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The interaction between components of the fibrinolytic system and GPIb/V/IX of platelets thrombus formation in mice

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1 The interaction of fibrinolytic components with GPIb/V/IX of platelets on thrombus formation, was investigated in mice deficient in tissue type (tPA−/−), urokinase type plasminogen activator (uPA−/−) or plasminogen activator inhibitor-1 (PAI-1−/−) and in their wild type control (tPA+/+, uPA+/+, PAI-1+/+).

2 A thrombus was induced in the murine carotid artery using a photochemical reaction. The times to occlusion after the initiation of endothelial injury in all wild type mice was within 12 min, and no significant changes in occlusion delay were observed in uPA−/− and tPA−/− mice compared to wild type mice, whereas that of PAI-1 mice were significantly prolonged (16.9 ± 2.9 min, $P<0.05$).

3 When high molecular weight aurintricarboxylic acid (ATA), an inhibitor of platelet glycoprotein Ib/V/IX, was administered, the time to occlusion was prolonged in a dose-dependent manner in all types of mice. However, when this compound was injected in tPA−/− mice, the most significant changes were observed: i.e. the estimated ED_{50} was 20.2 times higher than that in tPA+/+ mice, but the estimated ED_{50} in uPA−/− mice was not changed as compared with that of wild type mice. On the other hand, when ATA was injected in PAI-1−/− mice, the estimated ED_{50} was significantly decreased ($P<0.05$).

4 Platelet aggregation induced by botrocetin was not significantly different among all types of mice. The bleeding time was prolonged in a dose dependent-manner when ATA was injected in all types of mice.

5 In conclusion, the antithrombotic effect of inhibition of platelet GPIb/V/IX is severely affected by the absence or presence of tPA-production on thrombus formation and the inhibition of PAI-1 could enhance this antithrombotic effect.

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Abbreviations: ATA, aurinotrycarboxylic acid; GP, glycoprotein; PAI-1, plasminogen activator inhibitor-1; PRP, platelet-rich plasma; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator

Introduction

The development of thrombus formation plays an important role in the pathogenesis of several vascular diseases, including unstable angina pectoris, acute myocardial infarction and stroke (Fuster, 1994). These processes are the results of a complex interplay among blood components such as platelets, coagulation factors and fibrinolytic factors, which individually play an important role but furthermore also interact with each other *in vivo*. The blood fibrinolytic system, which degrades intravascular fibrin is activated by urokinase-type plasminogen activator (uPA) (Matsuo *et al*., 1986) or by tissue type plasminogen activator (tPA) (Matsuo *et al*., 1981) enzymes that convert plasminogen to fibrinolytic protease plasmin (Collen & Lijnen 1991). However, cofactors, inhibitors or other proteases may also contribute to the regulation of vascular fibrinolysis (Plow *et al*., 1995). Moreover, PAI-1 is also an important factor in fibrinolytic system. Several studies suggest that plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrinolysis by binding irreversibly to the active site of tPA or uPA, is a major determinant of the resistance of platelet rich clots to lysis by tPA *in vitro* and *vivo* (Levi *et al*., 1992; Braaten *et al*., 1993; Stringer *et al*., 1994). However, other studies suggest that PAI-1 plays only a minor role in regulating the lysis of platelet rich clots (Kunitada *et al*., 1992). These contrasting results probably resulted from the variable concentrations of platelets, tPA, PAI-1, and other factors that were used in different *in vitro* experiments.

On the other hand platelet adhesion to the vessel wall triggers thrombus formation. Several *in vitro* experiments have confirmed the involvement of the von Willebrand factor (vWF)–GPIb axis in the early phase of thrombus formation (Coller *et al*., 1983; Ikeda *et al*., 1991), but vWF was also reported to play a role during *in vivo* studies (Miller *et al*., 1991). Aurintricarboxylic acid (ATA), a triphenylmethyl dye compound, inhibits platelet adhesion by interfering with vWF binding to platelet glycoprotein (GP) Ib (Phillips *et al*., 1988), thus preventing thrombus formation *in vivo* (Strony *et al*., 1990; Takiguchi *et al*., 1996). ATA has multiple effects on platelet activation (Matsuno *et al*., 1998). Commercial ATA contains a large number of molecular size variants which have different inhibitory effects on vWF-GPIb (Phillips *et al*., 1988). We previously reported how fractionated ATA (mean

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molecular weight = 7500) selectively inhibited the aggregation of platelets induced by botrocetin and how it reduced neointima formation (Matsuno *et al*., 1997). The adhesion of platelets plays a trigger of the development of occlusive thrombus formation.

We previously reported a simple and reproducible thrombus model in rat femoral artery (Matsuno *et al*., 1992) which is useful for the development of thrombus formation in small animals. Recently we applied this system in mice and investigated the role of fibrinolytic components in the formation and removal of thrombus (Matsuno *et al*., 1999). Moreover, the interaction between the lack of tPA and either a GPIIb/IIIa antagonist or a thrombin inhibitor was investigated using mice deficient in tPA (Matsuno *et al*., 2000). Our previous data indicated that the inhibitory effect of a GPIIb/IIIa antagonist on thrombus formation is diminished by the lack of tPA in mice, however the antithrombotic action of a thrombin inhibitor was not affected by either presence or absence of tPA in mice. Therefore, in the present study, we focused on the antithrombotic effects of an inhibitor of platelet GPIb/V/IX, high molecular weight ATA fractionated by commercial ATA, in normal and in mice with targeted disruption of tPA, uPA or PAI-1 gene, since the prevention of function of platelet GPIb/V/IX or the inhibition of PAI-1 has become of major interest for antithrombotic therapy.

