in vivo is moderated by TAFI as well as by other plasma inhibitors (7, 17). The mechanism by which TAFI inhibition influences endogenous thrombolysis or spontaneous plasmin generation is unclear, however. Plasmin generation in these circumstances might be induced by tissue-type (tPA) and urokinase-type (uPA) plasminogen activators (18). Whether these mechanisms play a dominant role in endogenous thrombolysis in vivo, using our rat mesenteric arterial thrombolysis model.

Argatroban infusion enhanced endogenous thrombolysis. PKSI-527, anti uPA and anti tPA IgGs suppressed argatroban-induced thrombolysis. Also, the antibody IgG preparations suppressed endogenous thrombolysis in the absence of argatroban. In the presence of PKSI-527, anti tPA IgG was more effective than anti uPA IgG in suppressing argatroban-induced thrombolysis. The results suggested that both tPA and plasma kallikrein-mediated uPA activation and tPA release contribute to endogenous fibrinolytic or thrombolytic mechanisms.

Suppression of argatroban-induced endogenous thrombolysis by PKSI-527, and antibodies to TPA and UPA, evaluated in a rat arterial thrombolysis model

Masaru Hashimoto1,2, Kazuhiro Oiwa3, Osamu Matsuo4, Shigeru Ueshima4, Kiyotaka Okada4, Yoshio Okada2,6, Shosuke Okamoto5, John C. Giddings7, Junichiro Yamamoto1,2

1Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University, Kobe, 2High Technology Research Laboratory, Kobe Gakuin University, Kobe, 3Communications Research Laboratory, Kansai Advanced Research Centre, Kobe, 4Second Department of Physiology, Kinki University School of Medicine, Osaka, 5Kobe Research Projects on Thrombosis and Haemostasis, Kobe, 6Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe, Japan 7Department of Haematology, University of Wales College of Medicine, Cardiff, UK

Summary

We have previously confirmed, using a rat mesenteric arteriole thrombolysis model, that thrombin inhibition induces endogenous thrombolysis in vivo. In addition, we have shown that thrombin-activatable fibrinolysis inhibitor (TAFI) plays a role in the down regulation of endogenous thrombolysis. However, the mechanism of endogenous thrombolysis or spontaneous plasmin generation in vivo remains unclear.

It has been shown in an in vitro system that plasma kallikrein activates pro-urokinase (pro uPA) and/or plasminogen, resulting in plasmin generation. These findings suggest that spontaneous fibrinolysis might be mediated by tPA and plasma kallikrein-dependent uPA. The aim of the present study was to examine whether these mechanisms play a dominant role in endogenous thrombolysis in vivo, using our rat mesenteric arterial thrombolysis model.

Argatroban infusion enhanced endogenous thrombolysis. PKSI-527, anti uPA and anti tPA IgGs suppressed argatroban-induced thrombolysis. Also, the antibody IgG preparations suppressed endogenous thrombolysis in the absence of argatroban. In the presence of PKSI-527, anti tPA IgG was more effective than anti uPA IgG in suppressing argatroban-induced thrombolysis. The results suggested that both tPA and plasma kallikrein-mediated uPA activation and tPA release contribute to endogenous fibrinolytic or thrombolytic mechanisms.
minogen activators. In addition, in vitro studies have demonstrated that plasma kallikrein not only activates factor XII leading to fibrin formation but also activates pro-UK resulting in plasmin generation (18-21).

The aim of the present study was to investigate the role of plasma kallikrein, tPA and uPA in spontaneous plasmin generation in vivo using a standardised animal model of arterial thrombolysis established in our laboratory.

