Factors Contributing to Increased Vascular Fibrinolytic Activity in Mongrel Dogs

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Background. Numerous investigators have observed that pulmonary emboli are rapidly lysed in a canine model system. This study was undertaken to delineate the unique mechanism that accounts for the rapid dissolution of pulmonary emboli in mongrel dogs.

Methods and Results. Canine plasminogen activator (PA) activity (2.6±1.1 IU/mL acidified platelet-poor plasma [PPP], <0.3 IU/mL acidified whole blood serum [WBS], mean±SD; n=6) and PA inhibitor activity (6.1±2.6 U/mL PPP, 35.4±7.8 U/mL WBS; n=6) were determined in standard plasminogen-based chromogenic assays. Analysis of canine PPP, WBS, platelet lysates, and primary canine endothelial cell (EC) cultures by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fibrin autography revealed a plasminogen-dependent lytic zone at 45-kd relative molecular mass that was shown to be related to urokinase-type PA (u-PA) by its selective inhibition through amiloride. Analysis of canine platelets on standard 125I fibrin plate assays revealed a net fibrinolytic activity. In a clot lysis assay system, canine platelets were able to stimulate fibrinolysis when layered on the outside of fibrin clots containing autologous PPP. Moreover, net fibrinolytic activity of primary canine pulmonary artery endothelial cells was higher than the activities expressed by canine aortic or carotid artery endothelial cells.

Conclusions. Rapid lysis of pulmonary emboli in mongrel dogs appears to be a result of 1) the high u-PA activity in canine PPP and 2) the predominant association of u-PA activity with canine platelets and canine pulmonary artery endothelial cells. (Circulation 1993;87:1990–2000)

KEY WORDS • endothelium • thrombosis • plasminogen activators • platelets

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normal thrombus formation and dissolution are associated with several cardiovascular diseases, including atherosclerosis,1,2 and both hemorrhagic and thromboembolic conditions.3,4 Proteolytic degradation of fibrin clots is mediated by an active serine proteinase, plasmin. Plasmin is formed in the circulation from the zymogen plasminogen through the proteolytic action of plasminogen activators (PAs). PAs are highly specific serine proteinases that can be divided into two distinct enzymes, tissue-type PA (t-PA) and urokinase-type PA (u-PA) (for reviews, see References 5 and 6). Present data suggest that the physiologically relevant vascular PA in healthy humans is t-PA, whereas u-PA has been implicated in the regulation of extravascular proteolytic activity.7 However, the correlation of defective u-PA release with an incidence of deep venous thrombosis,8 as well as other data,9 suggests that the situation may be more complex. Thus, the role of u-PA known to be present in plasma, as well as associated with platelet membranes,10 in mediating intravascular fibrinolysis is not understood.

Within the past few years, it has become apparent that inhibitors of plasminogen activation play a critical role in the control of fibrinolysis (for reviews, see References 11–14). Several experimental observations support the hypothesis that PA inhibitor type 1 (PAI-1) is the principal physiological inhibitor of PAs (for reviews, see References 13 and 14). Elevated levels of PAI-1 have been found in a variety of cardiovascular diseases, including myocardial infarction,15–17 disseminated intravascular coagulation,1,18 deep venous thrombosis,19,20 and preeclampsia.21 The development of therapeutic approaches to facilitate thrombolysis requires the use of animal models that are appropriately analogous to clinical situations. Unfortunately, one of the problems of using animal models in thrombosis research is the lack of detailed knowledge concerning the differences among the species in the hemostatic and fibrinolytic systems.22

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Mongrel dogs have been widely used as animal models for thrombosis research (for reviews, see References 23–30). However, the use of these animals as models for chronic pulmonary arterial thrombosis has been difficult because of the rapid lysis of thrombi in their pulmonary vasculature.31–33 In fact, the remarkable efficiency of the canine fibrinolytic system has been observed by a variety of laboratories over the past 30 years. For example, Wessler et al34 studied the fate of serum-induced thrombi released into the canine vasculature and observed the complete degradation of thrombi in the canine pulmonary arterial tree within 6 weeks. Moreover, angiographic studies by Dalen et al35 indicated that a single experimentally induced embolus remained for only 5 days in the canine pulmonary vasculature. In comparison, resolution of massive pulmonary emboli in humans is usually incomplete after 1 year.36 Data are currently lacking on the PAs that account for the ability of the canine species to rapidly degrade fibrin clots. Therefore, experiments were designed to address this issue and define the mechanisms that account for the rapid dissolution of pulmonary emboli in mongrel dogs.

In this study, we provide evidence that u-PA is the major vascular PA in mongrel dogs and that its association with platelets and its production and secretion by pulmonary arterial endothelial cells account for the increased fibrinolytic activity of the canine pulmonary vasculature.