Methods

Animals

Gene-deficient mice were generated by homologous recombination in embryonic stem cells, as described previously (Carmeliet *et al*., 1993; 1994). Six mice were used in each group. All experiments were performed in accordance with institutional guidelines.

Reagents

Fractionated aurintricarboxylic acid (ATA) was obtained from commercial ATA (Aldrich) using gel permeation chromatography as described previously (Kawasaki *et al*., 1994). The average molecular weight was estimated by comparison with those of standard polystyrene polymers. The compound (average molecular weight = 7500, the index of dispersion = 1.5, higher molecular ATA) was selected and used for experiments. The other chemical substances were obtained from Sigma Chemical Co. Ltd. Botrocetin was purified from *bothrops jararaca* by Fujimura's methods (Fujimura *et al*., 1991).

Production of endothelial injury

The experimental procedure to induce an endothelial injury has been described in detail previously (Matsuno *et al*., 1991, 1999). Mice ($n = 6$, each) were anaesthetized by intraperitoneal injection of 44 mg kg^{-1} sodium pentobarbitone. In brief, the right common carotid artery, the left jugular vein and the right femoral artery were exposed under the anaesthesia with pentobarbitone. Catheters (ID = 0.5 mm, OD = 0.8 mm, polyethylene sp3, Natume Co. Ltd., Tokyo, Japan) were connected to the left jugular vein and right femoral artery for the injection of rose bengal (30 mg kg^{-1}) and for monitoring blood pressure and pulse rate using a pressure transducer (AP601G Nihon Koden, Tokyo, Japan) during experiments on day 0. Blood flow in the carotid artery was continuously monitored using a doppler flow probe (Model PDV-20, Crystal Biotech Co. Ltd., Tokyo, Japan) positioned proximally to the injured area of the carotid artery. Irradiation of green light (540 nm) was started and then rose bengal was injected as a bolus 10 min after the observation of control blood flow. The irradiation was continued for 15 min after the injection of rose bengal. This procedure results in destruction of endothelial cells in the irradiated area by oxygen radicals by photochemical reaction between rose bengal and green light. Our previous histological observations have revealed that platelet rich thrombus was obviously established when the blood flow was zero.

Infusion regimen to prevent in vivo *thrombus formation*

High molecular weight ATA was administered by continuous intravenous infusion using an infusion pump (TERMO STC-523, TERMO, Tokyo, Japan). The infusions were started 20 min before the initiation of endothelial injury and continued for 60 min thereafter. Animals were divided into a control group (saline infusion), a group treated with various doses of high molecular weight ATA (10, 30, 100, 300 or 1000 μg kg^{-1} h^{-1} for tPA + / + and tPA − / − mice, 1 3, 10 or 30 μg kg^{-1} h^{-1} for PAI-1 − / − mice, 1, 3, 10, 30, 100 or 300 μg kg^{-1} h^{-1} for PAI-1 + / + mice or 10, 30 100 or 300 μg kg^{-1} h^{-1} for the other mice).

Bleeding times

At the end of infusion, a bleeding time was performed as described (Carmaliet *et al*., 1993). A distal 2 mM segment of the tail was severed with a razor blade after the measurement of blood flow. The caudal extremity was immediately immersed in 0.9% saline at 37°C with the tip of the tail 5 cm below the body. The bleeding time was defined as the time required for cessation of blood flow.

Ex vivo *platelet aggregation*

At the end of each experiment, blood was collected by heart puncture on sodium citrate (3.3%) under ether anaesthesia and centrifuged for 12 min $190 \times g$ to obtain platelet-rich plasma (PRP). The platelets in PRP were then counted and finally adjusted to 4×10^8 cells ml^{-1} (final concentration) with platelet poor plasma. Platelet aggregation was induced by 4.0 μg ml^{-1} botrocetin or 3.3 μg ml^{-1} collagen and followed in an aggregometer (Aggrecorder II, DA-3220, Kyotodaiichi-Chemical, Japan) at 37°C with 800 r.p.m. stirring speed. Aggregation is expressed as a percentage of the maximum light transmission obtained in the absence of drugs. All counts were done in duplicate.

Electron microscopic observation

Vascular surfaces in each mouse were followed removing denudated segments of the carotid arteries. These segments were prepared without rinsing to leave any formed platelets intact, and were fixed 2.0% glutaraldehyde in 50 mM sodium phosphate buffer for 30 min. Each segment was cut open longitudinally to allow visual inspection for scanning electron microscopy (SEM) as described (Matsuno *et al*., 1992).

Statistics

All data are expressed as mean ± s.e.mean. The significance versus the each wild type mouse was determined by ANOVA

followed by Willcoxon's test for the time to occlusion *in vivo* (* and ** are $P<0.05$ and $P<0.01$, respectively).