**Materials and methods**

**Animals**

Male Wistar ST rats aged 8 weeks and weighing 230-270 g were obtained from SLC Co. Ltd. (Hamamatsu, Japan). The animals were allowed drinking water *ad libitum* and were fasted overnight prior to the thrombolysis experiments to suppress peristaltic movement of the intestine 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**

Plasma kallikrein synthetic inhibitor (PKSI-527) was prepared in our laboratory as previously described (22) and was dissolved and diluted in physiological saline immediately before use. Argatroban (Novastan, 0.5 mg/ml) was purchased from Mitsubishi Tokyo Pharmaceutical Co. Ltd. (Tokyo, Japan) and diluted in physiological saline. Antibodies against tPA and uPA were produced in our laboratory and were stored at −80 °C (23). Antibody titres were assessed by gel immunoprecipitation using the Ouchterlony technique (24), and were diluted to a standard concentration with phosphate buffered saline (PBS, pH 7.4) before use. Control IgG was purchased from Zymed Laboratoried Inc (San Francisco, USA) and diluted in PBS.

Argatroban was administered by continuous infusion at a dose of 2.0 mg/kg/h. PKSI-527 was given at a dose of 100 mg/kg/h. The rates of infusion were 4.0 ml/h and 7.7 ml/h respectively. Antibody IgG and control IgG were given at a standard concentration of 0.5 mg/kg in a volume of 1.54 ml/kg.

**Helium-Neon (He-Ne) laser-induced thrombosis and thrombolysis**

Microvascular thrombi were produced in rat mesenteric arterioles as described previously (7). Briefly, animals were anaesthetised with nembutal (70 mg/kg, i.m.) and canulae (PE50: inner diameter 0.58 mm, outer diameter 0.97 mm) were inserted into both femoral veins and the right jugular vein 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 seconds at 15 seconds interval until the extent of the thrombus reached 90% of the lumen diameter. The thrombus was then allowed to stabilise for 10 minutes before test substances were given through the femoral vein or right jugular vein. PKSI-527 and/or argatroban were infused for 60 minutes through the femoral vein or right jugular vein. Anti tPA IgG, anti uPA IgG or control IgG were given as bolus injections. Experiments were performed over as short a time-scale as possible to minimise possible inter-batch variations in the animal model.

**Figure 1:** Evaluation of thrombolysis using Image Analyst software. The thrombus within the lumen of the arteriole was enclosed in a rectangular box and a threshold level defining the thrombus area was set on a grey scale. The extent of thrombolysis was calculated from the following formula: An x Gn / Ao x Go, where Ao is the thrombus area immediately before infusion of the test agents (time 0) and An is the area of the thrombus at time intervals during thrombolysis (time n). Go is the grey reading at time 0 and Gn is the equivalent reading at time n.
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 by Image Analyst software (Automatix, USA), as previously reported (7, 17). The method used to calculate the dimensions of an individual thrombus is shown in figure 1. Briefly, 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 calculation provided an estimate of thrombus volume. Thrombus size during thrombolysis was expressed relative to that of the thrombus immediately before agent administration.

Blood pressure

Blood pressure was measured in the femoral artery at 0, 10 and 60 min using indwelling canulae and a disposable pressure transducer (model DT-XXAD, Viggo-Spectramed, USA). Mean blood pressure was calculated by the following formula; mean blood pressure = 1/3 (2 × minimum blood pressure + maximum blood pressure).

Statistical analysis

The results were analysed by multiple repeated ANOVA followed by Fisher PLSD post hoc test and were expressed as mean ± SEM. p <0.05 was considered as statistically significant.

Results

Effect of PKSI-527 on argatroban-induced endogenous thrombolysis

After stabilisation of the thrombus for 10 min, argatroban alone at a dose of 2.0 mg/kg/h was infused though a femoral vein. The results are shown in figure 2. Argatroban alone (saline + argatroban: ■) significantly accelerated thrombolysis compared with the control (saline + saline; ○: p < 0.001). PKSI-527 (100 mg/kg: PKSI-527 + argatroban: ▲) significantly suppressed this argatroban-induced thrombolysis (saline + argatroban: ■; p < 0.0001).

