Inhibition of Plasminogen Activator Inhibitor-1 Activity Results in Promotion of Endogenous Thrombolysis and Inhibition of Thrombus Extension in Models of Experimental Thrombosis

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Background. We investigated the effect of inhibition of plasminogen activator inhibitor-1 (PAI-1) activity by a murine monoclonal anti-human PAI-1 antibody (MAI-12) on in vitro thrombolysis and on in vivo thrombolysis and thrombus extension in an experimental animal model for thrombosis.

Methods and Results. Thrombolysis, mediated by recombinant tissue-type plasminogen activator (rt-PA), was studied in an in vitro system consisting of fibrinogen, plasminogen, and thrombin. Addition of purified platelets during clot formation inhibited rt-PA-mediated thrombolysis in a dose-dependent manner. Platelet-dependent thrombolysis resistance could be completely neutralized by the monoclonal antibody MAI-12, supporting the notion that the observed resistance is due to PAI-1 released from α-granules of platelets. Subsequently, we monitored in vivo thrombolysis and thrombus extension of human whole blood thrombi that were allowed to form in rabbit jugular veins. During thrombus formation, either MAI-12 or an irrelevant antibody was incorporated. Thrombolysis and thrombus extension were simultaneously measured during endogenous thrombolysis or upon administration of different dosages of rt-PA. We observed that endogenous thrombolysis was enhanced in the presence of MAI-12 compared with the control antibody. Significantly, thrombus extension was partially prevented by MAI-12 and not by the control antibody irrespective of whether exogenous rt-PA was systematically administered.

Conclusions. These observations further confirm the essential role of PAI-1 in the regulation of the thrombotic system and support the exploration of adjunctive therapy based on inhibition of PAI-1 activity in thrombolytic strategies. (Circulation 1992;85:305–312)

Studies with a canine model for coronary artery stenosis and thrombosis have demonstrated that reocclusion of initially reperfused vessels upon thrombolytic therapy is associated with the accumulation of activated aggregated platelets. Inhibition of aggregation by antibodies, directed against the major platelet receptor glycoprotein (GP) IIb/IIIa, largely prevents the occurrence of reocclusion. Likewise, clinical trials of patients suffering from acute myocardial infarction have shown improved long-term outcome if platelet activation is inhibited. In this study, we have attempted to identify a platelet component that may cause resistance to thrombolysis mediated by tissue-type plasminogen activator (t-PA). To that end, we focused on the function of plasminogen activator inhibitor-1 (PAI-1), the physiological, fast-acting inhibitor of t-PA. PAI-1 is present in the α-granules of platelets as well as in plasma and in the endothelial cell matrix. From the Center for Haemostasis, Thrombosis, Atherosclerosis and Inflammation Research (M.L., B.B., J.W.t.C.), Academic Medical Center, Amsterdam, The Netherlands, and the Department of Molecular Biology, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (A.-J.v.Z., H.F.), Amsterdam.

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Presented in part at the 10th International Congress on Fibrinolysis, August 1990, Indianapolis, Ind.

Received April 22, 1991; revision accepted August 27, 1991.

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PAI-1 belongs to the serine protease inhibitor (serpin) family and acts as a pseudosubstrate for t-PA, forming equimolar, inactive PAI-1/t-PA complexes. It has been reported that the number of PAI-1 molecules per platelet is 4,000–8,000. Consequently, activation and aggregation of platelets may cause a local concentration of PAI-1 of 50 to 100 μg/ml, being about four orders of magnitude higher than that in plasma. Furthermore, in vitro experiments have revealed that PAI-1 can specifically bind to fibrin and, moreover, that fibrin-bound PAI-1 is still able to form complexes with t-PA. Hence, platelet-rich thrombi are equipped with high concentrations of a fast-acting t-PA inhibitor that, by virtue of its fibrin-binding properties, is fixed within the thrombi.