Results

Antithrombotic effect of high molecular weight ATA in vivo

Times to occlusion of each group are shown in Figure 1. When saline was infused in wild type mice (uPA+/+, tPA+/+, PAI-1+/+), times to occlusion were 10.5 ± 1.1, 10.7 ± 1.8 and 11.2 ± 1.0 min, respectively. In the groups of uPA−/− or tPA−/− mice, the time to occlusion in the case of an infusion of saline was slightly but not significantly shortened to 10.1 ± 0.9 and 9.2 ± 2.2, respectively. On the other hand, in PAI-1−/− mice, time to occlusion in the case of an infusion of saline was significantly prolonged to 16.9 ± 2.9 min as compared with that of PAI-1+/+ mice ($P<0.05$).

In wild type mice, times to occlusion were prolonged by the treatment with high molecular weight ATA in a dose-dependent manner. Administration of high molecular weight ATA at a dose of 10 or 30 $\mu g\ kg^{-1}\ h^{-1}$ to uPA−/− mice, did not significantly change the time required to occlude the carotid artery. When high molecular weight ATA at a dose of 100 $\mu g\ kg^{-1}\ h^{-1}$ was given, the time to occlusion in uPA−/− mice was significantly prolonged as compared with that of wild type mice. An estimated ED_{50} for high molecular ATA in uPA−/− mice ($ED_{50}=53\ \mu g\ kg^{-1}\ h^{-1}$) was not different that of uPA+/+ mice ($ED_{50}=49\ \mu g\ kg^{-1}\ h^{-1}$). In contrast, in tPA−/− mice, when high molecular weight ATA was given at doses of 10, 30, 100 or 300 $\mu g\ kg^{-1}\ h^{-1}$, no significant prolongation of the time to occlusion was obtained. A significant prolongation to 19.8 ± 3.9 min was only observed when high molecular weight ATA was infused at a dose of 1.0 mg $kg^{-1}\ h^{-1}$. An estimated ED_{50} for high molecular ATA in tPA−/− mice was 788 $\mu g\ kg^{-1}\ h^{-1}$ which was 17.9 times higher than that needed for tPA+/+ mice ($ED_{50}=44\ \mu g\ kg^{-1}\ h^{-1}$).

In PAI-1−/− mice, doses as low as 3 $\mu g\ kg^{-1}\ h^{-1}$ resulted in a significant prolongation (versus PAI-1+/+ mice), whereupon after treatment with 10 $\mu g\ kg^{-1}$ three out of the six arteries examined did no longer occlude within the observation period, although the blood flow was slightly decreased. An estimated ED_{50} for high molecular ATA in PAI-1−/− mice was 3.8 $\mu g\ kg^{-1}\ h^{-1}$ which was 13.6 times lower than that needed for PAI-1+/+ mice ($ED_{50}=52\ \mu g\ kg^{-1}\ h^{-1}$). Mean times to occlusion in each group are shown in Figure 2.

Bleeding time

The bleeding times are shown in Table 1. No significant difference was observed between gene-deficient mice and wild type mice. When high molecular weight ATA was administered, bleeding time was prolonged in a dose-dependent manner in all types of mice. The bleeding time was statistically significantly changed when high molecular weight ATA was administered at doses of 0.3 and 1.0 mg kg^{-1} and was especially prolonged when high molecular weight ATA at a dose of 1 mg kg^{-1} was administered to tPA−/− or tPA+/+ mice.

Ex vivo *platelet aggregation*

Maximum aggregation obtained with platelets from each group are shown in Figure 2. The changes from the control (infusion of saline) value were statistically significant when high molecular weight ATA was given at doses over

