Plasminogen Activator Inhibitor-1 Suppresses Endogenous Fibrinolysis in a Canine Model of Pulmonary Embolism

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Background. Plasminogen activator inhibitor-1 (PAI-1), the specific, fast-acting inhibitor of tissue-type plasminogen activator (t-PA), binds to fibrin and has been found in high concentrations within arterial thrombi. These findings suggest that the localization of PAI-1 to a thrombus protects that same thrombus from fibrinolysis. In this study, clot-bound PAI-1 was assessed for its ability to suppress clot lysis in vivo.

Methods and Results. Autologous, canine whole blood clots were formed in the presence of increasing amounts of activated PAI-1 (0–30 μg/ml). Approximately 6–8% of the PAI-1 bound to the clots under the experimental conditions. Control and PAI-1-enriched clots containing iodine-125–labeled fibrinogen were homogenized, washed to remove nonbound elements, and delivered to the lungs of anesthetized dogs where the homogenates subsequently underwent lysis by the endogenous fibrinolytic system. I2125-labeled fibrin degradation products appeared in the blood of control animals within 10 minutes and were maximal by 90 minutes. PAI-1 reduced fibrin degradation product release in a dose-responsive manner at all times between 30 minutes and 5 hours (≥76% inhibition at 30 minutes, PAI-1 ≥6 μg/ml). PAI-1 also suppressed D-dimer release from clots containing small amounts of human fibrinogen. t-PA administration attenuated the effects of PAI-1, whereas latent PAI-1 (20 μg/ml) had no effect on clot lysis. Blood levels of PA and PAI activity remained unaltered during these experiments.

Conclusions. The results indicate that PAI-1 markedly inhibits endogenous fibrinolysis in vivo and, moreover, suggest that the localization of PAI-1 to a forming thrombus is an important physiological mechanism for subsequent thrombus stabilization. (Circulation 1991;84:287–292)

The fibrinolytic system maintains blood vessel patency by clearing the vasculature of thrombi. The initial step in fibrinolysis is the conversion of plasminogen to plasmin by plasminogen activators (PA) such as tissue-type plasminogen activator (t-PA).1 Plasmin is responsible for the subsequent degradation and solubilization of the insoluble fibrin clots. Fibrin regulates the fibrinolytic process by binding to and juxtaposing key members of this pathway. t-PA and plasminogen binding sites are revealed on fibrin after its conversion from fibrinogen.2,3 The resultant colocalization of both t-PA and plasminogen on fibrin results in a massive acceleration of plasmin generation in the vicinity of the clot.2,4 Conversely, fibrin also binds factors that oppose the fibrinolytic process including the specific plasmin inhibitor, α2-antiplasmin.5 Clots enriched in α2-antiplasmin resist lysis in vitro.5

Plasminogen activator inhibitor-1 (PAI-1) may play a critical role in governing the fibrinolytic system. PAI-1 is the fast-acting, plasma inhibitor of t-PA and urokinase.6 PAI-1 is synthesized by vascular endothelial cells and smooth muscle cells and is released from platelets upon activation.9,10 Thus, PAI-1 may be present in significant concentrations at localized sites of vascular injury and in forming thrombi. Clinical studies suggest an association between cardiovascular disease with elevated levels of PAI activity in the circulation.11 Higher levels of PAI-1 may lead to inhibition of fibrinolysis resulting in prolonged clot stability and a tendency to thrombosis.

Despite suggestive in vitro and clinical data, there is no direct evidence indicating that PAI-1 affects the fibrinolytic process in vivo. Wagner et al12 showed that PAI-1, like other important members of the fibrinolytic pathway, binds to fibrin. Other reports indicate that the concentrations of PAI-1 in human13

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and in experimentally induced porcine\textsuperscript{14} thrombi are two orders of magnitude higher than the levels found in plasma. These data suggest that the localization of PAI-1 within a thrombus protects that same thrombus from fibrinolysis. In this study, blood clot homogenates, which had incorporated PAI-1, were delivered to the microvasculature of the canine lung, and the subsequent effects of the endogenous fibrinolytic system were monitored. The PAI-1–enriched emboli resisted lysis in this in vivo setting.