The precise contribution of PAI-1 to the regulation of overall thrombolytic activity in the vasculature in vivo has not been fully documented, although an important regulatory role may be conceived from in vitro studies showing reduced plasminogen-activating activity and a prolonged clot lysis time in plasma containing high levels of PAI-1. Additional indirect evidence is revealed by the association between arterial thrombotic complications such as myocardial infarction and high plasma levels of PAI-1. These observations led to the hypothesis that enhanced inhibition of endogenous fibrinolysis may result in the occurrence of thrombotic events.

Massive activation of the fibrinolytic system is achieved by infusion of plasminogen-activating drugs such as streptokinase, recombinant t-PA (rt-PA), or recombinant single-chain urokinase (rscu-PA), ultimately resulting in the formation of plasmin and, subsequently, lysis of fibrin clots. Such thrombolytic treatments have been shown to reduce mortality in patients with acute myocardial infarction. However, the success of these thrombolytic strategies has been hampered by the frequent occurrence of thrombotic reocclusion of initially reperfused vessels. As noted above, reocclusion is thought to be mediated by adherence and activation of platelets at the site of the coronary artery stenosis. The role of PAI-1 in inhibition of overall fibrinolysis and subsequently in the development of thrombosis and the potential of PAI-1 to prevent t-PA-mediated thrombolysis prompted us to study the effect of inhibition of PAI-1 activity by an anti-human PAI-1 activity antibody (MAI-12) both on in vitro thrombolysis and on in vivo thrombolysis and thrombus extension in a rabbit experimental thrombolysis model.

Methods
Preparation of Plasma and Platelets
Blood was obtained from five healthy volunteers (age, 18–32 years) and from three New Zealand White rabbits.

For the measurement of PAI-1 activity in plasma, human or rabbit blood (9 vol) was collected in plastic syringes containing 1 vol EDTA (270 mmol/l), Na₂CO₃ (1.9 mmol/l), prostaglandin E₁ (282 nmol/l, Sigma Chemical Company), and theophylline (30 mmol/l, Sigma). The samples were immediately immersed in melting ice and subsequently centrifuged at 1,600g for 30 minutes at 4°C to obtain platelet-poor plasma. Plasma samples were stored at −70°C until used.

For the measurement of platelet-PAI-1 activity, blood (9 vol) from humans or rabbits was collected into plastic syringes preloaded with 3.6% (wt/vol) trisodium citrate (1 vol). Platelet-rich plasma was obtained by centrifugation at 180g for 10 minutes at room temperature. Gel-filtered platelets were prepared from platelet-rich plasma according to methods described before. Briefly, platelet-rich plasma was passed over an acetone-washed Sepharose CL-2B column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in calcium-free phosphate-buffered saline (pH 7.35) containing 0.25% (wt/vol) bovine serum albumin (Sigma) and 0.1% (wt/vol) glucose. Gel-filtered platelets were counted with a Coulter counter and diluted with calcium-free phosphate-buffered saline to a concentration of 3×10³/ml platelets. The gel-filtered platelets were either lysed with 0.1% (vol/vol) Triton X-100 (Sigma) or incubated for 10 minutes at 37°C with 1 IU/ml human thrombin (Roche, The Netherlands). Upon incubation, the samples were centrifuged at 4,000g for 30 minutes at 4°C to sediment the platelet remnants and to obtain platelet releasates. Platelet lysates and platelet releasates were stored at −70°C until assayed.

For the in vitro clot lysis experiments, pelleted platelets from human blood prepared as described previously were used.

Measurement of PAI-1 Activity in Plasma, Platelet Lysates, and Platelet Releasates
PAI-1 activity was measured with an amidolytic method as described previously. Briefly, samples to be tested were incubated for 1 hour with varying concentrations of the monoclonal anti-PAI-1 antibody (MAI-12 or MA-7D4, Biopool, Umeå, Sweden), which has been described previously, or with a control antibody (anti-pollen; Central Laboratory of The Netherlands Red Cross Blood Transfusion Service). Subsequently, the samples were incubated for 10 minutes at room temperature with an excess of t-PA. Part of the t-PA, inhibited by PAI-1 present in the sample, formed inactive complexes. Residual t-PA activity was determined by subsequent incubation with 0.13 μmol/l plasminogen (KabiVitrum), 0.12 mg/ml cyanogen bromide–digested fibrinogen fragments (t-PA stimulator, KabiVitrum), and 0.1 mmol/l S-2251 (KabiVitrum). The amount of plasmin generated in the incubation mixture, determined by the conversion of the chromogenic substrate, was inversely proportional to the PAI-1 activity in the sample. The results of the samples to be tested were related to the results of samples of PAI-1-depleted plasma (KabiVitrum) to which fixed amounts of t-PA were added. Results were expressed
In international units (IU), where 1 IU is the amount of PAI-1 that inhibits 1 IU t-PA (first international standard of the World Health Organization).