Methods

Fibrinolytic Reagents

Human t-PA was obtained from American Diagnostica Inc. (Greenwich, Conn.). A comparison of its activity against the t-PA International Standard (National Institute for Biological Standards and Controls [NIBSC], London, UK, Ref No. 83/517) revealed that this preparation exhibited a specific activity of 500,000 IU/mg. Commercially available urokinase ("Winknase," Sterling-Winthrop, Rensselaer, N.Y.) was purified further by affinity chromatography on columns of p-aminobenzamidine-Sepharose and its activity compared with World Health Organization reference 66/46, NIBSC. Human PAI-1 was purified from media conditioned by a transformed human lung fibroblast cell line (SV40 W138 VA13 2RA) according to procedures described previously.37 Human fibrinogen was purchased from American Diagnostica Inc. Human and bovine α-thrombin and canine fibrinogen were purchased from Sigma Chemical Co. (St. Louis, Mo.). Canine and human plasminogen were prepared by affinity chromatography on lysine-Sepharose.38

Antisera to human u-PA and t-PA were raised in New Zealand White rabbits according to procedures described previously.39 Antiserum to human urokinase was also purchased from American Diagnostica Inc. (product No. 389, protein-A-Sepharose-purified polyclonal antibody). Antiserum to human PAI-1 was prepared as described.39 Rabbit antisera against purified human plasminogen and human α2-antiplasmin were purchased from Calbiochem-Behring Corp. (La Jolla, Calif.).

Subjects

Thirty mongrel dogs of either sex (average weight, 20 kg) were studied. Samples were collected from two groups of dogs. Blood was withdrawn from a front leg vein in awake animals for the preparation of platelet-poor plasma (PPP), whole blood serum (WBS), and platelets. Canine pulmonary artery, aorta, and carotid artery were obtained during the harvesting of organs from a second group of dogs that served as donors for single lung transplants in a separate series of experiments by our group. These animals were anesthetized with sodium pentobarbital (30 mg/kg i.v.) with additional doses as required, intubated, and respired with room air (Harvard Respirator, Harvard Apparatus, South Natick, Mass.). Human PPP, WBS, and platelets were collected from the antecubital vein of six healthy volunteers free of aspirin or any related medication.

Preparation of Plasma and Serum

Whole human blood and canine blood were collected either into polypropylene tubes containing 200 mmol/L EDTA (final concentration, 20 mmol/L) or, for the acidified samples, into 0.13 mol/L trisodium citrate, immediately acidified with cold acetate buffer, and centrifuged (680g, 30 minutes, room temperature) to obtain PPP. WBS was obtained by collecting whole blood into glass tubes and incubating it for 1 hour at 37°C. Clots were removed by centrifugation at 680g for 20 minutes.

Platelet Preparation

For the preparation of platelets, canine blood was collected into acid citrate dextrose (0.025 mol/L citric acid, 0.85 mol/L sodium citrate, 2% dextrose; one part acid citrate dextrose to five parts whole blood). Platelet-rich plasma (PRP) was prepared by centrifugation of anticoagulated whole blood (200g, 15 minutes). The PRP was aspirated without agitating the buffy coat and then centrifuged (680g, 15 minutes). The pellet was washed three times with 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, 2.5 mmol/L EDTA, pH 7.4.40 The platelets were diluted to a concentration of 1 x 10⁵/mL and stored at −70°C. Gel-filtered platelets were prepared from human and canine PRP according to the method described by Berrettini et al.41 Briefly, platelets were obtained from PRP by centrifugation and then passed over a Sepharose CL-2B (Pharmacia, Piscataway, N.J.) column equilibrated with HEPES/Tyrode's buffer, pH 7.35, containing 1 mg/mL bovine serum albumin (Sigma Chemical Co.). The platelets were lysed either by repeated freezing and thawing cycles or by addition of Triton X-100 (1% final concentration). Before analysis, the samples were centrifuged at 680g for 30 minutes at 4°C to remove cell debris.

Tissue Culture

Canine vessels were collected into cold tissue culture medium M199 and processed immediately. Endothelial cells were isolated by a gentle scrape of the endothelial side of a vessel strip with a No. 15 blade and grown to confluence on fibronectin (Sigma) coated 33-mm culture dishes (Corning, N.Y.) in M199 supplemented with 20% pooled human plasma–derived serum and 0.4% endothelial growth factor prepared from bovine hypothalamus.42 Conditioned medium was prepared by washing the monolayers twice with phosphate-buffered saline (PBS) and then incubating the cultures for 24 hours in complete medium. The resulting conditioned medium was collected, centrifuged at 680g to remove...
floating cells and cellular debris and stored in 0.01% Tween 80 at −70°C until used. Endothelial cell lysates were obtained from primary endothelial cell cultures that had been grown for 3 days. Cells were detached by scraping, and the resulting endothelial cell sheets were broken up by pipetting. After three freeze–thaw cycles, the cells were centrifuged at 680g to remove debris, and a protein determination (BCA-assay, Pierce, Rockford, Ill.) of the cell lysates was carried out.

