Suppression of Thrombolysis in a Canine Model of Pulmonary Embolism

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Background  The brisk fibrinolytic response of canines has impaired efforts to develop a canine model of chronic thromboembolic pulmonary hypertension. Difficulties in retaining chronic embolic residuals were partially overcome by administration of tranexamic acid (TXA) (Circulation. 1991;83:1272-1279). In this study, we used type 1 plasminogen activator inhibitor (PAI-1), a major inhibitor of the endogenous fibrinolytic system, to determine its efficacy in the suppression of thrombolysis in canines.

Methods and Results  Thrombus was induced in the inferior vena cava of anesthetized mongrel dogs with thrombin and a special double-balloon catheter; 2 hours later, the thrombus was embolized. In one group of dogs, activated type 1 plasminogen activator inhibitor (PAI-1) (130 µg) was delivered directly into the forming thrombus; in another, TXA (110 mg/kg) was given intravenously before thrombus formation; in controls, thrombus was induced without inhibitors. Cross-linked fibrin degradation product (D-dimer) appeared in the blood of control animals within 1 hour of thrombus induction (176±62.5 versus 1.02±0.39 ng/mL baseline; mean±SEM), was maximal by 4 hours (413±110 ng/mL) and remained elevated at 24 hours (90.8±19.5 ng/mL). Compared with controls, PAI-1 and TXA suppressed D-dimer release by 80% and 85%, respectively, over the first 24 hours. One week later, animals were killed, and residual emboli were harvested. Perfusion scan defects persisted in all animals at this time, but there were no scan defect differences among groups. However, emboli recovered from animals receiving PAI-1 still harbored immunoreactive PAI-1 and were, on average, more than twofold greater in mass (393±56 mg) than emboli recovered from either controls (183±76 mg) or animals receiving TXA (180±80 mg).

Conclusions  Intravenous TXA and intrathrombus PAI-1 effectively suppress thrombolysis for 24 hours in canines. Thromboemboli enriched with PAI-1 appear to resist lysis for longer periods of time (up to 1 week). These findings are consistent with the hypothesis that PAI-1 remains associated with the embolus, where it continues to inhibit lysis, whereas TXA eventually diffuses out of the embolus, allowing lysis to ensue. (Circulation. 1994;90:3091-3097.)

Key Words: • plasminogen activator inhibitor • fibrinolysis • tranexamic acid • D-dimer

Clinical studies of patients surviving an acute pulmonary embolism have shown that vascular obstruction spontaneously resolves in the majority of cases.1-4 However, embolic obstruction fails to resolve in a small percentage of patients who subsequently develop chronic thromboembolic pulmonary hypertension.5-9 The mechanisms responsible for this failure to resolve are not known. To explore these mechanisms, we have developed a canine model of pulmonary embolism. This model has been used in the present investigation to determine whether type 1 plasminogen activator inhibitor (PAI-1) could (1) be incorporated into a thrombus formed in vivo and (2) suppress the lysis of that thrombus, after embolization, for a period of 1 week.

Prior investigations have demonstrated that, in the absence of inhibitors, the remarkably efficient fibrinolytic system of the dog rapidly lysed experimentally induced thromboemboli.10 We have previously used tranexamic acid (TXA), an inhibitor of plasmin, to suppress the endogenous fibrinolytic system.11 However, TXA is rapidly cleared from the circulation,11-13 and chronic administration often induces nausea and anorexia, making it difficult to sustain inhibitory blood levels.11

Recent reports have demonstrated that high circulating levels of PAI-1 can suppress clot dissolution in vivo.14-16 Moreover, PAI-1 can bind directly to fibrin in vitro and still retain its ability to inhibit both tissue-type and urokinase-type plasminogen activators.17,18 In this regard, whole blood clots that have incorporated PAI-1 ex vivo resist lysis when reintroduced into experimental animals.15,19 These data suggest that PAI-1 may play an important role in thrombus stabilization.

In the present study, we evaluated the endogenous lysis of thromboemboli induced either in the presence of circulating TXA or in the presence of PAI-1 that had been delivered directly into forming thrombi. The results showed that while both inhibitor treatments effectively suppressed thrombolysis for 24 hours, greater embolic residuals were recovered at the end of 1 week only from animals harboring thromboemboli enriched with PAI-1.