**Methods**

**Materials**

Two-chain t-PA (920 IU/\(\mu\)g), glu-plasminogen, and des-AA-fibrinogen were obtained from American Diagnostica, Greenwich, Conn. D-Val-Leu-Lys-pNA (S2251), D-Ile-Pro-Arg-pNA (S2288), and CNBr fibrinogen fragments were obtained from Helena Laboratories, Beaumont, Tex. Human fibrinogen was purchased from Calbiochem, La Jolla, Calif., and single-chain t-PA (Activase) was obtained from Gentech, South San Francisco, Calif. Guanidine HCl was obtained from Schwarz/Mann, Cleveland, Ohio. Recombinant, human PAI-1 was expressed in Saccharomyces cerevisae and purified to homogeneity as described by Gardell et al.\textsuperscript{15}

**PAI-1 Activity Assay**

The inhibitory activity of PAI-1 was determined by adding increasing volumes of PAI-1 to a constant amount of two-chain t-PA (0.938 IU) in the wells of microtiter plates (Microwell, Nunc, Inc., Naperville, Ill.) in 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.01% Tween 80 (TNT buffer). After 1 hour at 25°C, an equal volume of TNT buffer (100 \(\mu\)l) containing 0.5 \(\mu\)M glu-plasminogen, 50 \(\mu\)g/ml des-AA-fibrinogen, and 1 mM S2251 was added. Residual t-PA activity was determined by measuring the increase in optical density at 405 nm after 1.5 hours of incubation at 37°C. The linear portion of the inhibition curve was extrapolated to obtain the concentration of PAI-1 necessary for complete inhibition of the t-PA. One arbitrary unit (AU) is defined as the amount of PAI-1 required to inhibit one international unit of t-PA.

**Activation of PAI-1**

The purified PAI-1 was in a latent, inactive form that can be activated by treatment with guanidine HCl.\textsuperscript{7,15} Latent PAI-1 (<2 AU/\(\mu\)g) was exposed to 5.4 M guanidine HCl for 5 minutes at 25°C and was subsequently desalted by gel filtration in TNT buffer at 4°C. The activated PAI-1 was 35% active and possessed a specific activity of 509 AU/\(\mu\)g based on its activity against two-chain t-PA in the PAI-1 activity assay described above.

**Measurement of Endogenous Fibrinolysis In Vivo**

Laboratory bred mongrel dogs of either sex weighing 10–15 kg were anesthetized with sodium pentobarbital and respired with room air (Harvard Respi-
37°C, the clots were homogenized and washed as described above, and the radioactivity of each clot homogenate was determined.

**Determination of Plasma PAI-1 Activity**

PAI activity in plasma was determined essentially by the method of Chmielewska and Wiman as described. The t-PA activity of plasma was measured as described in the directions of the KabiVitrum, COA-SET t-PA kit.

**Statistical Analysis**

Statistical significance (p<0.05) of differences among treatments was determined by two-way repeated-measures analysis of variance and one-way analysis of variance of independent observations with Dunnnett's multiple comparison test. The significance of the difference between individual time points was determined by the method of two-sided least-significant difference.

**Results**

Wagner et al demonstrated that PAI-1 binds to purified fibrin in vitro. To establish whether PAI-1 also binds to whole blood clots ex vivo, canine blood was allowed to spontaneously clot in the presence of increasing amounts of activated PAI-1 and a trace amount of 125I-labeled PAI-1. The clots were homogenized, washed extensively to remove any unbound PAI-1, and the counts per minute of the final clot suspensions was determined. At initial PAI-1 concentrations of 1.2, 6, and 30 μg/ml, 0.10±0.02, 0.44±0.10, and 1.77±0.20 μg PAI-1 were associated with the washed clot homogenate (average±range from two experiments in duplicate). These data indicate that 6–8% of the PAI-1 added to the blood strongly associates with the final, insoluble clot suspension.

To determine whether PAI-1-containing clots were protected from lysis in vivo, autologous blood was clotted in the presence of vehicle or increasing amounts of activated, unlabeled PAI-1. Trace amounts of 125I-labeled fibrinogen were included as an eventual marker for fibrin degradation products. The clots were homogenized, washed, and after resuspension were delivered through the left jugular vein. Levels of radioactivity in tissue sections indicated that the clot homogenates dispersed and lodged throughout the entire lung. Radioactivity was not present along the route of delivery, and blood radioactivity levels from control and PAI-1 dogs were low 1 minute after clot delivery. In contrast, 125I-labeled fibrinogen, which had not been allowed to clot, entered the total blood volume immediately after delivery and subsequently cleared from the circulation with a half-time of approximately 8 hours. These data indicate that the clot homogenates, which contain 125I-labeled fibrinogen, lodge in the lung microvasculature immediately after administration.