Amidolytic Assay for PA and PAI-1 Activity Measurement

The method described by Verheijen et al was modified as follows. For measurement of PA activity, acidified PPP was obtained by withdrawing whole blood into cold 0.13 mol/L trisodium citrate (1:9) followed by immediate acidification with 4 mol/L acetic acid buffer (1:3). After spinning, the PPP was acidified with 20% acetic acid (1:20). The amidolytic assay was carried out with an incubation time of 12 hours at room temperature after an overnight incubation at 4°C. A standard curve was generated by the addition of increasing amounts of single-chain t-PA (sct-PA) to PAI-1-depleted plasma (American Diagnostica, Inc.). PA activity was determined by reaction with cyanogen bromide digest fragments of fibrinogen (CNBr-fibrinogen fragments, American Diagnostica, Inc.), Glu-plasminogen, and the chromogenic substrate H-D-Val-Leu-Lys p-nitroanilide (S-2251, Kabi-Vitrum, Franklin, Ohio). The values for PA activity in sample PPP were obtained by direct comparison with the measured absorbance (405 nm) detected by use of the purified sct-PA standards.

PAI-1 activity was determined by measuring the inhibition of amidolytic activity of a reference amount of t-PA as follows: 20 µL of sample was incubated with 20 µL of 40 IU/mL sct-PA for 8 minutes at 37°C in dilution buffer. Samples were acidified with 50 µL of acetate buffer (pH 3.5), diluted to a final volume of 2 mL, and brought to a 200-mmol/L final concentration with substrate solution containing CNBr-fibrinogen fragments, plasminogen, and the chromogenic substrate S-2251. One unit of PAI-1 is defined as the amount of PAI-1 activity that inhibits 1 IU of sct-PA in this system.

Functional Immunological Assay for PAI-1 Activity

PAI-1 activity was measured by use of immobilized t-PA to bind active PAI-1 in a sample, and the bound PAI-1 was immunologically detected by use of an alkaline-phosphatase/polyclonal anti-PAI-1–based system. Briefly, flat-bottom microtiter plates (Immuno II, Dynatech, Chantilly, Va.) were coated overnight at 4°C with t-PA (100 µL/well, 1 µg/mL). At this and each subsequent step, the plates were washed with 0.01 mol/L Tris-buffered saline supplemented with 0.1% Triton X-100. The wells were then incubated with Blotto (5% wt/vol skimmed milk powder in 0.01 mol/L Tris, pH 7.5; 200 µL/well) for 2 hours to block any remaining reactive sites on the plastic. Test samples and standard curves of purified human PAI-1 were diluted in Blotto containing 0.5% Triton X-100 and incubated on the t-PA–coated plates (100 µL, 1.5 hours, 37°C). Samples were assayed both before and after activation with a denaturant. Bound PAI-1 was detected by incubating the washed wells (1 hour, 37°C) with rabbit antiserum to PAI-1 (1:500 dilution, 100 µL/well) followed by incubation (30 minutes, 23°C) with alkaline phosphatase–labeled goat anti-rabbit IgG (1:2,000 dilution, 100 µL/well). After addition of the substrate p-nitrophenylphosphate, the resulting color change was measured at 405 nm for a period of 10 minutes.

For activation of latent PAI-1, samples were incubated in 0.1% sodium dodecyl sulfate (SDS) (1 hour, 37°C) followed by neutralization with a 50-fold excess of Triton X-100.

SDS Gel Electrophoresis

For SDS–polyacrylamide gel electrophoresis (SDS-PAGE), SDS-polyacrylamide slab gels and buffers were prepared as described by Laemmli. Samples were applied to gels composed of 10-cm resolving gels of 9% acrylamide and 2-cm stacking gels of 4% acrylamide and then subjected to electrophoresis at room temperature for 16 hours or until the dye reached the bottom of the gel. Gels were analyzed by fibrin autorgraphy or reverse fibrin autorgraphy.

