Plasminogen Activator Inhibitor 1: Development of a Radioimmunoassay and Observations on Its Plasma Concentration During Venous Occlusion and After Platelet Aggregation

By Egbert K.O. Kruijthof, Grazia Nicolosa, and Fedor Bachmann

To study the effect of plasminogen activator inhibitors (PAI) on fibrinolysis it is essential to be able to specifically measure these proteins in plasma. To this end PAI-1 was purified from cortisol-stimulated HT 1080 fibrosarcoma cells and antiserum raised in rabbits. The immunologic relationship of the purified inhibitor to PAI-1 in plasma and platelet extracts was established by immunoblotting and regular and reverse fibrin zymography. Furthermore, the purified product could be immunoprecipitated with antibodies to human or bovine endothelial cell-derived PAI-1. A radioimmunoassay was developed that measures both free and tissue-type plasminogen activator (t-PA)-bound PAI-1 in plasma and has an effective range of 8 to 250 ng/mL. PAI-1 antigen levels showed a twofold increase after 20 minutes of venous occlusion, partially due to hemocoagulation. Approximately one quarter of PAI-1 before and after venous occlusion is derived from platelets.

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The fibrinolytic system in plasma is a complex enzyme cascade system that degrades fibrin and thereby contributes to the prevention of thrombotic disease. A precise regulation of this system is needed to avoid nonspecific proteolytic degradation of plasma proteins and hemostatic plugs. Plasminogen activator inhibitors (PAIs) are important modulators of fibrinolytic activity. Two distinct PAIs have been purified and are well characterized: PAI-1 and PAI-2. PAI-1 is found in normal plasma and platelet releasates, whereas PAI-2 is observed in pregnancy plasma and in leukocytes. In a variety of clinical conditions increased levels of PAI activity have been observed, and it has been suggested that PAI activity behaves as an acute-phase reactant. PAI activity assays, however, do not measure the total PAI content of plasma but rather the level of free, active PAI-1. To evaluate correctly the contribution of PAI-1 to fibrinolytic activity it is, therefore, essential to complement PAI and PAI activity measurements with specific antigen determinations of tissue-type PA (t-PA) and PAI-1. To this end we have developed a radioimmunoassay (RIA) for PAI-1, applied this assay to the measurement of PAI-1 levels in the plasma of normal individuals before and after venous stasis, and studied the contribution of platelets to PAI-1 levels in plasma.

MATERIALS AND METHODS

Materials. Iodogen was obtained from Pierce, Oud Beijerland, The Netherlands; Na125I from New England Nuclear, Dreieich, FRG; Sephadex G25, Sephadex G100, CNBr-activated Sepharose 4B, concanavalin A (Con A)-Sepharose, and protein A-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden. All reagents for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and the peroxidase staining reagent were obtained from Bio-Rad Laboratories, Richmond, CA. The World Health Organization International Reference Preparation of human t-PA (NIBSC 83/517) was provided by Dr P.J. Gaffney from the National Institute for Biological Standards and Control, London, UK.

t-PA. Human t-PA was purified and antiserum against t-PA raised in rabbits as described. The activity of the t-PA preparation used in this study was calibrated in a fibrin plate assay against that of the international reference preparation.

PAI-1. PAI-1 was purified from the conditioned medium of cortisol (10-7 mol/L) stimulated human fibrosarcoma (HT 1080) cells. The cells were grown to confluency in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum in 175-cm2 culture flasks (Corning Glass Works, Corning, NY), washed twice with phosphate buffered saline (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na2HPO4, 2 H2O, 0.2 g/L KH2PO4) and stimulated with 10-7 mol/L cortisol (Solu-Cortef, Upjohn, Puurs, Belgium) in DME. Five-liter batches were concentrated 20-fold in a Millipore membrane concentrator equipped with a 4,650-cm2 Pellikon membrane and passed over a 70 mL Con A-Sepharose column. After washing with 40 mL 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4, and 40 mL 0.05 mol/L Tris-HCl, 1 mol/L NaCl, pH 7.4, PAI-1 was eluted with 0.05 mol/L Tris-HCl, 0.5 mol/L NaCl, 0.5 mol/L methyl-α-mannoside, pH 7.4. The protein-containing fractions of the methylmannoside eluate (approximately 150 mL) were dialyzed against 2 x 4 L of deionized water and, after the addition of 0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 8.4, passed over a 1.5-mL column of diethyl aminoethyl-Sepharose preequilibrated with 0.02 to 1 mol/L NaCl in the same buffer. The column was washed with 10 mL of the same buffer followed by a stepwise gradient of 0.02 to 1 mol/L NaCl in the same buffer. PAI-1, as judged from...
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reverse fibrin zymography, and immunoblotting eluted at a NaCl concentration of 0.1 mol/L. The PAI-1-containing fractions (3 mL) were further purified by gel filtration on a 60 x 1.6-cm column of Sephadex G-100 preequilibrated with 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, and 0.0001% merthiolate, pH 7.4. The final product showed one band on reduced and nonreduced SDS-PAGE. The protein concentration of the pure product was determined by the method of Bradford. 24

