Plasminogen Activator Inhibitor-1 Is a Major Determinant of Arterial Thrombolysis Resistance

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Background—Platelet-rich thrombi are resistant to lysis by tissue plasminogen activator (tPA). Plasminogen activator inhibitor-1 (PAI-1), a rapid inhibitor of tPA, may contribute to arterial thrombolysis resistance. However, few data are available regarding the effect of PAI-1 on arterial thrombolysis in animals. We used a murine carotid injury model to test the hypothesis that PAI-1 inhibits thrombolysis mediated by pharmacological concentrations of tPA.

Methods and Results—Platelet-rich thrombi were induced in wild-type mice (PAI-1+/+; n=11) and PAI-1–deficient mice (PAI-1−/−; n=11) with ferric chloride. Baseline carotid blood flows and mean occlusion times did not differ between PAI-1+/+ and PAI-1−/− mice. Clot lysis was induced by infusion of heparin (200 U/kg bolus, 70 U·kg⁻¹·h⁻¹ drip), human plasminogen (50 mg/kg), and tPA at 20 (n=10) or 100 (n=12) µg·kg⁻¹·min⁻¹. Mean plasma tPA antigens were 2.7 µg/mL (tPA infusion, 20 µg·kg⁻¹·min⁻¹) and 5.5 µg/mL (tPA infusion, 100 µg·kg⁻¹·min⁻¹), with no significant differences between PAI-1+/+ mice and PAI-1−/− mice. Reperfusion after tPA 20 µg·kg⁻¹·min⁻¹ occurred in 1 of 5 PAI-1+/+ mice versus 5 of 5 PAI-1−/− mice (P=0.0006). Reperfusion occurred in all mice that received tPA 100 µg·kg⁻¹·min⁻¹, but reperfusion times were significantly shorter in PAI-1−/− mice (17.8±2.6 minutes, n=6) than in PAI-1+/+ mice (35.7±5.1 minute, n=6; P=0.01). Histological analyses confirmed that carotid thrombi were platelet rich and that PAI-1 was distributed uniformly throughout thrombi from PAI-1+/+ mice. Lysates of PAI-1+/+ platelets inhibited human tPA, whereas PAI-1−/− platelet lysates did not.

Conclusions—PAI-1 is a major determinant of the resistance of platelet-rich arterial thrombi to lysis by pharmacological concentrations of tPA. Strategies to inhibit or resist PAI-1 may enhance thrombolysis. (Circulation. 1999;99:3050-3055.)

Key Words: thrombosis • thrombolysis • platelets • plasminogen activators

Coronary artery thrombosis is usually triggered by rupture of an atherosclerotic plaque. Because of high shear forces present in diseased arteries and local generation of strong platelet agonists such as thrombin, arterial thrombi are frequently platelet rich. Tissue plasminogen activator (tPA) commonly is administered to patients with acute myocardial infarction to lyse occlusive thrombi. However, platelet-rich thrombi are highly resistant to lysis by tPA.1 Plasminogen activator inhibitor-1 (PAI-1),2 the primary endogenous inhibitor of tPA and urokinase, may play an important role in inhibiting arterial clot lysis. Platelets and arterial thrombi contain high concentrations of PAI-1,3–5 and antibodies to PAI-1 accelerate clot lysis in vitro.6 However, the majority of platelet PAI-1 is inactive, and some in vitro studies have concluded that PAI-1 does not play a significant role in thrombolysis resistance.7,8 Furthermore, the very high plasma concentrations of tPA attained during thrombolytic therapy have been predicted to overcome the inhibitory effects of plasma and thrombus-associated PAI-1.9,10 The true role of PAI-1 in regulating the clearance of platelet-rich thrombi can only be assessed by in vivo experiments. Mice deficient in PAI-1 have been generated by gene targeting.11 In the present study, we have developed a murine model of arterial thrombolysis and applied it to wild-type (PAI-1+/+) mice and PAI-1-deficient (PAI-1−/−) mice to test the hypothesis that PAI-1 inhibits arterial thrombolysis initiated by pharmacological concentrations of tPA.