**t-PA-Mediated In Vitro Clot Lysis**

The clot lysis assay was performed essentially as described previously. Briefly, 1 mg human fibrogen (KabiVitrum), 125I-radiolabeled fibrinogen (20,000 cpm, Radiochemical Centre Amersham), 20 µg glu-plasminogen (purified from human plasma as described before), 1.5 ng two-chain Bowes melanoma t-PA (Biopool), and increasing amounts of the anti-PAI-1 monoclonal antibody (from 0 to 100 µg) were mixed in a volume of 1 ml in Tyrode’s buffer (137 mmol/l NaCl, 2.68 mmol/l KCl, 2 mmol/l CaCl2, 1 mmol/l MgCl2, 0.36 mmol/l NaH2PO4, 11.9 mmol/l NaHCO3, 5 mmol/l HEPES, 0.1% [wt/vol] glucose, and 0.35% [wt/vol] bovine serum albumin, pH 7.35). To constitute platelet-rich clots, 2.5x10^6 purified human platelets were added to this mixture. Subsequently, clot formation was induced by addition of 100 µl of this mixture to 10 µl human thrombin (10 IU/ml). The clots were incubated for increasing periods at 37°C and were then centrifuged for 4 minutes at 40,000g. Clot lysis was determined by measuring the radioactivity in the supernatant and relating this value to the radioactivity of the mixture without added thrombin.

**In Vivo Thrombolysis and Thrombus Extension**

Thrombolysis and thrombus extension of human thrombi formed in the jugular vein of rabbits was simultaneously assessed. For that purpose, we used New Zealand White rabbits weighing approximately 2.5 kg. Anesthesia was induced intramuscularly with 0.1 mg/kg atropine, 1.0 mg/kg diazepram, and 0.3 ml/kg Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 mg/ml fentanyl). If necessary, anesthesia was maintained with 4 mg/kg i.v. tiopental. The carotid artery and jugular vein were exposed through a medial incision in the neck. The carotid artery was cleared and a cannula (baby feeding tube, 1.6 mm Φ) was introduced up to the left ventricle of the heart. The jugular vein was cleared over a distance of 2 cm, and small side branches were ligated. The vein was clamped both proximally and distally to isolate the vein segment. Citrated human blood was then mixed with 125I-radiolabeled fibrinogen (final radioactivity, approximately 25 mCi/ml). Subsequently, the monoclonal anti-human PAI-1 (MAI-12; 75 µg/ml) or a control monoclonal antibody (anti-pollen, 75 µg/ml) was added; 150 µl of this blood was aspirated in a 1-ml syringe containing 25 µl thrombin (3.75 IU) and 45 µl 0.25 mol CaCl2, and 200 µl of the clotting blood was immediately injected into the isolated jugular vein segment. In all instances, the thrombus formed quickly and was allowed to age for 30 minutes before both vessel clamps were removed and blood flow was restored. 125I-radiolabeled fibrinogen (approximately 5 µCi) was then injected through the cannula in the carotid artery, followed by the injection of either a bolus dose rt-PA (0.5 mg/kg or 0.25 mg/kg) or a control solution. For each group of rabbits (no rt-PA, 0.25, and 0.5 mg/kg rt-PA) we analyzed four thrombi both in the presence of MAI-12 and the control monoclonal antibody. The extent of thrombolysis was assessed by measurement of the remaining 125I-fibrinogen in the thrombus and comparing this with the initial radioactivity of the thrombus. Comparing 125I-related blood radioactivity with 125I-related thrombus radioactivity, thrombus growth could be measured by calculating the blood volume accreted on the clot. Thrombolysis and thrombus extension were monitored 60 and 120 minutes after thrombus formation and were expressed as percentage of the initial thrombus volume.