Effect of antibodies against plasminogen activators on argatroban-induced endogenous thrombolysis

After stabilisation of the thrombus, bolus injections of anti tPA IgG (■), anti uPA IgG (▲) or control IgG (○) were administered followed immediately by argatroban. The results are shown in figure 3. Both antibodies significantly and equally prevented argatroban-induced endogenous thrombolysis (control IgG + argatroban vs anti uPA IgG + argatroban: p <0.005 and vs anti tPA IgG + argatroban: p <0.005). Specific reactions against uPA

Figure 2: Argatroban-induced endogenous thrombolysis and prevention of its thrombolysis by plasma kallikrein synthetic inhibitor (PKSI-527). Argatroban or saline (2.0 mg/kg/h) and PKSI-527 or saline (100 mg/7.7 ml/kg/h) were infused through femoral veins at a rate of 4.0 ml/h and 7.7 ml/h, respectively. control: saline + saline (○); argatroban: saline + argatroban (■); argatroban + PKSI-527 (▲). n = 5~6 rats in each group. §§: p <0.001, ††: p <0.0001.

Figure 3: Inhibition of argatroban-induced endogenous thrombolysis by antibodies against plasminogen activator. Argatroban (2.0 mg/kg/h) was infused through a femoral vein at a rate of 4.0ml/h. Anti tPA IgG (0.5 mg/kg), anti u-PA IgG (0.5 mg/kg) or control IgG (0.5 mg/kg) was administered as a bolus injection in the other femoral vein immediately before argatroban infusion. control IgG + argatroban (○), anti tPA IgG + argatroban (■), anti uPA IgG + argatroban (▲) and baseline (saline + saline: ○) n = 5~6 rats in each group. ***: p <0.01, §: p <0.001.
Regulation of endogenous plasminogen activation by plasma kallikrein and tPA were confirmed by the Ouchterlony technique. No cross-reactive immunoprecipitation was observed.

**Effect of antibodies against plasminogen activators on argatroban-induced endogenous thrombolysis in the presence of PKSI-527**

To investigate the relationship between the plasminogen activators and plasma kallikrein in endogenous thrombotic mechanisms, anti tPA IgG, anti uPA IgG or control IgG were given as bolus injections immediately before infusion of PKSI-527 or argatroban. The results, shown in figure 4, confirmed that argatroban-induced endogenous thrombolysis, (compare control IgG + saline + saline: ○ with control IgG + argatroban + saline: ◊; p <0.01), was suppressed by PKSI-527 (control IgG + argatroban + PKSI-527: ●; p <0.0001). Anti tPA (anti tPA IgG + argatroban + PKSI-527: ▲) but not anti uPA (anti uPA + argatroban + PKSI-527: ▲) further suppressed the endogenous thrombolysis significantly compared with control IgG (●; p <0.05).

**Effect of PKSI-527 on blood pressure**

Mean blood pressure was measured at 0, 10 and 60 min after PKSI-527 infusion. The results are shown in figure 5. Blood pressure was not affected significantly by PKSI-527.

**Discussion**

A variety of models have been established to examine mechanisms of thrombolysis in vivo. (14-17, 25). Procedures of this nature designed to study endogenous thrombolysis, need to be especially sensitive and reproducible, however, because intravascular responses to naturally occurring plasminogen activators (PAs) may be considerably weaker than those induced by exogenous fibrinolytic activators such as streptokinase. We have previously established a highly sensitive, reproducible and quantitative platelet-rich thrombolysis model using rat mesenteric arterioles and demonstrated that endogenous thrombolysis was induced in the presence of argatroban, a specific thrombin inhibitor. Lysis was suppressed by the specific plasmin inhibitor, tranexamic acid (7, 26). In the present study we have extended this model to investigate precise mechanisms of endogenous plasmin generation.