Methods

Animals
C57BL/6J mice were purchased from Jackson Labs, Bar Harbor, Me. PAI-1–deficient mice were generated as described previously.11 PAI-1−/− mice used in experiments were the product of ≥8 backcrosses to the C57BL/6J genetic background. Genotyping of mice was performed by polymerase chain reaction analysis of tail DNA.12 All experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Thrombolysis Protocol
Mice (6 to 8 weeks old; weight, 20 to 25 g) were subjected to a previously described carotid artery injury model that was modified to study thrombolysis.12 After intraperitoneal injection of sodium pentobarbital 110 mg/kg, the right internal jugular vein was exposed and cannulated with a catheter (L-Cath, Luther Medical Products) con-
Histological Analyses

Carotid arteries of selected mice were perfusion fixed and retrieved for histological analysis.12 Immunohistochemical staining for PAI-1 was performed with murine anti-human plasminogen monoclonal antibody (American Diagnostica) for 1.5 hours, then developed with the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim).

In Vitro Experiments

Plasma tPA antigen

Plasma was prepared by centrifugation of citrated blood (15 minutes, 1500g). tPA antigen was measured by ELISA (Imubind tPA, American Diagnostica).

Western Blotting

Citrated plasma samples were diluted 10-fold with 0.01 mol/L Tris-HCl, 0.14 mol/L NaCl, pH 7.4 (TBS), subjected to nonreducing SDS-PAGE, and transferred to polyvinylidene difluoride membranes with the Phast-Transfer semidyry system (Pharmacia). Blocked membranes were incubated with murine anti-human plasminogen monoclonal antibody (American Diagnostica) for 1.5 hours, then developed with the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim).

Results

Vascular Injury/Thrombolysis Studies

Carotid thrombi were induced in PAI-1−/− mice (n=11) and PAI-1+/+ mice (n=11) as shown in Figure 1. Baseline carotid artery blood flow was 0.68±0.07 mL/min in PAI-1−/− mice and 0.72±0.09 mL/min in PAI-1+/+ mice (P>0.6). Mean occlusion time after initiation of injury was 17.4±2.0 minutes in PAI-1+/+ mice and 16.5±1.6 minutes in PAI-1−/− mice (P>0.6). Figure 2 shows a carotid artery flow tracing from a typical experiment. After occlusive thrombus formation, 10 mice (5 PAI-1−/−, 5 PAI-1+/+; 3 males in each group) received tPA 20 µg·kg⁻¹·min⁻¹, and 12 mice (6 PAI-1−/−, 6 PAI-1+/+; 4 males in each group) received tPA 100 µg·kg⁻¹·min⁻¹. Reperfusion occurred 90 minutes after administration of tPA 20 µg·kg⁻¹·min⁻¹ was begun in 1 PAI-1+/+ mouse, whereas reperfusion did not

Figure 1. Thrombolysis protocol. Ventral neck is viewed through dissecting microscope (mouse head situated superiorly). Flow probe is present on left carotid artery. Filter paper saturated with FeCl₃ is positioned on artery proximal to flow probe. Right jugular vein is cannulated with polypropylene tubing.
occur in 4 of 5 PAI-1 +/- mice during 2 hours of flow monitoring. In contrast, carotid blood flow was restored in 5 of 5 PAI-1 +/- mice that received tPA 20 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) (mean reperfusion time, 52.2 \( \pm \) 9.5 minutes; \( P=0.0006 \) versus PAI-1 +/- mice; Table). In response to tPA 100 \( \mu g \cdot kg^{-1} \cdot min^{-1} \), the carotid arteries in all animals in both groups were reperfused. However, mean reperfusion times were shorter in PAI-1 +/- mice (17.8 \( \pm \) 2.6 minutes) than in PAI-1 +/- mice (35.7 \( \pm \) 5.1 minutes; \( P=0.01 \)). Reocclusion after thrombolysis was not observed in either tPA dosing group. Mean plasma tPA antigen during infusion of tPA 20 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) was 2.7 \( \pm \) 0.3 \( \mu g/mL \) for PAI-1 +/- mice and 2.7 \( \pm \) 0.4 \( \mu g/mL \) for PAI-1 +/- mice (\( P>0.9 \)). Mean plasma tPA antigen during infusion of tPA 100 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) was 5.5 \( \pm \) 1.6 \( \mu g/mL \) for PAI-1 +/- mice and 5.5 \( \pm \) 2.0 \( \mu g/mL \) for PAI-1 +/- mice (\( P>0.9 \)). aPTTs were measured in the 10 mice that received tPA 20 \( \mu g \cdot kg^{-1} \cdot min^{-1} \). All values were >150 seconds. The aPTT of normal mouse plasma was 29.5 \( \pm \) 1.1 seconds (\( n=4 \)).