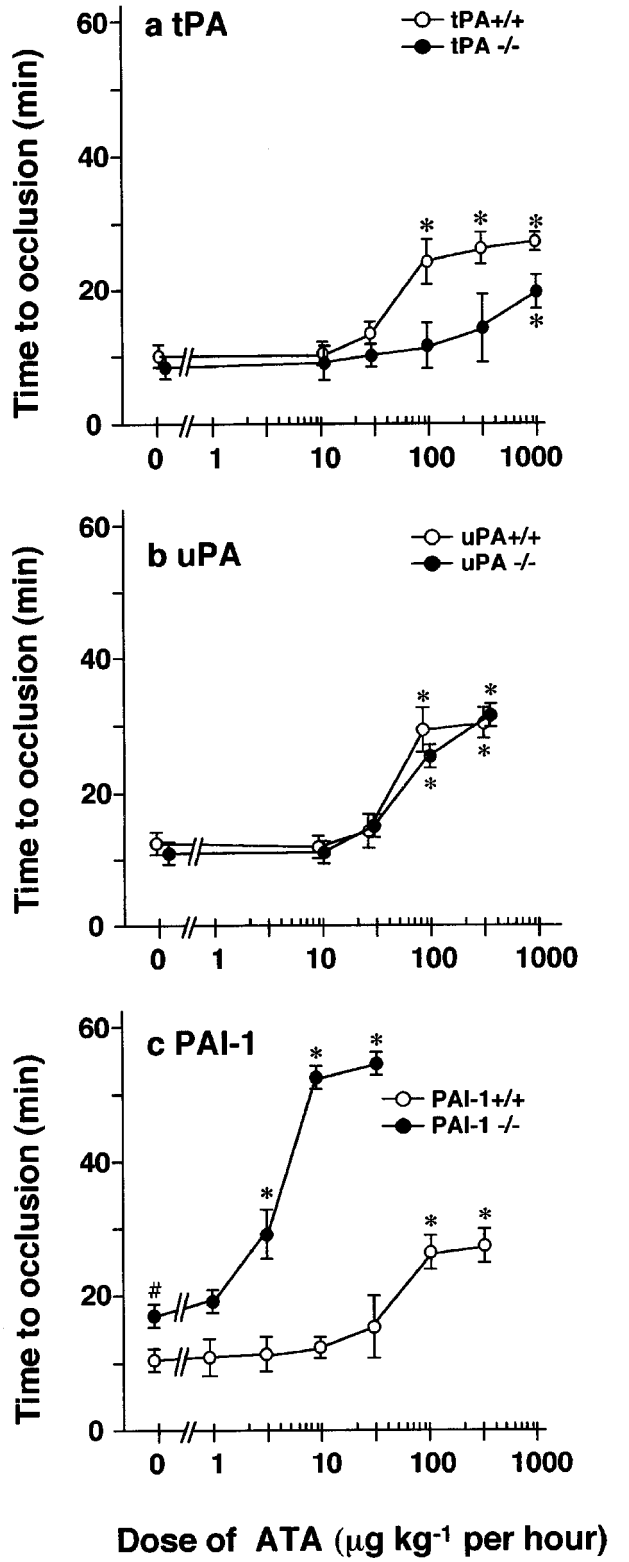

Figure 1 Inhibitory effect of high molecular weight ATA on thrombus formation in the carotid artery of tPA−/−, uPA−/−, PAI-1−/− or wild type mice. The carotid arterial blood flow was continuously monitored for 60 min after the initiation of endothelial injury using a photochemical reaction. Time to occlusion is given as mean±s.e.mean in each experiment. Times greater than 60 min are given as 60 min for calculation of mean±s.e.mean $*P<0.05$, $**P<0.01$ versus each control (infusion of saline). $\#P<0.05$ versus PAI-1+/+ mice.

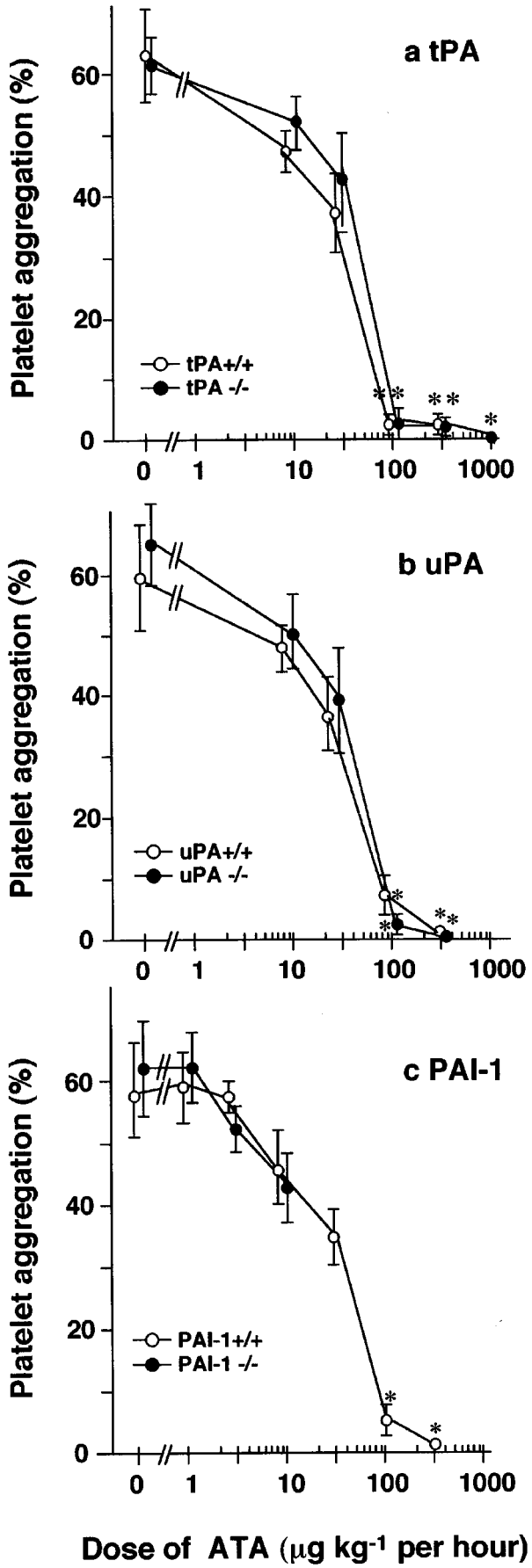

Figure 2 Dose dependent inhibition by high molecular weight ATA of platelet aggregation in PRP of tPA−/−, uPA−/−, PAI-1−/− and their wild type mice. Platelet aggregation was induced by botrocetin (4.0 μg ml^{-1}). Data are represented as mean±s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$ versus each control (infusion of saline).