Direct and Reverse Fibrin Autorgraphy

Fibrin autorgraphy was performed as previously described. Briefly, fibrin–agar indicator films were prepared containing 1% agarose, fibrinogen (2.4 mg/mL), human plasminogen (25 µg/mL), and α-thrombin (0.5 IU/mL) (all final concentrations). The solution was mixed and poured onto prewarmed glass plates. After solidification of the fibrin–agar film, the SDS gel, which had been soaked for 1.5 hours in 2.5% Triton X-100, was layered on the film and incubated in a humid chamber. Plates were read several times within the following hours of incubation at 37°C. Lysis was complete after 6–8 hours. Plasminogen-free plates were prepared as described by Lassen and Schumacher and Schill. In brief, fibrin–agar plates were heated for 60 minutes at 80°C in a humid chamber.

For reverse fibrin autorgraphy, fibrin–agar indicator films were prepared as described above. Indicator films also contained 0.05 IU/mL urokinase, which converts plasminogen to plasmin, thus hydrolyzing the fibrin. Development of opaque, lysis-resistant zones in the otherwise clear indicator film reveals PAI activity. The gel and indicator were incubated in a moist chamber for 2 hours at 37°C and then photographed in indirect light.

Immunoprecipitation Procedures

Protein A-Sepharose CL-4B (Pharmacia) was rehydrated in 0.14 mol/L NaCl, 10 mmol/L sodium phosphate buffer containing 0.1% bovine serum albumin, 0.05% Tween 20, and 0.02% sodium azide (immunoprecipitation buffer, pH 7.4). Washed beads (100 µL, representing 8 µg of coupled Sepharose beads) were incubated with rabbit antiserum (40 µL) against human and bovine PAI-1, t-PA, u-PA, α2-antiplasmin, plasminogen, and nonimmune rabbit serum (40 µL) for 15 minutes at room temperature. The beads were washed three times with immunoprecipitation buffer containing 0.5 mol/L NaCl, and PPP or WBS (35 µL of either) or platelet lysates (100 µL, representing 106 platelets) were added. The mixtures were incubated for 1 hour at room temperature and centrifuged, and the resultant pellets were extracted with sample buffer. Extracts from the immunoprecipitates were fractionated by SDS-PAGE and analyzed by fibrin autorgraphy.
**Fibrin Plate Assay**

[125I]NaI (100 mCi/mL) was obtained from Amersham Corp. (Arlington Heights, Ill.). 125I-labeled fibrinogen was prepared by the solid-state lactoperoxidase method to a specific activity of 2x10⁶ cpm/µg protein (<95% clottable protein). 125I-fibrinogen (10,000 counts per well, 20 µg protein per well) was dried on 24-well tissue culture dishes and clotted with 0.5 IU/mL α-thrombin for 1 hour at 37°C. Freshly prepared platelets as well as lysed platelets were incubated on the washed wells with 100 µL plasminogen (40 µg/mL; total volume, 1 mL per well) for 4 hours, and the radioactivity released into the supernatant was counted in a gamma counter at hourly intervals.

**Measurement of Plasin-Induced Clot Lysis**

The in vitro lysis of canine and human clots was performed according to the procedure described by Sakata et al. In brief, 3.7 mL of bovine fibrinogen (10 mg/mL, lot 90X038, Calbiochem, La Jolla, Calif.) were mixed with 120 µg of radiolabeled fibrinogen. Of this solution (final concentration, 1.6x10⁶ cpm/mL), 265 µL was mixed with 10 µL of plasminogen (2 mL) and 10 µL of either canine or human PPP (<500 platelets/mL). Increasing concentrations of autologous washed platelets (10⁶/mL to 5x10⁸/mL, all final concentrations) were added. Final volume was adjusted to 1 mL by addition of 0.1 mol/L Tris-HCl, pH 8.1, containing Tween 80 (final concentration, 0.01%) and bovine serum albumin (final concentration, 1 mg/mL). The mixture was clotted with α-thrombin (final concentration, 0.05 IU/mL) and CaCl₂ (final concentration, 2.5 mmol/L). The mixture was allowed to clot for 1 hour at 37°C, and the clot was freed from the wall of the tube by rimming with a toothpick. To measure fibrinolysis, 10 µL of serum was removed from around the clot at intervals for counting the soluble radioactivity. Results were expressed as percent release of total radioactivity. In a second series of experiments, 10 µL of platelets (10⁹/mL) was layered on the outside of clots aged for 1 hour containing autologous PPP. To reduce platelet aggregation and allow the exogenously added platelets to interact with the surfaces of the preformed clots, hirudin (1 unit/mL final concentration; Sigma) was first incubated (10 minutes, 37°C) with the 1-hour-old rimmed clots to neutralize residual solution-phase thrombin, and then the autologous platelets were added to the tubes. Controls were processed similarly but did not contain any exogenously added platelets.