Animal studies were approved by the Animal Review Board of the Academic Medical Center. Care and handling of the animals conformed to the guiding principles of the American Physiological Society and the Dutch Law for Experimental Animals.

**Statistical Analysis**

Statistical analysis was performed with analysis of variance and the Newman-Keuls test using the NUMBER CRUNCHER STATISTICAL PACKAGE (NCSS). Analysis was performed at the level of p=0.05.

**Results**

**Inhibition of PAI-1 Activity by the Monoclonal Anti-PAI-1 Antibody**

Several investigators have reported that platelets represent a major inducible reservoir of PAI-1. Activation of platelets (e.g., with thrombin) results in release of PAI-1 from the α-granules. To study the inhibition of PAI-1 activity by the monoclonal anti-PAI-1 antibody MAI-12, we first determined the PAI-1 content in platelet-poor plasma, platelet lysates, and platelet releasates. For the indicated preparations, values of 12.4 IU/ml (SEM, 3.7), 52.7 IU/ml (3x10^6 platelets; SEM, 4.0), and 55.4 IU/ml (3x10^6 platelets; SEM, 4.2), respectively, were found. These observations demonstrate that virtually complete release of PAI-1 from the platelets upon thrombin stimulation is obtained and stems with previous measurements of the number of PAI-1 molecules per platelet. Subsequently, we measured the inhibition of PAI-1 activity by increasing concentrations of MAI-12. The results are given in Figure 1. MAI-12 prevented PAI-1 activity in a dose-dependent manner, attaining already significant inhibition at a concentration of 5 µg/ml. Addition of a control monoclonal antibody did not affect the inhibitory activity of PAI-1. Incubation of human platelet releasate and lysate with the anti-PAI-1 antibody also resulted in inhibition of PAI-1 activity, although it was somewhat less effective compared with the inhibition of PAI-1 activity in human plasma, which is presumably due to differences in binding of plasma and platelet PAI-1 to stabilizing proteins such as vitronectin.
PAI-1 activity in rabbit plasma was 20.3 IU/ml (SEM, 4.2), whereas in rabbit releasates and lysates of $3 \times 10^9$/ml platelets, PAI-1 activity of 71.6 IU/ml (SEM, 7.2) and 74.4 IU/ml (SEM, 6.8), respectively, was detected. Remarkably, PAI-1 activity in rabbit plasma was also inhibited by the anti-human PAI-1 antibody, although higher concentrations of the antibody were required to achieve similar inhibition of

PAI-1 activity as in human plasma (Figure 1). PAI-1 activity in rabbit platelet releasates and platelet lysates was partly inhibited by high concentrations (up to 75 μg/ml) of the monoclonal anti-PAI-1 antibody. Again, no effect of the control antibody on PAI-1 activity in rabbit plasma or platelets was detected.

**Figure 2.** Graph shows effect of the monoclonal anti-plasminogen activator inhibitor-1 (PAI-1) antibody MAI-12 on recombinant tissue-type plasminogen activator (t-PA) - mediated clot lysis of platelet-rich clots. Clots were formed by mixing $^{125}$I-labeled fibrinogen with thrombin in the presence of plasminogen, t-PA, and platelets and were subsequently incubated in buffer. Clot lysis is shown as free radioactivity in the supernatant after centrifugation, representing lysed $^{125}$I-labeled fibrin vs. incubation time. Free radioactivity is expressed as percentage of total radioactivity incorporated in the clot. Clot lysis is shown in the presence of the monoclonal anti-PAI-1 antibody at concentrations of 0 (○), 0.5 (△), 2 (□), 10 (▼), 50 (●), and 100 (■) μg/ml, respectively.