Methods

Materials  Single-chain tissue-type plasminogen activator (TPA) (Bowes melanoma, 607 IU/µg), glu-plasminogen, and CNBr fibrinogen fragments were obtained from American Diagons...
tica. d-Val-Leu-Lys-pNA (S2251) was purchased from Kabi Vitrum. Human fibrinogen (>95% clottable) was obtained from Calbiochem. Topical thrombin (bovine) was obtained from Armour Pharmaceuticals. Ultrapure guanidine-HCl was from BRL, and tranexamic acid was from Sigma Chemical Co. Recombinant human PAI-1 (rPAI-1), expressed in Saccharomyces cerevisiae and purified to homogeneity as described by Gardell et al., was a generous gift of Merck Sharp and Dohme Research Laboratories. Biotinylated goat anti-rabbit immunoglobulin and peroxidase-conjugated streptavidin were purchased from DAKO Corporation, and 3,3',5-diaminobenzidine tetrahydrochloride was obtained from Zymed Laboratories.

rPAI-1 Activity Assay

The inhibitory activity of rPAI-1 was determined by adding increasing volumes of the inhibitor to 10 IU of TPA in a final volume of 100 μL of 50 mmol/L Tris-HCl, pH 7.4, 0.1 mol/L NaCl, 0.01% Tween 80 (TNT) buffer containing 0.5% bovine serum albumin (TNTB). The mixtures were incubated for 30 minutes at 37°C and then further diluted by adding 400 μL of TNTB. Twenty-five microliters of each diluted sample were then added to triplicate microtiter plate wells, each containing 200 μL of 0.25 mmol/L glu-plasminogen, 100 μg/mL CNBr fibrinogen fragments, and 0.3 mmol/L S2251. Residual TPA activity was determined by measuring the increase in absorbance at 405 nm after 2-hour incubation at 37°C. The linear portion of the inhibition curve was extrapolated to obtain the amount of rPAI-1 necessary for complete inhibition of the TPA. One unit of activity is defined as the amount of PAI-1 required to inhibit 1 IU of TPA.

Activation of rPAI-1

Recombinant PAI-1 was provided in a highly purified but inactive latent form that was activated by treatment with guanidine-HCl. Briefly, 215 μg of latent rPAI-1 (<2 U/μg) was exposed to 4 mol/L guanidine-HCl in TNT buffer for 5 minutes at 25°C and then desalted by gel filtration against TNT buffer at 4°C. The activated rPAI-1 possessed a specific activity of 514 ± 70.7 U/μg (mean ± SD). This activity is 60% of the theoretical value calculated for fully active rPAI-1 (840 U/μg), assuming 1:1 stoichiometry between TPA (Mr ~ 70 000; specific activity, 600 IU/μg) and rPAI-1 (Mr ~ 50 000).

Evaluation of Thrombolysis In Vivo

Conditioned female mongrel dogs (weight, 17 to 33 kg) were anesthetized with intravenous Biotal (thiamylal sodium; Boehringer Ingelheim, 15 to 20 mg/kg), intubated, and respirated with room air using a mechanical ventilator. Anesthesia was maintained by inhalation of halothane (1.0% to 1.5% in room air). Heart rate and mean systemic pressure were continuously monitored by a femoral arterial line. Blood pH, PaO2, and PaCO2 were periodically monitored to maintain PaCO2 between 35 and 40 mm Hg. A modified Swan-Ganz catheter consisting of a central port opening between two inflatable balloons 10 cm apart was used to form thrombi. The catheter was introduced through a saphenous vein cutdown and advanced, under fluoroscopic monitoring, into the inferior vena cava (IVC) until the balloons were positioned between the renal veins and the iliac junction. The balloons were then inflated against the vessel wall, thereby creating an intraballoon chamber ~10 mL in volume. The integrity of the chamber was fluoroscopically confirmed by injecting a small amount of contrast medium (Renografin-76, Squibb Diagnostics) into the intraballoon chamber. Next, with balloons inflated, 10 mL of blood was withdrawn from the intraballoon chamber into a sterile plastic syringe containing 1.0 mL (20 mg) of human fibrinogen and either 1.5 mL of TNT buffer (control group, n = 5) or 1.5 mL of TNT buffer containing ~130 μg of activated rPAI-1 (PAI group, n = 5). This mixture was delivered, along with 150 U of thrombin, back into the intraballoon chamber, thereby inducing the thrombus. The amount of rPAI-1 was chosen to give a theoretical final concentration of ~10 μg/mL in the forming thrombus, a concentration shown to be effective in suppressing the lysis of whole blood clot homogenates in vivo. Another group of animals received a single bolus dose of intravenous TPA (110 mg/kg) immediately before thrombus induction (TXA group, n = 5). Thirty minutes after thrombus induction, the distal balloon was deflated and the thrombus was aged an additional ~1.5 hours. The thrombus was then embolized by deflating the proximal balloon and removing the catheter. A venogram was performed to confirm the presence of a thrombus before embolization and was repeated after embolization to verify the release of the thrombus from the IVC. Ten hours after embolization, the animals were extubated, allowed to recover, and returned to their cages.