100 μg kg^{-1} h^{-1} in all types of mice. The estimated IC_{50}-values in all mice-types were not significantly different. High molecular weight ATA did not affect platelet aggregation induced by collagen.

Histological observation

After the initiation of endothelial injury, thrombus formation was clearly observed using scanning electron microscopy in all types of mice. The thrombus included a lot of activated platelets, fibrin net and red blood cells (Figure 3a,d). However the thrombus formation was quantitatively different in PAI-1−/− mice compared to the others. In PAI-1−/− mice, fibrin nets are rarely observed in thrombi formed after endothelial injury (Figure 3c,f). On the other hand, in tPA−/− mice, local microthrombus developed by adherent platelets was observed even if an endothelial injury was not applied. Indeed the vascular surface is smooth without endothelial injury, however platelets are adhered on intact vascular surface (Figure 3b,e). These phenomena were not observed in the other type of mice.

Discussion

The present study was carried out to investigate the interaction between fibrinolytic components and an inhibition of platelet adhesion by interfering with vWF binding to platelet GPIb/V/IX, on thrombus formation *in vivo* using mice deficient in tPA, uPA or PAI-1.

Temporary prevention of GPIb/V/IX availability, representing a trigger of platelet activation, is expected to decrease thrombus formation resulting from multiple proaggregatory platelet stimuli. Indeed the presence of a GPIb/V/IX antagonist has been shown to decrease the time required to attain vascular reperfusion combined with thrombolytic drugs and subsequently maintained the arterial blood flow (Ito *et al.*, 1999). In this study, we could demonstrate that high molecular weight ATA has a significant antithrombotic effect in all types of mice. However the compound was markedly less effective in preventing thrombus formation in tPA−/− mice even though the inhibitory action of *ex vivo* platelet aggregation induced by botrocetin was not different among all the types of mice. These findings indicate that the antithrombotic effect of an interfering with vWF binding to platelet GPIb/V/IX *in vivo* closely depends on the availability of tPA. Very recently, we also reported that the antithrombotic effect of a GPIIb/IIIa antagonist was diminished in mouse deficient in tPA, but a thrombin inhibitor was not affected by the lack of tPA (Matsuno *et al.*, 2000). These results also indicated that lack of tPA significantly affects the antithrombotic effect by antiplatelet agents.

Therefore, we speculate that the physiologic importance of such responses to the reduction of antithrombotic effect by antiplatelet agents in mice deficient of tPA might be as follows; In the artery, tPA is one of the important factors in thrombolysis and its production by quiescent endothelial cells may promote vascular patency. In our study, endothelial cells in the thrombus area are injured and hence have lost antithrombotic properties, including the production of tPA. However, in the periphery of the injured area, antithrombotic mechanisms in the artery could still be operative to fulfil the antithrombotic function. Additionally, after endothelial injury, the vascular surface was not smooth and a significant shear stress might be continuously presented in the injured area. Shear stress is an important factor in the process of platelet activation. Indeed, our histological observations showed that

Table 1 Bleeding time in deficient and wild type mice

Dose of ATA (μg ml^{-1})	*uPA*+/+	*uPA*−/−	*tPA*+/+	*tPA*−/−	*PAI-1*+/+	*PAI-1*−/−
0	51±11	52±10	51±9	54±11	52±12	57±11
1	NA	NA	NA	NA	50±9	54±10
3	NA	NA	NA	NA	48±12	55±12
10	52±14	49±9	46±8	51±10	52±11	58±14
30	54±11	51±7	51±9	52±11	55±12	61±10
100	66±16	59±11	64±14	68±9	62±14	NA
300	74±12*	79±15*	81±11*	81±12*	74±13*	NA
1000	NA	NA	132±19**	129±22**	NA	NA

Data are represented as mean±s.e.mean. *$P<0.05$, **$P<0.01$ versus control (infusion of saline). NA, not applicable.