Figure 1 indicates that 125I-labeled fibrin degradation products began to appear in the blood of the vehicle-treated groups within 10 minutes of clot delivery and were near maximal by 90 minutes. Clots formed in the presence of PAI-1 appeared to resist lysis (Figure 1). 125I-labeled fibrin degradation products in blood at PAI-1 levels of 6 and 30 μg/ml were significantly less (p<0.0001) than the levels in the controls. The lowest level of PAI-1 used (1.2 μg/ml) also suppressed clot lysis, but the effect was not statistically significant (p=0.06). At the 30-minute time point, when fibrin degradation product levels were increasing rapidly in control dogs, PAI-1 at 6 and 30 μg/ml suppressed clot lysis by 76% and 84%, respectively, as judged by computing the area under the curves. The half-maximal effective dose of PAI-1 was 2.7 μg/ml at this time. The inhibitory effects of PAI-1 were temporal in nature. Even at the highest dose of PAI-1 used (30 μg/ml), more radioactivity was present in the blood at later times than at earlier times. At the end of the experiments described above (at 300 minutes), the radioactivity remaining in the lungs was determined (Table 1). Relative to the control animals, significantly more 125I-labeled fibrinogen remained in the dogs that had received the PAI-1-containing clots (p=0.016 by analysis of variance).

In control experiments, dogs received clots that were formed in the presence of latent, unactivated
PAI-1 (20 µg/ml, n=3). However, there was no difference in 125I-labeled fibrin degradation product release between these clots and the vehicle-treated clots at any time between 0 and 300 minutes (data not shown).

D-Dimer is a defined fibrin degradation product that is cleaved from cross-linked fibrin by plasmin. Because antibodies are available that recognize human D-dimer, the release of this specific fragment from clots that contained small amounts of human fibrinogen was measured (Figure 2). The release of D-dimer from the vehicle-treated clots was apparent at 10 minutes and was maximal by 60 minutes. After 90 minutes, the levels of D-dimer decreased. In contrast, significantly less D-dimer was released from the PAI-1-treated clots at all times between 30 and 300 minutes (p<0.05).

Plasma levels of PA and PAI activity did not change during these experiments. Levels of PA activity before and at 30, 60, 120, and 240 minutes after clot administration in two control and two high-dose PAI-1 (30 µg/ml) dogs ranged from 2.7 to 5 IU/ml and from 2.5 to 4.9 IU/ml, respectively. PAI activity levels before and at 30, 60, 120, and 240 minutes after clot administration ranged from 16 to 19 AU/ml and from 16 to 20 AU/ml in the same vehicle and PAI-1 dogs, respectively.

High levels of exogenously added t-PA should reverse the effects of PAI-1, if PAI-1 suppresses clot dissolution by inhibiting PAs in the lung. To test this hypothesis, t-PA was administered immediately after delivery of vehicle- or PAI-1–treated clots (Figure 3). The concentration of PAI-1 used (6 µg/ml) suppresses clot dissolution in the absence of exogenous t-PA (Figure 1). t-PA administration resulted in rapid clot dissolution as determined from the appearance of 125I-labeled fibrin degradation products in blood (Figure 3). Moreover, PAI-1–containing clots lysed as rapidly as the control clots. The percentage of administered radioactivity remaining in the lungs at the end of the experiments was similar in both groups (vehicle, 1.56%; PAI-1, 1.68%).

**Discussion**

The effects of PAI-1 on fibrinolysis in an in vivo setting were investigated in a canine model of pulmonary embolism. The initial binding studies indicated that when increasing concentrations of PAI-1 were added to forming whole blood clots ex vivo, 6–8% of the PAI-1 bound to the final clot. Presumably, binding to the clot reflects the binding of PAI-1 to fibrin within the clot. Vehicle- and PAI-1–containing clot homogenates were subsequently delivered to the lung microvasculature, and the release of 125I-labeled fibrin degradation products into the circulation were measured. Relative to the vehicle treatment, PAI-1 significantly inhibited 125I-labeled fibrinogen D-dimer release. Clot homogenates, which contained vehicle or PAI-1 and small amounts of human fibrinogen, were delivered to lungs at time 0. Blood samples were withdrawn at indicated times. Amount of D-dimer present in the plasma was determined with an immunoassay that measures human D-dimer. Canine plasma did not interfere with the assay. •, Vehicle, n=2; ○, 6 µg/ml PAI-1, n=2. Results are mean±range. Concentration of PAI-1 refers to the concentration of PAI-1 per milliliter of blood before clot homogenization.
fibrin degradation product release in a dose-dependent manner. Moreover, the levels of $^{125}$I-labeled fibrinogen (fibrin) that remained in the lungs at the end of the experiments were significantly higher for the PAI-1–treated clots. The release of D-dimer was also significantly reduced by PAI-1. These latter findings suggest that the differences in blood radioactivity between the control and PAI-1 treatment groups reflect different rates of clot lysis and are not due to different rates of fibrin degradation product clearance from the circulation. The protective effects of PAI-1 were transient because the release of $^{125}$I-labeled fibrin degradation products and D-dimer from the PAI-1–containing clots increased slowly over time. Latent PAI-1 did not protect the clots from dissolution; therefore, activation of PAI-1 is essential for its clot stabilizing activity. Whether this reflects a reduced fibrin-binding capacity of latent PAI-1 or its inability to inhibit PAs requires further investigation. Together, the data indicate that PAI-1 exerts a marked inhibitory influence on the endogenous fibrinolytic system of the canine lung.