**Statistical Analysis**

The general selection of material and population sampling was based on standard procedures. The arithmetic mean, SD, and SEM were calculated on all groups of data. All data are shown as mean±SEM unless otherwise indicated. Comparisons were made either by Student's t test (paired t test) or variance analysis. A three-factor ANOVA was used in the interpretation of the clot lysis experiments. In these experiments, the variance of lysis expressed as the log percent of total lysis was analyzed according to the factors of time, concentration of platelets associated with the clots, and canine versus human subjects. Significance was accepted at the p<0.05 level for all analyses.

**Results**

**Characterization and Identification of Canine Vascular PAs**

PA activity in acidified canine PPP was measured by means of a standard plasminogen-based amidolytic assay and found to be significantly higher than the levels detected in human PPP (2.5±0.9 IU/mL versus 0.5±0.03 IU/mL, p<0.002; Figure 1A). In nonacidified samples, PA activity was not detected in the chromogenic assay in canine or human PPP (data not shown). To identify the relative molecular masses (M₀) (molecular weight) of the PAs present in canine blood, the samples were subjected to SDS-PAGE and the fibrinolytic enzymes localized by fibrin autography. Analysis of canine PPP, WBS, or platelets (Figure 2A, lanes 1–3, respectively) revealed a M₀ 45-kd lytic zone that was not present in corresponding human blood fractions (Figure 2A, lanes 4–6, respectively). The presence and size of the lytic zone were independent of whether human or canine plasminogen was used as substrate in the indicator gels (data not shown). A second prominent lytic zone (i.e., M₀ 90–110 kd) was present in both canine and human blood samples (Figure 2A, lanes 1–3 and 4–6, respectively). Analysis of canine samples on plasminogen-depleted fibrin agar indicator plates revealed that the enzymatic activity responsible for the 45-kd lytic zone was dependent on the presence of plasminogen in the fibrin–agar overlay in comparison to the enzymatic activity responsible for the 90–110-kd lytic zone, which did not appear to require the presence of plasminogen in the fibrin–agar overlay (Figure 2B, compare lanes 1 and 1').

To further characterize the different lytic zones in canine PPP, immunoprecipitation experiments were
performed with antisera to human PAs (i.e., t-PA, urokinase), as well as antisera to human plasminogen. Immunoprecipitates were fractionated by SDS-PAGE and the lytic zones revealed by fibrin autography. The Mr, 90–110-kd lytic zone was found to result from the activity of plasmin/plasminogen (Figure 2C, lane 2) and the activity of t-PA inhibitor complexes (Figure 2C, lane 3) by immunoprecipitation with the respective antisera. Analysis of the plasminogen-immunodepleted samples on plasminogen-free fibrin–agar plates did not reveal a 90–110-kd lytic zone (data not shown), ruling out the possibility that a nonspecific protease was present at this molecular weight. Lytic zones were not observed in the lanes containing immunoprecipitates obtained by use of either commercial antisera to human u-PA (Figure 2C, lane 4) or an antisera to u-PA prepared in our laboratory (data not shown). Because the antibodies generated to human u-PA may react with only a weak avidity to the canine molecule, we used amiloride (i.e., a specific enzymatic inhibitor of human u-PA\(^{22}\)) to investigate the presence of u-PA in canine PPP. Figure 2D indicates that the enzymatic activity responsible for the 90–110-kd lytic zone (lane 1) was unaffected by the presence of amiloride in the indicator gel. Analysis of 0.05 units of human urokinase resulted in complete inhibition of the activity of this PA on the indicator plate containing amiloride (compare lanes 2 and 3), whereas no inhibition occurred when t-PA (0.05 IU) was studied (compare lanes 3 and 3').