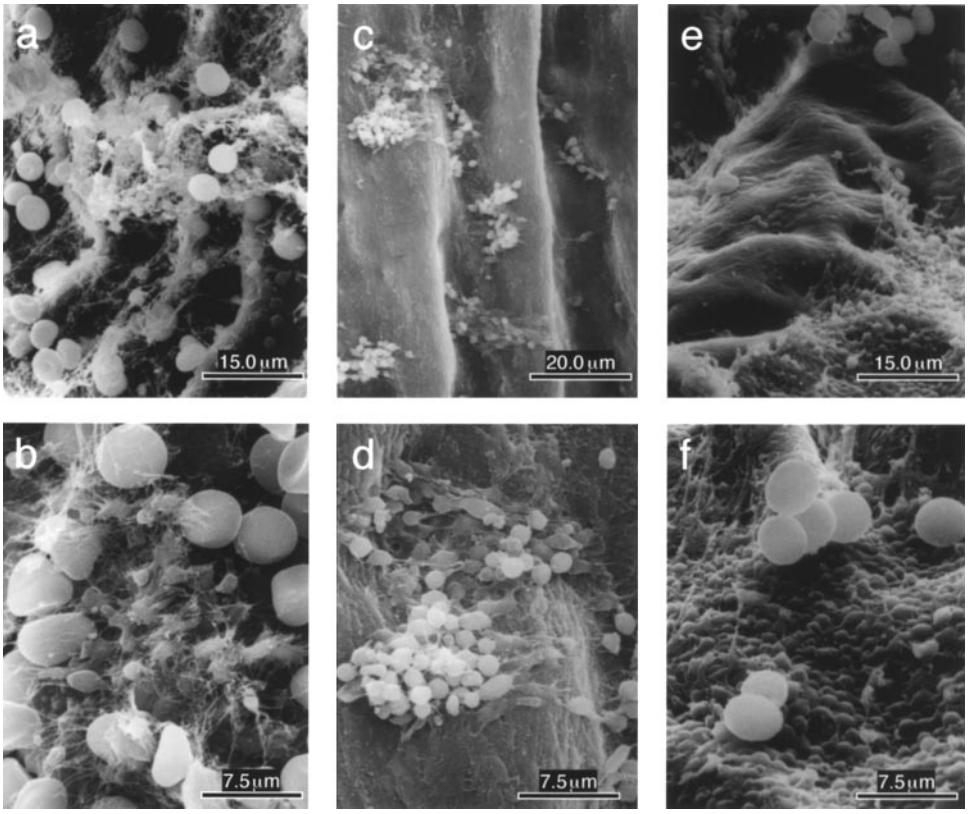

Figure 3 Scanning electron micrographs from a wild type mouse (a and b), a mouse deficient tPA (c and d) and a mouse deficient PAI-1 (e and f). (a) thrombus formation after endothelial injury in a wild type mouse. A mural thrombus including a lot of activated platelets, fibrin net and red blood cells. (b) Intact arterial surface in a tPA−/− mice. Locally activated platelets were observed. Adherent platelets consist of microthrombus formation on non-injured endothelial surface. (c) thrombus formation after endothelial injury in a PAI-1−/− mouse. Activated platelets aggregate on injured surface with red blood cells. However fibrin networks were rarely observed in thrombus formation. d, e and f are representative of high magnification of a, b and c, respectively.

the lack of tPA promotes microthrombus on non-injured endothelial surface. These adhered platelets could play a significant role in reducing the antithrombotic efficacy by the interfering with vWF binding to platelet GPIb/V/IX using high molecular weight ATA since high molecular weight ATA had a little effect in preventing thrombus formation when the compound was administered after vascular injury. Moreover, tPA inhibits platelet activation in response to pathological shear stress by altering the multimeric composition of von-Willebrand factor (Kamat *et al*., 1995).

On the other hand, uPA also plays a role in the fibrinolytic system, but our data indicate that the lack of uPA dose not really modulate the efficacy of the antithrombotic therapy by an agent interfering with vWF binding to platelet GPIb/V/IX *in vivo*. This finding was also observed when a GPIIb/IIIa antagonist was treated in uPA−/− mice (Matsuno *et al*., 2000). From these data we can conclude that tPA plays a more prominent role in thrombosis-mediated vascular occlusion than uPA.

PAI-1 also participates in the development of thrombus formation. Collectively, recent data suggested that PAI-1 plays an important role in haemostasis, thrombosis, thrombolysis and possibly in the progression of atherosclerosis (Carmeliet *et al*., 1993). In these experiments, PAI-1 deficiency affected both the development of the thrombus and the enhancement of antiplatelet effect by high molecular weight ATA since our results have clearly demonstrated that compared with wild type mice, uPA−/− mice or tPA−/− mice, mice lacking PAI-1 exhibit a significant prolongation of the time to occlusion by thrombus. These results were supported by the histological observation using the scanning electron microscopy since there were few fibrin networks in mural thrombus

on injured area. Indeed, the potential antithrombotic effect of an inhibitor of PAI-1 has been indicated from *in vitro* as well as from *in vivo* experiments (Ohtani *et al*., 1997; Berry *et al*., 1998) and a monoclonal antibody against PAI-1 was shown to inhibit thrombus growth and to enhance clot lysis in a rabbit model of venous thrombosis (Biemond *et al*., 1995). Therefore, the antithrombotic effect of combined inhibition of PAI-1 with the interfering with vWF binding to platelet GPIb/V/IX may be useful in preventing vascular occlusion *in vivo*.

On the other hand, antithrombotic interventions may easily be complicated by hemorrhagic events. Inactivation of the single fibrinolytic factors did not significantly affect haemostasis, whereas delayed rebleeding after trauma or surgery is a consistent clinical observation in patients with reduced PAI-1 levels (Dieval *et al*., 1991; Lee *et al*., 1993). In our experiments, in PAI-1−/− mice, bleeding time was slightly prolonged, but not significantly. In the case of treatment with high molecular weight ATA in tPA or uPA deficient mice, the antithrombotic effect was paralleled by a prolongation of the bleeding time. The bleeding tendency was furthermore moderately more pronounced in wild type control mice and uPA−/− mice. However, the effective dose of high molecular weight ATA in order to prevent the vascular occlusion by thrombus in tPA−/− mice elicited the most marked prolongation of the bleeding time. These results indicate that the prevention of vWF binding to platelet GPIb/V/IX in the condition of tPA deficiency may result in a significant risk for hemorrhagic events when aiming to obtain a sufficient antithrombotic effect. On the contrary, under the condition of lack of PAI-1, it would be easy to get sufficient antithrombotic activity by the inhibition of vWF binding to platelet GPIb/V/IX without bleeding risk. These findings indicated that PAI-1 could be a different approach to fibrinolysis in either the development of thrombus formation or haemorrhagic events.