Clot lysis from both vehicle- and PAI-1–treated clots occurred without changes in the circulating levels of PA or PAI activity. Thus, the lytic process does not appear to be due to large increases in PA released during the experiments, nor can the protective effects of PAI-1 be ascribed to large-scale inhibition of circulating PA activity. The inhibitory effects of PAI-1 appear to be due to the PAI-1 that was initially delivered with the clot and are not due to PAI-1 released into the circulation that can occur after endotoxin administration or as an acute-phase response.

PAI-1 most likely suppressed clot dissolution above by inhibiting PAs present in the lung. This notion is supported by the finding that high levels of exogenous t-PA reversed the effects of PAI-1. The endogenous PA(s) responsible for clot lysis in this model may be either urokinase or t-PA, produced locally or recruited from the circulation. Wagner et al. showed that PAI-1, bound to fibrin, dissociates in the presence of t-PA and urokinase and directly inhibits these proteinases by forming inactive, 1:1 molar complexes. In addition, the PAI-1–t-PA complexes were capable of rebinding to fibrin through t-PA-binding sites; an action that would suppress the binding of active t-PA and the resulting augmentation of plasminogen activation.

The initial blood levels of PAI-1 required for the subsequent half-maximal protection of the clots at the 30-minute time point were approximately 2.7 μg/ml. These concentrations of PAI-1 are considerably greater than the levels of PAI-1 encountered in the normal circulation (<20 ng/ml). However, it is important to note that less than 10% of the added PAI-1 actually bound to the forming clot and would have been delivered with the clot homogenate to the lungs. Thus, PAI-1 concentrations that confer clot stabilization in vivo appear to be less than 300 ng/ml. These levels may be physiologically relevant because the amount of PAI-1 in serum is greater than 200 ng/ml and PAI-1 is synthesized and released from endothelial cells in response to a variety of inflammatory stimuli including endotoxin and transforming growth factor-β.

The low binding efficiency of PAI-1 toward forming blood clots observed ex vivo may have been due to our use of latent PAI-1, which was activated with guanidine HCl and is only partially capable of recognizing fibrin. This situation may not occur in vivo where naturally occurring PAI-1 may be fully capable of binding to fibrin. It is also possible that other factors, present in blood or platelets, regulate the extent of PAI-1 binding to a thrombus.

Our model may best approximate a physiological setting in which high levels of PAI-1, released from platelets or the vascular wall, accrue within a growing thrombus presumably by binding to fibrin. This idea is supported by recent findings that indicate that human arterial thrombi contain 155- and 213-fold more PAI-1, respectively, than an equivalent volume of plasma. The accumulation of PAI-1 within a
thrombus would not only prevent the rapid clearance of PAI-1 from the circulation but would maintain PAI-1 in its active form. High local concentrations of PAI-1 would prevent premature thrombus dissolution by inhibiting PAI activation of the fibrinolytic system. The PAI-1 would eventually be overwhelmed by PAs, released locally or recruited from the circulation, and lysis would ensue. It is interesting to speculate that the greater resistance of platelet-rich thrombi than platelet-poor thrombi to lysis by exogenous t-PA is mediated by platelet-derived PAI-1. The results indicate that PAI-1 is a potent inhibitor of fibrinolysis in vivo and, moreover, suggest that the net balance between PAI-1 and PAs at the thrombus interface dictates the rate of thrombus dissolution or, potentially, of thrombus formation. It remains to be determined whether elevated levels of PAI-1 in the circulation, such as have been measured in survivors of a first myocardial infarction who later have another infarction, can also lead to greater thrombus stability.

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