The dose of argatroban required to stimulate the fibrinolytic effect in rats was higher than that used clinically in humans.
(180 µg/kg/h; 27). Nevertheless, no adverse side effects were observed in our current studies and there was no excessive bleeding from intravenous sites. We have previously observed that rats were relatively insensitive to the effects of ticlopidine compared with man (28) and it seems likely that our animal model is also less sensitive to direct thrombin inhibition by argatroban. The precise mode of action of argatroban in these circumstances remains to be determined, however. We have also shown that TAFI plays a role in moderating endogenous fibrinolysis (7, 17). Delayed activation of TAFI by inhibition of thrombin might have promoted thrombolysis in our experiments. In addition, impaired activation of factor XIII might have rendered fibrin-thrombi more fragile, and depressed thrombin-induced platelet interactions might have contributed to an enhanced susceptibility to thrombolysis.

We have now demonstrated that antibodies against tPA and uPA suppressed argatroban-induced thrombolysis, indicating that both these activators contributed to endogenous fibrinolysis. Moreover, the plasma kallikrein inhibitor, PKSI-527, also suppressed argatroban-induced thrombolysis. Anti tPA but not anti uPA further suppressed argatroban-induced endogenous thrombolysis in the presence of PKSI-527. These results suggest that endogenous uPA might be generated by plasma kallikrein-mediates activation of pro uPA. Plasma kallikrein regulates blood pressure and although PKSI-527 tended to raise blood pressure in the present study, the differences between the treated and control animals were not statistically significant. It seems unlikely, therefore, that PKSI-mediated changes in blood pressure contributed significantly to the observed fibrinolytic responses. Plasma kallikrein is also known to mediate the generation of bradykinin (BK) from kininogen, and BK has been shown to stimulate the release of tPA from endothelial cells (29, 30). The inhibition of endogenous thrombolysis by PKSI-527 might have been due, therefore, to a combined effect on uPA production and tPA release. Anti-tPA but not anti-uPA, however, further suppressed fibrinolysis in the presence of PKSI-527, and it appeared, therefore, that tPA-induced plasmin generation might have been influenced by both kallikrein-dependent and kallikrein-independent mechanisms.

Previous studies have shown that tPA has a high affinity for fibrin, approximately 20-fold greater than pro uPA (31), suggesting that tPA might be more active than uPA in fibrinolysis. In the present study, however, both anti tPA and anti uPA equally inhibited argatroban-induced thrombolysis, indicating that both activators play a significant role in fibrinolytic mechanisms. Furthermore, we have previously demonstrated, by transmission electron microscopy, that He-Ne laser-induced thrombi are mainly composed of platelets (7), and studies in vitro have determined that pro uPA binds to platelet membranes (32). It is known that pro uPA is activated by plasmin and plasma kallikrein (18-21), and it has been shown that prekallikrein activation by its endothelial cell activator on cells in culture or on a cell matrix leads to kinetically favourable pro uPA activation. (20, 21). Furthermore, it has been suggested that platelet-bound pro uPA is involved in platelet-clot lysis (33-36). Our data extend these earlier findings and indicate that both naturally occurring uPA and tPA significantly induce dissolution of platelet-rich thrombi.

Other endothelial interactions might also influence localised fibrinolytic mechanisms. Laser-irradiation of the luminal blood vessel surface does not cause morphological damage to the endothelium in the model (7, 37, 38), but might disturb functional properties including the release of tPA and plasminogen activator inhibitor (PAI-1). In addition, we consistently observe leukocyte-rolling on the vessel wall and incorporation into developing platelet-rich-thrombi in our video images, and it may be that white cell enzymes such as cathepsins affect thrombus pathology. The nature of these complex reactions was outside the scope of the present study, however, and remains to be fully explored. Moreover, we recently demonstrated that endogenous thrombolysis was enhanced not only by argatroban but also by activated protein C and a specific inhibitor of factor Xa (DX-9065a; 7, 17, 39). Overall, therefore, there appears to be a number of haemostatic reactions that may be involved in the suppressed fibrinolytic activity reported in patients with cardiovascular diseases (40, 41). Further studies in this field could lead to the development of challenging new strategies for thrombolytic therapy.

In conclusion, our findings show that spontaneous or argatroban-induced thrombolysis is regulated by both endogenous tPA and plasma kallikrein-related uPA mechanisms.

References