In conclusion, the present study shows that tPA, but not uPA, significantly contributes to the antithrombotic efficacy of the interfering with vWF binding to platelet GPIb/V/IX. The lack or reduced levels of tPA in vessels could severely attenuate the antithrombotic action of antiplatelet agents. Additionally, the inhibition of PAI-1 markedly enhances the antithrombotic therapy by antiplatelet drugs without bleeding risk. These findings could be part of a new supportive therapeutic concept in the treatment of cardiovascular diseases.

References

BERRY, C.N., LUNVEN, C., LECHAIRE, I., GIRARDOT, C. & O'CONNOR, S.E. (1998). Antithrombotic activity of a monoclonal antibody inducing the substrate form of plasminogen activator inhibitor type 1 in rat models of venous and arterial thrombosis. *Br. J. Pharmacol.,* **125,** 29–34.

BIEMOND, B.J., LEVI, M., CORONEL, R., JANSE, M.J., CATE, J.W. & PANNEKOEK, H. (1995). Thrombolysis and reocclusion in experimental jugular vein and coronary artery thrombosis. Effects of a plasminogen activator inhibitor type 1-neutralizing monoclonal antibody. *Circulation,* **91,** 1175–1181.

BRAATEN, J.V., HANDT, S., JEROME, W.G., KIRKPATRICK, J., LEWIS, J.C. & HANTGAN, R.R. (1993). Regulation of fibrinolysis by platelet-released plasminogen activator inhibitor 1: light scattering and ultrastructural examination of lysis of a model platelet-fibrin thrombus. *Blood,* **81,** 1290–1299.

CARMELIET, P., KIECKENS, L., SCHOONJANS, L., REAM, B., VAN NUFFELEN, A., PRENDERGAST, G., COLE, M., BRONSON, R., COLLEN, D. & MULLIGAN, R.C. (1993). Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J. Clin. Invest.,* **92,** 2746–2755.

CARMELIET, P., SCHOONJANS, L., KIECKENS, L., REAM, B., DEGEN, J., BRONSON, R., DE VOS, R., VAN DEN OORD, J.J., COLLEN, D. & MULLIGAN, R.C. (1994). Physiological consequence of loss of plasminogen activator gene function in mice. *Nature,* **368,** 419–424.

COLLEN, D. & LIJNEN, H.R., (1991). Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood,* **78,** 3114–3124.

COLLER, B.S., PEERSCHKE, E.I., SCUDDER, L.E. & SULLIVAN, C.A., (1983). Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. *Blood,* **61,** 99–110.

DIEVAL, J., NGUYEN, G., GROSS, S., DELOBEL, J. & KRUITHOF, E.K. (1991). A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1. *Blood,* **77,** 528–532.

FUJIMURA, Y., TITANI, K., USAMI, Y., SUZUKI, M., OYAMA, R., MATSUI, T., FUKUI, H., SUGIMOTO, M. & RUGGERI, Z.M. (1991). Isolation and chemical characterization of two structurally and functionally distinct forms of botrocetin, the platelet coagglutinin isolated from the venom of Bothrops jararaca. *Biochemistry,* **30,** 1957–1964.

FUSTER, V. (1994). Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation,* **90,** 2126–2146.

IKEDA, Y., HANDA, M., KAWANO, K., KAMATA, T., MURATA, M., ARAKI, Y., ANBO, H., KAWAI, Y., WATANABE, K. & ITAGAKI, I. (1991). The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J. Clin. Invest.,* **87,** 1234–1240.

ITO, T., MATSUNO, H., KOZAWA, O., NIWA, M., SAKAI, N. & UEMATSU, T. (1999). Comparison of the antithrombotic effects and bleeding risk of fractionated aurin tricarboxylic acid and the GPIlb/IIIa antagonist GR144053 in a hamster model of stenosis. *Thromb. Res.,* **95,** 49–61.

KAMAT, S.G., MICHELSON, A.D., BENOIT, S.E., MOAKE, J.L., RAJASEKHAR, D., HELLUMS, J.D., KROLL, M.H. & SCHAFER, A.I. (1995). Fibrinolysis inhibits shear stress-induced platelet aggregation. *Circulation,* **92,** 1399–1407.

KAWASAKI, T., KAKU, S., KOHINATA, T., SAKAI, Y., TANIUCHI, Y., KAWAMURA, K., YANO, S., TAKENAKA, T. & FUJIMURA, Y. (1994). Inhibition by aurintricarboxylic acid of von Willebrand factor binding to platelet GPIb, platelet retention, and thrombus formation in vivo. *Am. J. Hematol.,* **47,** 6–15.