Blood samples (5 mL) were collected in siliconized glass tubes containing 0.1 volume of 3.2% sodium citrate immediately after embolization and at intervals thereafter for determination of PAI-1 antigen, PAI activity, d-dimer, and TXA plasma levels. Blood was centrifuged at 2000g for 15 minutes at 4°C to obtain plasma, which was stored at ~7°C until assayed. Four-view perfusion lung scans (anterior, postero, right lateral, and left lateral) using 99mTc-labeled macroaggregated albumin were obtained in 3 of the 5 animals in each group at the following times: 1 day before thrombus induction (baseline), 2 hours after embolization, and at the conclusion of the study (1 week after embolization). After the final scan, these animals were euthanized with intravenous Nembutal (pentobarbital sodium; Abbott Labs, 120 mg/kg) and autopsied to determine the precise location and mass of residual emboli.

This protocol was approved by the University of California at San Diego Animal Subjects Committee. Care and handling of experimental animals conformed to the standards established by the University of California at San Diego Department of Veterinary Services, which are in compliance with Federal recommendations.

Plasma PAI-1 Antigen and PAI Activity Assay

The plasma concentration of human PAI-1 antigen was determined by a monoclonal antibody sandwich enzyme-linked immunosorbent assay (Technoclone). The assay reportedly measures free, complexed, and latent PAI-1 and is not affected by other plasminogen activator inhibitors. Control experiments revealed that activated PAI-1 and human PAI-1 standard were essentially indistinguishable in this assay (data not shown). Fast-acting PAI activity in plasma was determined by a two-stage, plasminogen-based assay.

Plasma d-Dimer Assay

The plasma concentration of d-dimer was determined by a sandwich enzyme-linked immunosorbent assay (Diagnostica Stago). The specificity of this assay for human d-dimer necessitated the incorporation of human fibrinogen into the induced thrombus as described above. Control experiments confirmed that (1) canine d-dimer indeed was not detected by this assay and (2) TXA (up to 500 μg/mL) did not interfere with the measurement of d-dimer in plasma (data not shown).

Plasma TXA Assay

The plasma concentration of TXA was determined by ion-exchange, thin-layer chromatography as previously described. Briefly, after an initial extraction, TXA from 1 mL of plasma, samples and TXA standards of varying concentrations were spotted on thin-layer plates coated with Polygram Ione-X-25 SA-Na (Macherey-Nagel). Plates were developed sequentially with 0.2 mol/L sodium acetate (pH 5.2) and 0.1 mol/L sodium phosphate (pH 7.0) and then sprayed with ninhydrin (0.2% in ethanol) to reveal the TXA zones. TXA standards and the comigrating sample zones were scraped, eluted with n-propanol/water (1:1 vol/vol), and the absorbance of the eluates at 570 nm was measured. The TXA concentra-
tion in the samples was determined by comparison to a curve constructed from the TXA standards.