**t-PA-Mediated In Vitro Clot Lysis**

To study the effects of inhibition of PAI-1 activity by the monoclonal antibody MAI-12 on in vitro clot lysis, we incorporated increasing amounts of MAI-12 into platelet-rich clots. In agreement with previous studies, lysis of platelet-poor clots was strictly dependent on the presence of t-PA. In the absence of t-PA, no clot lysis was observed after at least 4 hours of incubation, whereas 42% lysis and 89% lysis at 30 and 60 minutes, respectively, was observed 60 minutes after addition of t-PA. The addition of $2.5 \times 10^9$/ml of purified platelets to forming clots caused a substantial resistance toward t-PA-mediated clot lysis, resulting in only 15% clot lysis after an incubation of 2 hours (Figure 2). The subsequent addition of MAI-12 to platelet-rich clots generated a relief of platelet-dependent clot lysis inhibition. Under these conditions, 45% t-PA-mediated clot lysis was achieved after a 2-hour incubation with 0.5 μg/ml of MAI-12, whereas 85% clot lysis was elicited after a 1-hour incubation with 2 μg/ml of MAI-12.
**In Vivo Thrombolysis and Thrombus Extension**

We simultaneously measured thrombolysis and thrombus extension of human thrombi formed in rabbit jugular veins under different conditions. Specifically, either the monoclonal anti-PAI-1 antibody MAI-12 or a control monoclonal antibody was incorporated during thrombus formation. The effect of either one of the antibodies on the efficiency of thrombolysis and on thrombus extension was assessed both for endogenous thrombolysis and upon systemic administration of 0.25 or 0.50 mg/kg of exogenous human rt-PA, respectively. Incorporation of MAI-12 resulted in a significant increase of endogenous thrombolysis (Figure 3). Under these conditions, lysis of MAI-12-containing thombi was 14% (SEM, 1.5) at 60 minutes and 16% (SEM, 3.9) at 120 minutes, whereas only 3% (SEM, 0.5) and 8% (SEM, 0.8) was observed in the control antibody group, respectively (p<0.05, ANOVA and Newman-Keuls test).

In the groups receiving thrombolytic treatment, no significant difference in thrombolysis was observed between clots in which the monoclonal anti-PAI-1 antibody was incorporated versus control clots. In the rabbits treated with 0.25 mg/kg rt-PA, 24% (SEM, 4.3) of the initial clot containing the monoclonal anti-PAI-1 antibody had lysed at 60 minutes and 39% (SEM, 5.6) at 120 minutes versus 22% (SEM, 5.3) and 27% (SEM, 5.0) of the control clots (Figure 4). Thrombolysis induced by 0.5 mg/kg rt-PA of anti-PAI-1 antibody containing clots was 36% (SEM, 4.0) after 60 minutes and 82% (SEM, 3.3) after 120 minutes compared with 32% (SEM, 1.8) and 79% (SEM, 2.4) of control clots, respectively (Figure 5).

Thrombus extension of clots containing MAI-12 was significantly reduced, compared with thrombi...
with the control antibody. This finding was observed irrespective of the addition of exogenous rt-PA to the rabbits. In the absence of thrombolytic treatment, thrombi containing the control antibody showed a fibrinogen accretion of 36% (SEM, 3.0) at 60 minutes and of 43% (SEM, 3.5) at 120 minutes, compared with thrombi containing the anti-PAI-1 antibody of only 16% (SEM, 3.9) and of 26% (SEM, 3.0), respectively (p<0.05, ANOVA and Newman-Keuls test, Figure 3). Thrombus extension upon 0.25 mg/kg rt-PA was significantly reduced in anti-PAI-1-containing thrombi: 8% (SEM, 1.5) at 60 minutes and 14% (SEM, 1.5) at 120 minutes compared with control thrombi, 18% (SEM, 2.0) at 60 minutes and 28% (SEM, 4.3) at 120 minutes (p<0.05, ANOVA and Newman-Keuls test, Figure 4). Similar results were observed in the rabbits receiving 0.5 mg/kg rt-PA (Figure 5).