**Figure 2.** Electrophoretic analysis and identification of plasminogen activators (PAs) in canine blood by different fibrin autography techniques. Panel A: PAs in platelet-poor plasma (PPP), whole blood serum (WBS), and platelets. PPP (10 µL/lane; lanes 1 and 4), WBS (10 µL/lane; lanes 2 and 5), and platelets (unwashed, 10⁵/lane; lanes 3 and 6) from either a mongrel dog (lanes 1–3) or a healthy human volunteer (lanes 4–6) were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by fibrin autography for PA activity as described in "Methods." Lanes 7 and 8 contain purified human urokinase and tissue-type PA (t-PA) (0.05 IU/lane, respectively). Panel B: Plasminogen-dependent and plasminogen-independent fibrinolysis of canine PPP. Canine PPP (10 µL/lane, lanes 1 and 1') and purified PAs (0.05 IU t-PA/lane, lanes 2 and 2'; 0.05 IU urokinase/lane, lanes 3 and 3') were fractionated by SDS-PAGE. Lanes 1–3 were analyzed on a plasminogen-containing fibrin–agar plate, whereas lanes 1'–3' were analyzed on a plasminogen-free fibrin–agar plate. Panel C: Immunological identification of lytic zones in canine PPP. Canine PPP (5 µL, lane 1) was mixed and incubated with Sepharose-protein A beads that had been preincubated with either rabbit antisera against human plasminogen (lane 2), t-PA (lane 3), urokinase-type PA (u-PA) (lane 4), and normal rabbit serum (lane 5). The beads were extracted with sample buffer and were fractionated by SDS-PAGE followed by fibrin autography. Lanes 6 and 7 show purified human urokinase and t-PA (0.05 IU/lane, respectively). Panel D: Identification of canine u-PA through its selective inhibition by amiloride. Canine PPP (5 µL, lanes 1 and 1') and purified PAs (0.05 IU urokinase/lane, lanes 2 and 2'; 0.05 IU t-PA/lane, lanes 3 and 3') were fractionated by SDS-PAGE and analyzed by fibrin autography. Amiloride (1 mmol/L) was incorporated into the fibrin film in the right panel (lanes 1'–3').
Identification and Characterization of Canine PAIs

To complement the experiments on the PAIs present in canine blood, we also analyzed the samples for PAI activity. Another indication of the high PA activity in canine PPP was the low PAI activity in the canine species compared with human PPP (6.1±2.6 U/mL versus 10.5±4.2 U/mL, p=0.001; Figure 1B). In canine WBS, PAI activity levels were comparable to the levels found in human WBS (22.5±3.3 U/mL versus 19.1±4.6 U/mL, p=0.1; Figure 1B). To identify canine PAIs, reverse fibrin autography was used to localize the molecular weights of the fibrinolytic inhibitors after electrophoresis. A prominent 80-kd lysis-resistant zone was consistently found in canine WBS and was identified as α2-antiplasmin by immunoprecipitation (Figure 3, panel A). Interestingly, a 50-kd lysis-resistant zone was detected in the pellet after immunoprecipitation with antiserum to PAI-1 that could not be seen in the nonimmunoprecipitated WBS (Figure 3A). Canine platelets displayed a 50-kd lysis-resistant zone comigrating with human PAI-1 that was immunoprecipitated with antiserum to PAI-1 (Figure 3B).

Balance Between PA and PAI Activities of Canine Platelets

The above data obtained from direct fibrin autography and reverse fibrin autography indicate that both PA and PAI activities were associated with canine platelets. A number of possibilities could account for this observation. For example, canine platelet PAI-1 could be stored solely in a nonactive (latent) form, or canine u-PA could be associated with platelets as a proenzyme. To clarify this issue, canine platelet lysates were incubated in the presence and absence of an active PA (i.e., human t-PA), and the formation of a high-molecular-weight zone that would result from the formation of complexes was analyzed. SDS-PAGE/fibrin autography analysis of platelet lysates incubated with human t-PA revealed a 110-kd zone of lysis (Figure 4A, lane 3) that was not present in lysates incubated in the absence of t-PA (Figure 4A, lane 2). We complemented the experiments by using platelets washed by gel filtration, a procedure known to remove plasma proteins that are weakly associated with platelets.41 Figure 4A indicates that the 45-kd zone was present in both washed (lane 2) and gel-filtered (lane 4) canine platelets, suggesting that the enzyme responsible for this lytic zone was tightly associated with the platelets, whereas the 90-kd plasminogen-related lytic zone was not present in the platelets that were washed by gel filtration (compare lanes 2 and 4). Furthermore, incubation of t-PA with lysates obtained from gel-filtered canine platelets also resulted in the appearance of a 110-kd zone of lysis (Figure 4A, lane 5). Because these data suggested that a PAI was present in an active form in the platelet lysates, we decided to immunologically quantify the ability of PAI-1 in canine platelets to form complexes with t-PA. Figure 4B indicates that canine platelet lysates express

FIGURE 3. Electrophoretic analysis of fibrinolytic inhibitors in canine whole blood serum (WBS) and platelets. Canine WBS (panel A, 5 µL/lane) and lysed canine platelets (panel B, 106/lane) were incubated with Sepharose-protein A beads that had been preincubated with rabbit antisera to either human plasminogen activator inhibitor-1 (PAI-1) (lanes 2 and 6), α2-antiplasmin (lanes 3 and 7), and nonimmune IgG (lanes 4 and 8). The beads were extracted with sample buffer and fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by reverse fibrin autography. Lanes 1 and 5 contain the starting material before immunoprecipitation (i.e., WBS and platelet lysates). Lane 9 contains 100 ng of purified human PAI-1.
PAI-1 activity (1.7±0.23 U/10⁸ platelets) that is quantitatively comparable to the PAI-1 activity present in lysates of human platelets (1.76±0.36 IU/10⁸ platelets). Moreover, the PAI-1 activity detected after denaturant treatment of the canine platelet lysates (3.49±1.63 IU/10⁸ platelets) was comparable to the PAI-1 activity after denaturant treatment of human platelet lysates (3.8±0.55 U/10⁸ platelets).