**PAI-1 Immunohistochemistry**

Residual emboli recovered at autopsy were immediately fixed for 24 hours in 0.05 mol/L cold sodium phosphate buffer, pH 7.4, containing 4% paraformaldehyde. Specimens were then rinsed in 70% ethanol, embedded in paraffin blocks, and sectioned at 2-μm thickness onto polylysine-coated slides. Immunohistochemical staining of the specimens for PAI-1 was performed as previously described. Briefly, PAI-1 was localized using affinity-purified polyclonal rabbit anti-human PAI-1 (4 μg/mL) as the primary antibody followed by sequential treatment with biotinylated goat anti-rabbit immunoglobulins and a streptavidin-peroxidase conjugate. A reddish-brown deposit after development with the chromogenic substrate (3,3′-diaminobenzidine tetrahydrochloride) was indicative of positive immunoreactivity. Negative controls were prepared by omitting the primary antibody or alternatively by substituting the primary antibody with nonimmune rabbit IgG in the staining procedure. Specimens were counterstained with hematoxylin and photographed under the light microscope.

**Statistical Analysis**

All data are presented as mean±SEM unless otherwise noted. Differences between measured values at different time points within a treatment group were evaluated by repeated-measures ANOVA. Differences between treatment groups were evaluated by one-way ANOVA of independent groups. In both of these analyses, the Tukey-Kramer multiple comparison test was used to evaluate the significance of multiple comparisons. Differences yielding \( P<.05 \) (two-sided) were considered significant.

**Results**

**Plasma PAI-1 Antigen and PAI Activity Levels After Thrombus Induction**

Plasma PAI-1 antigen and PAI activity levels were used to monitor the appearance of PAI-1 in the circulation after thrombus induction in control animals and in animals receiving intrathrombus rPAI-1 (Fig 1). The monoclonal antibodies used to quantitate PAI-1 antigen apparently did not cross-react with endogenous canine PAI-1, as evidenced by the extremely low baseline plasma levels (Fig 1A, \( t=0 \)). Thus, the PAI-1 antigen levels shown in Fig 1 predominantly reflect rPAI-1. As shown, the PAI-1 antigen levels of the control group did not change at any time before or after thrombus induction. In contrast, there was a significant rise in the PAI-1 antigen level of the PAI group 1 hour after thrombus induction (16.2±1.8 ng/mL versus 0.9±0.3 ng/mL baseline, \( P<.001 \)). This rise was transient, as noted by the rapid fall to near baseline levels within 2 to 3 hours. The plasma PAI-1 antigen profile of the PAI group is consistent with an early appearance of rPAI-1 in the circulation followed by normal clearance.

The plasma PAI-1 antigen levels of the PAI group were essentially mirrored by the corresponding plasma PAI activity levels (Fig 1B). Bear in mind that unlike the PAI-1 antigen levels shown in Fig 1A, the PAI activity levels shown in Fig 1B reflect the activities of both endogenous PAI-1 and rPAI-1. Although the control group exhibited a broad rise and fall in plasma PAI activity over the first 24 hours, the only time the PAI group was significantly elevated compared with controls was 1 hour after thrombus induction.

**D-Dimer Release From Thromboemboli**

Plasma levels of d-dimer were used to monitor the lysis of thromboemboli in vivo. Because the d-dimer assay is specific for human d-dimer, only the lysis of experimentally induced thrombi (for example, those supplemented with human fibrinogen) was detected.

The plasma d-dimer levels of each group after thrombus induction are presented in Fig 2. In the control group, d-dimer levels were significantly elevated above baseline (\( t=0 \)) as early as 1 hour after thrombus induction (176±62.9 versus 1.02±0.39 ng/mL); the level was maximal 4 hours after thrombus induction (413±110 ng/mL) and was still significantly elevated 24 hours later (90.8±19.5 ng/mL). The d-dimer levels of both the TXA and PAI groups were significantly lower compared with controls at all times between 1 and 10 hours after thrombus induction. At 24 hours, the d-dimer levels of all three groups were elevated compared with baseline but not significantly different from one another (\( P=.261 \)). One week after thrombus induction, the d-dimer levels of all three groups were still slightly elevated but not significantly different from baseline (data not shown). By determining the area under the curves in Fig 2, it can be estimated that the rPAI-1 and TXA treatments resulted in an 80% and 85% suppression of d-dimer release, respectively, during the first 24 hours.
The TXA group exhibited a small but significant rise in D-dimer level 4 to 6 hours after thrombus induction. During this time interval, the plasma TXA level fell from 14.9 to 11.5 \( \mu \text{g/mL} \) (Fig 3), suggesting that the plasma TXA concentration required to inhibit systemic fibrinolysis in canines is approximately 15 \( \mu \text{g/mL} \).