To examine the frequency of spontaneous thrombolysis in this model, carotid artery thrombi were induced in PAI-1 +/- mice (\( n=3 \)) and PAI-1 +/- mice (\( n=3 \)), after which heparin and human plasminogen were infused (ie, exogenous tPA omitted). No animals became reperfused during 2 hours of monitoring. Similarly, reperfusion was not observed in 3 PAI-1 +/- mice that received heparin but not tPA or human plasminogen. To examine the effects of exogenous human plasminogen on thrombolysis, 3 PAI-1 +/- mice received tPA (100 \( \mu g \cdot kg^{-1} \cdot min^{-1} \)) and heparin after thrombus formation but no human plasminogen. No reperfusion was observed during 2 hours of monitoring. To examine the circulating concentrations of human plasminogen attained in mice, blood samples were collected from mice (\( n=3 \)) 5 minutes, 1 hour, and 2 hours after infusion of human plasminogen (50 mg/kg). Plasma was subjected to Western blotting with a monoclonal antibody that recognized human but not murine plasminogen. Human plasminogen concentrations in mouse plasma were similar to those in human plasma (Figure 3).

To study the effects of PAI-1 on thrombolysis when tPA is administered as a bolus and 2-step infusion, carotid artery thrombi were induced in 10 mice (5 PAI-1 +/-, 5 PAI-1 +/-). Ten minutes after occlusion, human plasminogen was infused. Ten minutes later, tPA and heparin were infused. Heparin was administered as a bolus (71 U/kg) followed by a constant infusion (14.3 U \( \cdot \) kg \( ^{-1} \) \( \cdot \) h \(^{-1} \)). tPA was administered as a bolus (0.21 mg/kg) followed by a continuous infusion of 25 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) for 30 minutes and then 8.3 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) for 60 minutes, after which it was discontinued. On a weight basis, these infusion protocols for heparin and tPA were the same as those typically administered to a 70-kg human with acute myocardial infarction.17 Carotid blood flow was monitored until reperfusion, then for an additional 30 minutes. If reperfusion occurred during tPA infusion, blood flow was monitored for 30 minutes after tPA was discontinued. If reperfusion did not occur by 90 minutes after completion of the tPA infusion, the experiment was stopped. Reperfusion was observed in 2 PAI-1 +/- mice (90 and 118 minutes after tPA administration was begun), whereas 3 PAI-1 +/- mice did not undergo reperfusion. Reperfusion was observed in 5 of 5 PAI-1 +/- mice (140, 40, 99, 55, and 54 minutes after tPA administration was begun; mean, 77.6 \( \pm \) 18.5 minutes; \( P=0.027 \) versus PAI-1 +/- mice). Reocclusion after tPA was stopped was not observed in any of the 7 mice that experienced reperfusion.

**Histological Analyses**

Injured carotid arteries from 6 mice that received tPA 20 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) and 6 that received tPA 100 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) were excised, and hematoxylin and eosin–stained cross sections (4 per artery) were examined histologically. Of animals that received lower-dose tPA, occlusive, platelet-rich thrombus was present in 2 of 3 PAI-1 +/- mice, whereas residual

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**Table 1. Reperfusion Times (min) of PAI-1 +/- Mice and PAI-1 +/- Mice**

<table>
<thead>
<tr>
<th>TPA 20 ( \mu g \cdot kg^{-1} \cdot min^{-1} )</th>
<th>TPA 100 ( \mu g \cdot kg^{-1} \cdot min^{-1} )</th>
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<tbody>
<tr>
<td>PAI-1 +/-</td>
<td>PAI-1 +/-</td>
</tr>
<tr>
<td>No lysis</td>
<td>25</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>No lysis</td>
<td>26</td>
</tr>
<tr>
<td>No lysis</td>
<td>17</td>
</tr>
<tr>
<td>52.2 ( \pm ) 9.5**</td>
<td>35.7 ( \pm ) 5.1</td>
</tr>
</tbody>
</table>

*Values in last line are mean \( \pm \) SEM.