PAI-1 was radioiodinated by the iodogen technique. 25 Radioiodinated inhibitor was separated from free 125I by passage over a 10-mL column of Sephadex G-25. Its specific radioactivity was between 15 and 50 nCi/ng (corresponding to 15 to 50 molecules of 125I incorporated per 100 molecules of PAI-1). After radioiodilution, approximately 70% of the inhibitor had retained its ability to bind to anti-PAI-1 antibodies. Under the conditions of the RIA using different antiserum dilutions and the recommended amount of cellulose-bound donkey antibodies to rabbit IgG (Sac-Cel, Wellcome, Beckenham, UK) maximal binding was only 30% at an antiserum dilution of 1:200. This low binding was found to be due to the limited capacity of the Sac-Cel preparation. When immunopurified antibodies and twice the amount of Sac-Cel were used, 70% of the radiolabeled antigen was immunoprecipitated.

Preparation of antiserum to PAI-1. For the immunization of rabbits with PAI-1, the gel-filtered PAI-1 preparation was subjected to further purification by SDS-PAGE in a 10% gel. 26 The stained PAI-1 bands were cut out and mixed by passage through hypodermic needles of different diameter with 0.3 mL 0.2% SDS and 0.5 mL Freund’s adjuvant (complete for the first immunization, incomplete for subsequent immunizations).

Total anti-PAI-1 IgG was prepared by passing 1 mL antiserum over 1 mL protein A-Sepharose. After washing with 10 mL 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.5, the IgG was eluted with 0.1 mol/L glycine-HCl, pH 2.2. Immediately after elution the IgG solution was neutralized by addition of 0.1 vol of 1 mol/L Tris. For preparing immobilized anti-PAI-1 IgG, the IgG was dialyzed overnight against two changes of 1 L 0.1 mol/L sodium bicarbonate, pH 8.1, and coupled to 1 mL CNBr-activated Sepharose 4B. 13,14

Immunopurified anti-PAI-1 antibodies were prepared from total IgG by immunoadsorption to immobilized PAI-1 (150 μg pure PAI-1 coupled to 300 μL CNBr-activated Sepharose) and elution with glycine-HCl as described earlier. Biotinylated anti-PAI-1 IgG was prepared from the immunopurified IgG by incubating for two hours at room temperature 200 μL IgG (0.3 mg/mL in sodium bicarbonate) with 10 μL N-hydroxysuccinimidobiotin (Sigma Chemical Co, St Louis; 1 mg/mL in dimethylsulfoxide). Incorporated biotin was separated from free biotin by centrifugation through a 0.6-mL spin column of Sephadex G-25. Immobilized immunopurified anti-PAI-1 IgG as well as control IgG was prepared either by direct coupling of 0.5 mg IgG/mL CNBr-activated Sepharose or by incubating for one hour at room temperature 10 μg IgG with 100 μL cellulose-bound donkey antibodies to rabbit IgG (Sac-Cel) followed by removal of the supernatant by centrifugation (five minutes, 1,000 g). For comparative purposes antisera to bovine PAI-1 and human PAI-1 were obtained from Drs Loktoff (Scripps Clinic, La Jolla, CA), and Sprenger (Gaubius Institute, Leiden, The Netherlands), respectively.