### Perfusion Lung Scans and Autopsy Findings

Perfusion lung scans were performed on 3 of the 5 animals in each group at the following times: before thrombus induction (baseline), 2 hours after embolization, and 1 week later (before the animals were killed). All baseline lung scans were normal. Two hours after embolization, significant perfusion defects (for example, defects in at least two of the four views) were evident in all animals. Right lower lobe defects were commonly observed, occurring in 7 of 9 (78\%) animals studied (Table). At 1 week, perfusion defects were still evident in all animals, but the scans had normalized considerably compared with the corresponding 2-hour postembolization scans (Table). Qualitatively, there were no apparent differences between groups with regard to embolic residuals detected by perfusion scan before the animals were killed. There was, however, excellent correlation between the region of the lung showing a perfusion defect and the actual location of the emboli recovered at autopsy.

All animals killed still harbored residual emboli. On gross examination, the emboli fell into two groups based on physical appearance: larger emboli (>200 mg) were typically blackish-red in color and quite firm in texture; smaller emboli (<200 mg) were lighter in color, firm in texture, and frequently attached to the arterial wall.

The total embolic mass recovered from each animal varied considerably. As shown in the Table, the two largest masses (405 and 484 mg) were recovered from animals in the PAI group. The two smallest masses (19 and 31 mg) were recovered from animals in the TXA and control groups, respectively. The mean embolic mass of each group is presented in Fig 4. As shown, there was a tendency toward larger embolic residuals in the PAI group (388 mg) as compared with either the control group (183 mg; \( P=.046 \), one-sided \( t \) test) or the TXA group (180 mg), whereas the embolic masses of the control and TXA groups were nearly identical (183 mg versus 180 mg, respectively).

### Demonstration of Immunoreactive PAI-1 Within Recovered Emboli

Specimens recovered at autopsy were subjected to immunohistochemical staining for PAI-1 antigen (Fig 5). Diffuse, somewhat heterogeneous positive staining...
of the entire clot matrix was characteristic of specimens from the PAI group (Fig 5B). In contrast, essentially no staining of the clot matrix was observed in specimens from either the control group or the TXA group (compare Figs 5A and 5C with Fig 5B). Instead, positive staining in these groups appeared to be associated with cells at the clot surface. These cells appeared to be endothelial cells and/or fibroblasts, both of which are capable of synthesizing PAI-1.\(^\text{(21,23,24)}\) Although cell-associated PAI-1 is probably of canine origin, we have previously shown that canine PAI-1 does cross-react with the polyclonal rabbit anti-human PAI-1 antibody used in these experiments.\(^\text{25}\) In control experiments, no positive staining was observed when the primary (anti-PAI-1) antibody was omitted from the procedure or was substituted with non-immune IgG (not shown).

**Discussion**

We have demonstrated here for the first time that biologically active rPAI-1 can incorporate into a forming thrombus in vivo and suppress the release of a fibrin-specific degradation product (D-dimer) by approximately 80% over the first 24 hours (Fig 2). Moreover, rPAI-1–enriched thromboemboli recovered after 1 week still harbored immunoreactive PAI-1 (Fig 5) and were, on average, approximately two times greater in mass than thromboemboli without rPAI-1 (Fig 4). Our results support the notion that localization of relatively high concentrations of active PAI-1 within a thrombus can provide an important thrombus stabilizing effect, presumably by inhibiting plasminogen activators released locally as well as those recruited from the circulation.

The early protective effects of rPAI-1 observed shortly after thrombus induction may have been mediated, in part, by rPAI-1 that did not incorporate directly into the thrombus but instead found its way into the general circulation. This circulating component, however, was rapidly cleared, as shown by changes in plasma PAI-1 antigen levels shortly after thrombus induction (Fig 1A). The protective effects seen later on (4 to 24 hours), when the plasma PAI-1 levels had returned to baseline, were most probably due to rPAI-1 that had incorporated directly into the thrombus, presumably bound to fibrin. The plasma half-life of the circulating...
component was estimated to be approximately 45 minutes, in close agreement to the reported clearance of activated rPAI-1 from the circulation of rats\(^{15}\) and rabbits.\(^{26}\) Several investigators have shown that high circulating levels of rPAI-1 alone can suppress thrombolysis in animal models,\(^{14,16}\) but much higher levels than those observed in our study were required.