*\( P=0.0006 \) vs PAI-1 +/- mice; \( P=0.01 \) vs PAI-1 +/- mice.
thrombus was detected in none of the 3 PAI-1 −/− mice. No residual thrombus was observed in cross sections from mice (3 PAI-1 +/+, 3 PAI-1 −/−) that received tPA 100 μg · kg⁻¹ · min⁻¹. Injured carotid arteries of control mice that did not receive tPA were subjected to immunohistochemical analysis. PAI-1 staining was observed throughout thrombi from 2 PAI-1 +/+ mice but was undetectable in thrombi from 2 PAI-1 −/− mice (Figure 4).

**In Vitro Experiments**

Animal studies of tPA-catalyzed thrombolysis generally have used human tPA. We compared the capacity of human tPA to generate plasmin in mouse, rabbit, dog, and human plasma in the presence of soluble fibrin fragments. Compared with human plasma, plasmin formation was inefficient in mouse, rabbit, and dog plasma (Figure 5). However, addition of purified human plasminogen (18 μg/mL) to mouse plasma accelerated plasmin formation to levels greater than those observed in rabbit or dog plasma. To determine whether mouse platelets contained PAI-1 activity that inhibited human tPA, murine platelet lysates were incubated with human tPA (125 pg). Platelet lysates from PAI-1 +/+ mice (n=4) inhibited human tPA activity by 41.5%, whereas platelet lysates from PAI-1 −/− mice (n=4) did not inhibit tPA (residual tPA activity was 104% that of control samples lacking platelet lysates).

**Discussion**

In these studies, we used a murine model of arterial thrombosis to probe the role of PAI-1 in mediating the resistance of platelet-rich thrombi to lysis by pharmacological concentrations of tPA. In response to a tPA infusion rate of 20 μg · kg⁻¹ · min⁻¹ we observed carotid artery reperfusion in only 1 of 5 wild-type mice, results consistent with those of Jang et al,1 who initially described the resistance of platelet-rich thrombi to lysis in rabbits. In contrast, thrombolysis was observed in 5 of 5 PAI-1 −/− mice. Similar antithrombolytic effects of PAI-1 were observed when tPA was administered to mice in a bolus/2-step infusion protocol mimicking “front-loaded” tPA administration to humans with acute myocardial infarction.17 When higher tPA concentrations (100 μg · kg⁻¹ · min⁻¹) were administered, reperfusion was observed in all PAI-1 +/+ mice; however, reperfusion times were significantly longer than those of PAI-1 −/− mice. These results strongly suggest that PAI-1 is an important determinant of thrombolysis resistance and that under controlled experimental conditions, endogenous PAI-1 can account for the failure
of thrombolytic therapy to restore arterial patency. The role of PAI-1 in thrombolysis resistance has been a subject of controversy. PAI-1 is a potent inhibitor of tPA and urokinase, and clot lysis by physiological concentrations of tPA is inhibited by platelet PAI-1 in vitro. However, the plasma concentrations of tPA attained during thrombolytic therapy (≈2 μg/mL) are ≈3 orders of magnitude higher than basal tPA concentrations (≈2 ng/mL),20 which suggests that plasma PAI-1 (generally <20 ng/mL) is insufficient to significantly attenuate thrombolysis by pharmacological concentrations of tPA. Consistent with this hypothesis, Lucore and Sobel showed that endogenous tPA circulates predominantly in complex with PAI-1, whereas during thrombolytic therapy, exogenous tPA saturates plasma PAI-1 and circulates predominantly as free tPA or in complexes with other protease inhibitors. In contrast to the plasma compartment, PAI-1 released locally at sites of arterial injury by platelets and endothelial cells may be an important determinant of the thrombolytic response to exogenous tPA, because PAI-1 concentrations are very high in platelet-rich thrombi.4,5,22 However, the majority of platelet PAI-1 is inactive, and several groups have concluded that PAI-1 does not contribute substantially to platelet-dependent inhibition of clot lysis in vitro when tPA is present in pharmacological concentrations.7,8,10 Therefore, our studies, which strongly support an important role of PAI-1 in arterial thrombolysis resistance, underscore the importance of in vivo experiments in defining protein function. We hypothesize that the discordant effects of PAI-1 on clot lysis in vitro and in vivo are explained primarily by 2 factors. First, thrombi that form at sites of arterial injury typically consist almost entirely of dense platelet aggregates.23 Therefore, their platelet and PAI-1 content are considerably higher than those of platelet-rich clots formed in vitro. Second, thrombin and products released from platelets, such as transforming growth factor-β, stimulate release of PAI-1 from vascular endothelial and smooth muscle cells, thereby enriching thrombus PAI-1 content.24 This blood-vessel-wall pool of PAI-1 was absent in the previously described in vitro models.7,8 There are few other in vivo studies that address the impact of PAI-1 on arterial thrombolysis. Biemond et al used a monoclonal antibody to PAI-1 to augment tPA-mediated fibrinolysis in a canine coronary artery thrombosis model. However, thrombi were formed by injection of whole blood and thrombin into an occluded segment of coronary artery. Therefore, they were more reflective of venous thrombi that form during stasis, as opposed to platelet-dense thrombi that form in arteries under conditions of high shear stress.