KUNITADA, S., FITZGERALD, G.A. & FITZGERALD, D.J. (1992). Inhibition of clot lysis and decreased binding of tissue-type plasminogen activator as a consequence of clot retraction. *Blood,* **79,** 1420–1427.

LEE, M.H., VOSBURGH, E., ANDERSON, K. & MCDONAGH, J. (1993). Deficiency of plasma plasminogen activator inhibitor 1 results in hyperfibrinolytic bleeding. *Blood,* **81,** 2357–2362.

LEVI, M., BIEMOND, B.J., VAN ZONNEVELD, A.J., TEN CATE, J.W. & PANNEKOEK, H. (1992). Inhibition of plasminogen activator inhibitor-1 activity results in promotion of endogenous thrombolysis and inhibition of thrombus extension in models of experimental thrombosis. *Circulation,* **85,** 305–312.

MATSUNO, H., KOZAWA, O., NIWA, M., TANABE, K., ICHIMARU, K., TAKIGUCHI, Y., YOKOTA, M., HAYASHI, H. & UEMATSU, T. (1998). Multiple inhibition of platelet activation by aurintricarboxylic acid prevents vascular stenosis after endothelial injury in hamster carotid artery. *Thromb. Haemost.,* **79,** 865–871.

MATSUNO, H., KOZAWA, O., NIWA, M. & UEMATSU, T. (1997). Inhibition of von Willebrand factor binding to platelet GP lb by a fractionated aurintricarboxylic acid prevents restenosis after vascular injury in hamster carotid artery. *Circulation,* **96,** 1299–1304.

MATSUNO, H., KOZAWA, O., NIWA, M., UESHIMA, S., MATSUO, O. & COLLEN, D. (1999). Differential role of components of the fibrinolytic system in the formation and removal of thrombus induced by endothelial injury. *Thromb. Haemost.,* **81,** 601–604.

MATSUNO, H., KOZAWA, O., UESHIMA, S., MATSUO, O., COLLEN, D. & UEMATSU, T. (2000). Lack of tPA significantly affects antithrombotic therapy by a GPIIb/IIIa antagonist, but not by a thrombin inhibitor in mice. *Thromb. Haemost.,* **83,** 605–609.

MATSUNO, H., UEMATSU, T., NAGASHIMA, S. & NAKASHIMA, M. (1991). Photochemically induced thrombosis model in rat femoral artery and evaluation of effects of heparin and tissue-type plasminogen activator with use of this model. *J. Pharmacol. Methods,* **25,** 303–317.

MATSUNO, H., UEMATSU, T., UMEMURA, K., TAKIGUCHI, Y., WADA, K. & NAKASHIMA, M. (1992). Effects of vapiprost, a novel thromboxane receptor antagonist, on thrombus formation and vascular patency after thrombolysis by tissue-type plasminogen activator. *Br. J. Pharmacol.,* **106,** 533–538.

MATSUO, O., BANJO, H., OKADA, K., TANAKA, K., SUCKED, M., IDA, Y. & ARMOUR, H. (1986). Thrombolytic effect of single-chain pro-urokinase in a rabbit jugular vein thrombosis model. *Thromb. Res.,* **42,** 187–194.

MATSUO, O., RIJKEN, D.C. & COLLEN, D. (1981). Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. *Nature,* **291,** 590–591.

MILLER, J.L., THIAM-CISSE, M. & DROUET, L.O. (1991). Reduction in thrombus formation by PG-1 F(ab')2, an anti-guinea pig platelet glycoprotein lb monoclonal antibody. *Arterioscler. Thromb.,* **11,** 1231–1236.

OHTANI, A., MURAKAMI, J. & HIRANO-WAKIMOTO, A. (1997). T-686, a novel inhibitor of plasminogen activator inhibitor-1, inhibits thrombosis without impairment of hemostasis in rats. *Eur. J. Pharmacol.,* **330,** 151–156.

PHILLIPS, M.D., MOAKE, J.L., NOLASCO, L. & TURNER, N. (1988). Aurintricarboxylic acid: a novel inhibitor of the association of von Willebrand factor and platelets. *Blood,* **72,** 1898–1903.

PLOW, E.F., HERREN, T., REDLITZ, A., MILES, L.A. & HOOVER-PLOW, J.L. (1995). The cell biology of the plasminogen system. *FASEB J.,* **9,** 939–945.

STRINGER, H.A., VAN SWIETEN, P., HEIJNEN, H.F., SIXMA, J.J. & PANNEKOEK, H. (1994). Plasminogen activator inhibitor-1 released from activated platelets plays a key role in thrombolysis resistance. Studies with thrombi generated in the Chandler loop. *Arterioscler. Thromb.,* **14,** 1452–1458.

STRONY, J., PHILLIPS, M., BRANDS, D., MOAKE, J. & ADELMAN, B. (1990). Aurintricarboxylic acid in a canine model of coronary artery thrombosis. *Circulation,* **81,** 1106–1114.

TAKIGUCHI, Y., SHIMAZAWA, M. & NAKASHIMA, M. (1996). A comparative study of the antithrombotic effect of aurintricarboxylic acid on arterial thrombosis in rats and guinea-pigs. *Br. J. Pharmacol.,* **118,** 1633–1638.

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