Although the amount of rPAI-1 initially bound to the thrombus could not be determined directly, an estimate obtained by extrapolating the PAI-1 antigen curve (Fig 1A) back to the 30-minute time point indicated that approximately 50% of the delivered rPAI-1 had incorporated into the thrombus. This estimate was based on the observation that the extrapolated level was approximately 50% of the expected level of a bolus intravenous injection of 130 \(\mu\)g rPAI-1, assuming a 10% blood volume to weight ratio and a uniform distribution of rPAI-1 in the circulation. Reilly et al\(^{29}\) reported that while only 6% to 8% of rPAI-1 added to forming canine whole blood clots ex vivo remained tightly bound after homogenization and extensive washing, these clots were still more resistant to lysis in vivo compared with control clots formed without rPAI-1.

The suppression of D-dimer release mediated by intrathrombus rPAI-1 over the first 24 hours was roughly equivalent to that attained by administering a single bolus dose of intravenous TXA immediately before thrombus induction (Fig 2). A small but significant rise in the plasma D-dimer level was observed when the plasma TXA level fell below 15 \(\mu\)g/mL (Fig 3). This suggests that a minimal TXA plasma concentration of approximately 15 \(\mu\)g/mL is required to inhibit systemic fibrinolysis in canines, in good agreement with the reported level of 10 \(\mu\)g/mL for inhibition of systemic fibrinolysis in humans.\(^{13}\) Interestingly, we observed no further increase in plasma D-dimer, even when circulating TXA levels continued to fall below 15 \(\mu\)g/mL (Fig 3). This might be due to prolonged inhibition by TXA that had not yet diffused from the core of the thrombus.

Based on the significant perfusion scan defects observed in all animals 2 hours after embolization, it may be concluded that neither TXA nor rPAI-1 had any adverse effects on thrombus formation. Ironically, despite early differences in D-dimer levels, there were no apparent differences in residual scan defects between treatment groups after 1 week, suggesting that the thromboemboli had all resolved to a similar degree. Since it has been observed that perfusion scans often underestimate the actual size of subacute and chronic emboli,\(^{11,27}\) we relied on embolic masses recovered at autopsy to provide a more definitive measure of potential differences in thrombus resolution among treatment groups. We found that thromboemboli recovered from the PAI group were notably “larger” than thromboemboli recovered from either the control or TXA groups (Fig 4). This finding is consistent with the hypothesis that rPAI-1 remains associated with the embolus, where it continues to inhibit fibrinolysis, whereas TXA eventually diffuses out, allowing lysis to ensue. Indeed, immunoreactive PAI-1 was still detected within week-old thromboemboli recovered from the PAI group (Fig 5B).

It is important to note that no more than 5% of the original thrombus mass (estimated to be \(\approx 10\) g) was recovered from any animal. Although much of this apparent weight loss may be attributed to clot retraction, a significant degree of thrombolysis also appears to have occurred even in thromboemboli enriched with rPAI-1. While we have demonstrated that immunoreactive PAI-1 is still present within rPAI-1–enriched thromboemboli (presumably bound to fibrin), this PAI-1 may have been inactivated at some point due to complex formation with plasminogen activators or to conversion from the active to the latent form of PAI-1.\(^{28}\) Although PAI-1 bound to vitronectin in plasma and in the subcellular matrix of endothelial cells is apparently stabilized in the active form,\(^{29}\) the long-term functional stability of PAI-1 bound to fibrin is not known.

**Conclusions**

The focus of the present study has been on thrombus resolution rather than thrombus induction. Although the possible role of t-PA–PAI-1 imbalance in the pathogenesis of venous thrombosis remains controversial,\(^{30}\) our results indicate that PAI-1 is a potent thrombolytic inhibitor in vivo which, unlike TXA, appears to enhance thrombus stability by remaining associated with the thrombus, where it can inhibit plasminogen activators within the thrombus as well as at the thrombus interface. Whether thromboemboli markedly enriched in PAI-1 will be sufficiently stable to allow establishment of a model of chronic thromboembolic pulmonary hypertension remains to be determined.

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