We did not observe reocclusion after successful thrombolysis in our model. This probably reflects the continuous infusion of tPA throughout the protocol or the continued infusion of heparin when tPA was stopped after 90 minutes. Therefore, the potential role of PAI-1 in reocclusion after thrombolysis was not addressed by our experiments. Modification of our protocol (eg, lower heparin doses and/or more intense vascular injury) should facilitate the study of reocclusion. It is of interest that occlusion times did not differ between PAI-1 +/+ mice and PAI-1 −/− mice in our model. We believe this is because FeCl₃ is a strong stimulus for thrombosis, such that during clot formation, activation of platelets and the coagulation system greatly exceeds that of the endogenous fibrinolytic system. Our results are consistent with the normal response to hemostatic challenge observed in PAI-1 −/− mice.11 However, in other models in which thrombi form more slowly, PAI-1 potentially may affect thrombosis times by downregulating fibrinolysis.

The species specificity of fibrinolytic proteins is relevant to our studies. As with virtually all animal thrombolysis studies, we used human tPA because it is readily available as a recombinant protein. Korminger and Collen showed that clots formed in vitro from dog or rat plasma were lysed by human tPA only 30% and 10% as rapidly, respectively, as human clots. Studies by Lijnen et al in mice suggest that the relative inefficiency of human tPA in nonprimates results predominantly from the species-dependent interaction between tPA and plasminogen. These investigators studied the interactions of purified human and mouse fibrinolytic proteins. They showed that the following protein-protein interactions were not significantly affected across species: tPA–fibrin, plasminogen, and tPA–PAI-1. The second-order rate constant for the inhibition of human tPA by mouse PAI-1 was very similar to that for the inhibition of human tPA by human PAI-1, results that are consistent with our studies showing that platelet lysates from PAI-1 +/+ mice inhibited human tPA. However, the catalytic efficiency with which human tPA activated mouse plasminogen was 40-fold lower than that for human plasminogen. These results are consistent with our experiments examining human tPA-catalyzed plasmin formation in human, dog, rabbit, and mouse plasma. We showed that human tPA catalyzed plasmin formation in mouse plasma relatively inefficiently and that plasmin formation was enhanced substantially by addition of human plasminogen, resulting in rates of plasmin formation that exceeded those in dog or rabbit plasma. Our animal experiments demonstrate that physiological concentrations of human plasminogen can be attained in mice during thrombolysis experiments and that circulating human plasminogen is necessary for the thrombolytic efficiency of exogenous human tPA. In addition, the mean plasma concentration of human tPA attained in mice during infusion at 20 μg · kg⁻¹ · min⁻¹ (ie, 2.7 μg/mL) is consistent with that
observed in patients who receive front-loaded tPA for acute myocardial infarction (mean, 2.3 μg/mL).20

Our studies are relevant to thrombolytic therapy in humans with acute myocardial infarction. Even with aggressive tPA-dosing regimens, reperfusion is not achieved in ≈20% of patients, and complete reperfusion (ie, TIMI 3 flow) is attained in only half.29 Our results suggest that PAI-1 is a major determinant of the failure of thrombolytic therapy and that inhibition of PAI-1 by antibodies or synthetic compounds may improve thrombolysis.30–33 Our studies also support the use of PAI-1–resistant tPA mutants to accelerate thrombolysis.34 Although thrombolysis resistance is likely multifactorial in origin, our results suggest that PAI-1 plays a major role compared with other potential mediators such as α2-antiplasmin, factor XIII, or factor V.8,35,36 Given that multiple blood coagulation and fibrinolysis factors have been genetically modified in mice, our model should prove useful in studying the roles of other factors in arterial thrombolysis resistance.

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References