Because the net PA activity is a combination of the individual PA and PAI activities that are present in a particular sample, we extended our analysis of canine platelets by experiments designed to measure net platelet-associated fibrinolytic activity by use of a standard 125I-fibrin plate. Figure 4C shows the time-dependent fibrinolytic activity of canine platelet lysate on 125I-fibrin plates in the presence of purified plasminogen. In the presence of 1 mmol/L amiloride, lytic activity was suppressed significantly (Figure 4C). In comparison, human platelets did not induce any measurable fibrinolysis either in the presence or in the absence of amiloride during the 4-hour incubation period (Figure 4C).

**Contribution of Canine Platelets to Fibrinolysis**

To clarify the effect of canine platelets on clot lysis, we used a standard in vitro clot lysis assay system.50 Fibrin clots supplemented with PPP from canine and human subjects were prepared with autologous platelets at concentrations ranging between 10⁴/mL and 5×10⁷/mL, and the release of radioactivity in the supernatant was monitored as an indicator of clot lysis. Clots prepared with canine blood fractions were observed to lyse significantly faster than the respective clots prepared with human blood fractions (p<10⁻⁶; Figure 5, panel A versus panel B). Platelet-rich clots (i.e., clots prepared in the presence of >10⁷ platelets/mL) lysed significantly (p<10⁻¹²) more slowly than platelet-poor clots regardless of the species. However, clots prepared in the presence of canine PPP and 10⁸ platelets/mL lysed significantly faster than clots prepared only in the presence of canine PPP (p=0.003). In contrast, clots prepared in the presence of human PPP and 10⁸ platelets/mL did not lyse significantly faster than the control clots prepared only in the presence of human PPP (p=0.53; Figure 5B). Because platelets are known to actively interact with fibrin deposits during their transit through the circulation, a relevant question concerned the ability of platelets to bind and subsequently influence the lysis of preformed fibrin clots. Therefore, we performed additional experiments layering platelets on the outside of clots aged for 1 hour. Canine platelets significantly enhanced clot lysis under these conditions (p=0.0002; Figure 6, panel A). For comparison, clot lysis was delayed when human platelets were layered on clots prepared with autologous PPP (p=0.0001; Figure 6, panel B).

**Contribution of Canine Endothelial Cells to Fibrinolysis**

Because experimentally induced thrombi in the canine pulmonary arterial system are resolved considerably faster than thrombi in other canine blood vessels,32 we decided to investigate whether pulmonary vascular endothelial cells contribute to this process. Three distinct blood vessels (i.e., pulmonary artery, aorta, and carotid artery) were harvested from a series of mongrel dogs (n=4), and the endothelial cells of these vessels were isolated and cultured in vitro for 3 days. Fibrinolytic activities expressed by these cells were initially examined by analyzing both the condi-
conditioned medium and cell lysates by SDS-PAGE/fibrin autography. Figure 7 shows the data obtained from a representative mongrel dog and indicates that 3-day primary pulmonary artery conditioned medium and the corresponding lysates exhibit prominent 45-kd lytic zones (Figure 7, lanes 1 and 4, respectively) in comparison to the conditioned medium or lysates obtained from either the carotid artery (lanes 2 and 5) or the aorta (lanes 3 and 6) of a mongrel dog. Quantification of net PA activity in an $^{125}$I-fibrin plate assay demonstrated higher levels of PA activity associated with lysates of pulmonary artery endothelial cells (0.46 mIU/μg cell protein) compared with aortic (0.18 mIU/μg cell protein) or carotid artery (0.13

**Figure 5.** Graphs showing effect of canine and human platelets on clot lysis in vitro. Fibrin clots were prepared in the presence of canine (panel A, n=7) and human (panel B, n=4) platelet-poor plasma supplemented with the following concentrations of autologous platelets: no additional platelets (open squares), $10^5$ platelets/mL (closed triangles), $10^7$ platelets/mL (closed squares), or $5 \times 10^7$ platelets/mL (closed circles). Clot lysis is indicated as $^{125}$I% release over time. At each level of platelet concentration, the log transform of $^{125}$I% release provided a good fit to a linear regression against time ($p<10^{-4}$ for each data set). Levels of significance were calculated by ANOVA as described in "Methods."