Discussion

We investigated the effect of MAI-12, which inhibits the activity of both human and rabbit PAI-1, on experimental thrombolysis in vitro and in vivo. First, it is demonstrated that the presence of MAI-12 can fully prevent platelet-dependent inhibition of t-PA-mediated clot lysis. This observation is explained by the inhibition of PAI-1 activity released from the α-granules of the platelets in the presence of MAI-12. Second, it is shown that endogenous thrombolysis of human thrombi formed in rabbit jugular veins is significantly enhanced when MAI-12 had been incorporated during thrombus formation. Furthermore, we confirm that platelets constitute the major reservoir of PAI-1 because platelet releasates or platelet lysates contain three- to fourfold more PAI-1 than the amount present in the corresponding volume of plasma. Hence, we assume that the effect of MAI-12 on endogenous thrombolysis is mainly exerted by preventing the action of PAI-1 released from α-granules of activated platelets. Third, both in the absence and in the presence of systemically administered t-PA, thrombus extension is clearly restricted by the presence of MAI-12. This finding implies that, during the process of thrombus extension, platelets accumulate that originate either from the human thrombus or from rabbit blood. Because rabbit platelets are in large excess over human platelets in this system, it is assumed that mainly rabbit platelets will contribute to thrombus extension. As reported in this article, MAI-12 also inhibits rabbit PAI-1, although to a lesser extent than human PAI-1 (see Figure 1). Consequently, we favor the interpretation that restriction of thrombus extension by MAI-12 is due to inhibition of PAI-1 released from activated rabbit platelets. In view of the lower reactivity of MAI-12 toward rabbit PAI-1, it is tempting to speculate that the efficiency of the antibody to limit thrombus extension would be even more pronounced in a homologous human system. On the other hand, because a deficiency of PAI-1 has been associated with a hemorrhagic tendency, and bleeding upon administration of t-PA and aspirin can be reversed by systemic administration of PAI-1, inhibition of PAI-1 may eventually enhance the risk of bleeding.

The increase in endogenous lysis of thrombi containing MAI-12 compared with control thrombi further illustrates the importance of PAI-1 in the regulation of endogenous thrombolytic activity. These results may confirm the hypothesis of a causal relation between a high PAI-1 activity in plasma, apparently resulting in impaired fibrinolytic activity, and the occurrence of thrombosis, as indicated in previous studies. In contrast to the enhanced endogenous thrombolysis in the presence of MAI-12, we found no effect of the antibody when, additionally, 0.25 or 0.50 mg/kg of rt-PA was administered. This observation might eventually be explained by the vast excess of the initial plasma levels of rt-PA compared
with both the PAI-1 content in rabbit plasma and in human thrombus. Under these conditions, the relatively small amount of human PAI-1 within the thrombus will be rapidly "consumed" through the formation of inactive t-PA/PAI-1 complexes, and a demonstrable difference in thrombolysis between thrombi incorporated with MAI-12 and control thrombi cannot be expected. It should be considered, however, that the half-life of human rt-PA in rabbits is extremely short and has been estimated to be 5 minutes.31,32 Consequently, it is conceivable that a sustained action of the antibody could be particularly relevant for processes (i.e., thrombus extension and reocclusion) that may require a longer period than the clearance of t-PA. Indeed, monitoring thrombus extension at 1 or 2 hours after the onset of t-PA-mediated thrombolytic therapy clearly demonstrates that MAI-12 partially prevents thrombus extension.

Our model permitted us to concomitantly detect both thrombolysis and thrombus extension. Apparently, simultaneously with thrombolysis, resistance to lysis manifested as thrombus extension occurred. This finding is reminiscent of the reocclusion phenomenon observed in a canine coronary artery stenosis and thrombosis model. Whether the monoclonal anti-PAI-1 antibody will be effective in reducing reocclusion upon thrombolytic therapy cannot be answered on the basis of this study. First, our observations were made in a system in which the monoclonal anti-PAI-1 antibody was incorporated during thrombus formation. To further assess a potential clinical use, experiments should be performed in which the monoclonal PAI-1-inhibiting antibody or its Fab fragment is systemically administered. Second, we have used the rabbit jugular vein thrombosis model, which is not considered to be an optimal model to study coronary artery stenosis and thrombosis. For this purpose, more sophisticated animal models have been developed in which the anti-PAI-1 approach should be confirmed.1,33

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**KEY WORDS** • fibrinolysis • plasminogen activator inhibitor-1 • platelets • myocardial infarction
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