**Figure 6.** Graphs showing effect of platelets layered on the outside of canine or human plasma clots on clot lysis. Washed canine (panel A, n=4) and human (panel B, n=3) platelets ($10^7$ platelets/mL, final concentration) were layered on the outside of rimmed fibrin clots aged 1 hour containing autologous platelet-poor plasma. Results (closed squares) are presented as fold increase over the $^{125}$I% release of a parallel sample in the absence of platelets (open squares).
mIU/μg cell protein) lysates from endothelial cells that were obtained from mongrel dogs (n=4).

Discussion

Mongrel dogs have been known for over three decades to possess a very potent fibrinolytic system.31-34 This hyperfibrinolytic state is exemplified by the recent observation that even recombinant PAI-1 that is incorporated into thrombi is unable to completely suppress clot lysis in a canine model of acute pulmonary embolism.53 Although canine thrombosis models are widely used, the mechanisms underlying the ability of mongrel dogs to degrade thrombi rapidly are unknown. Therefore, initial experiments by our group confirmed and characterized a high net PA activity in canine blood fractions compared with human blood fractions (Figure 1). Further analysis of canine PPP, WBS, platelets, and endothelial cells identified the PA activity as related predominantly to u-PA (Figure 2). In light of these data, it should be noted that three groups have previously reported a canine PA in the perfusate of isolated canine tissues54-56 or cultured canine endothelial cells57 that was not further identified. Highsmith58 isolated a PA from canine venae cavae that was a single-chain peptide (M, 48 kDa) with a pH 5.9 and a pH optimum between 8.0 and 9.0. The sole report presented in the literature identifying the PA activity associated with canine-derived cells is from the group of Wilkinson et al.,59 who used cultured canine keratinocytes to demonstrate that the only secreted and cell-associated PA activity was a 57-kd u-PA.

Although platelets are believed to play a major role in the stabilization of thrombi because of their ability to store and release PAI-1, platelet-enhanced fibrinolysis has been observed for more than 30 years in human platelets in vitro.60-65 However, the mechanisms have not been determined. Our results indicate that canine platelets, when analyzed by direct and reverse fibrin autography, reveal both PA and PAI activity, whereas human platelets exhibit primarily PAI-1 activity. Moreover, gel-filtered canine platelets induced a dose- and time-dependent lysis of a 125I-fibrin plate (Figure 4C), corresponding to an activity of 75 mIU of urokinase per 10⁶ platelets, which is about 50-fold more than has been reported for isolated human platelet membranes.10 The presence of active PAI-1 within the platelet lysates was demonstrated by addition of t-PA, which resulted in the formation of high-molecular-weight complexes (Figure 4A). Analysis of canine PAI-1 by a functional immunnoassay revealed that, like human66,67 and porcine platelets,68 canine platelets contain PAI-1 primarily in a latent form that can be reactivated with denaturants (Figure 4B). Although canine platelet PAI-1 readily reacts with exogenous t-PA, it does not bind and complex u-PA that is associated with the platelets, suggesting that canine platelet u-PA is in a single-chain form. Previous studies on u-PA in human plasma indicating that circulating u-PA is in a proform support this finding.69 In vivo, the generation of plasmin facilitates the conversion of u-PA to its active two-chain form.69 Thus, the association of u-PA with canine platelets may lead to an increase in the fibrinolytic degradation of a thrombus in vivo. It is important to note that our data clearly indicate that both canine and human platelet-rich clots lyse more slowly than platelet-poor clots (Figure 5); however, canine platelets appear to have the capability of enhancing clot lysis under certain conditions (e.g., at low concentrations within the clot [Figure 5A] or when layered on the outside of an in vitro clot [Figure 6A]). Further experiments are required to determine the conditions in which canine platelets have the ability to enhance and/or modulate clot dissolution in vivo and to define the role of canine platelet-associated u-PA in vascular hemostasis.

In primary cultures of canine endothelial cells, u-PA was found to be the major cell-associated and cell-secreted PA activity. Moreover, quantification of PA activity by a fibrin plate assay and by fibrin autography revealed that pulmonary artery endothelial cells display significantly more of this u-PA activity than endothelial
cells harvested from two other major vessels in the dog (Figure 7). Therefore, our results suggest that platelet-associated u-PA, as well as the secretion of this molecule from pulmonary artery endothelial cells, may play a major role in destabilizing thrombi in the pulmonary vascular bed of mongrel dogs. Previous data by coworkers in our group32 revealed that clots in the canine pulmonary vasculature lyse at a faster rate than in the canine inferior vena cava. Investigators using mongrel dogs in model systems of thrombosis and thrombolysis have to consider these characteristic features of the canine fibrinolytic system in the interpretations of their experiments.

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