Plasmas. Blood was obtained by venipuncture from healthy volunteers in the age range of 26 to 59 years before and after ten or 20 minutes of venous occlusion induced by an arm cuff inflated midway between the systolic and diastolic pressures. Women using oral contraceptives were excluded from the study. The blood was collected on ice, anticoagulated with buffered citrate (nine parts of blood were mixed with one part of 0.1 mol/L sodium citrate/citric acid, pH 4.5), and centrifuged for 15 minutes at 1,800 g and 4°C. Plasma was collected and stored at -70°C. Platelet-rich plasma (PRP) was obtained by a ten-minute centrifugation at 200 g and 20°C; the platelet concentration was adjusted to 200 x 10⁸ platelets/mL by dilution with the homologous plasma. Platelet aggregation in PRP was induced by collagen (Horm, Hormon Chemie, Munich, FRG; 5 μg/mL final concentration). After a five-minute incubation at 37°C the plasma was centrifuged (one minute, 25°C, 10,000 g) and stored at -70°C as described before. 13 Only those PRPs that exhibited normal aggregation curves in an aggrerometer were used in the study. A normal plasma pool was prepared from the blood of 50 healthy blood donors.

An extract of washed platelets was made as described. 13

Immunoradiometric assay of t-PA in plasma. t-PA antigen levels in human plasma were determined by using an immunoradiometric assay essentially as described by Rijken et al. 24 Standard curves were made by using a t-PA preparation standardized against the international reference preparation of t-PA. The latter has a specific activity of 500,000 IU/mg protein (1 IU = 2 ng).

RIA of PAI-1 in plasma. One hundred microliters of plasma, diluted fivefold with immunodilution buffer (0.05 mol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 0.01 mol/L EDTA, 0.1% bovine serum albumin, and 0.05% NaN₃) was mixed with 10 μL anti-PAI-1 antiserum that had been diluted such (1:1,500) that, in the absence of cold PAI-1, half-maximal binding of the 125I-labeled PAI-1 to the antibodies occurred. After a two-hour incubation at 37°C, 50 μL of 125I-PAI-1 (8,000 cpm) was added and incubated overnight at 4°C. One hundred microliters of a suspension of donkey antirabbit IgG antibodies coupled to cellulose (Sac-Cel) was then added and incubated for 30 minutes at room temperature. Two milliliters of 0.15 mol/L NaCl was added and the mixture centrifuged for five minutes at 200 g. The pellet was washed twice with 2 mL 0.15 mol/L NaCl and counted in a gamma counter. Radioactivities after deduction of nonspecific binding of 125I-PAI-1 to Sac-Cel in the absence of added antiserum (usually less than 30 cpm) were compared in a log-log plot with a standard curve of PAI-1.

Determination of the fibrinolytic activity of the euglobulin precipitate. After 10 minutes thawing in a 37°C waterbath, 1 vol of plasma was diluted ten times with ice-cold water and acidified by the addition of a 0.75 vol of 0.25% acetic acid (resulting in a pH of approximately 5.9). After 30 minutes at 0°C the preparation was centrifuged for 20 minutes at 1,200 g and 4°C and the pellet redissolved in a 0.5 vol of 0.1 mol/L Tris-HCl, pH 7.5. Fibrinolytic activity of the euglobulin fractions was determined on plasminogen-rich bovine fibrin plates as previously described. 27 For each sample, 30 μL of the euglobulin solution and 5 μL of 17.5 mmol/L sodium flufenamate (Aldrich Chemical Co, Steinheim, FRG) was applied on the fibrin plates.

Electrophoretic-zymographic techniques. Fibrin zymography and reverse fibrin zymography were performed after electrophoretic separation of plasma and platelet proteins by SDS-PAGE in a 10% gel. 28 Immunoblotting was executed as follows: after SDS-PAGE the proteins were electrophoretically (16 hours, 30 V, 100 mA) transferred to nitrocellulose. The membrane was treated with Blotto (5% wt/vol skimmed milk powder in 0.025 mol/L Tris, 0.5 mol/L NaCl, pH 7.5 (TBS)), incubated for three hours at room temperature in 5 mL of biotinylated anti-PAI-1 IgG (2 μg/mL in Blotto), washed three times for 15 minutes with 100 mL Blotto and twice for ten minutes with TBS, incubated one hour at room temperature with a conjugate of horseradish peroxidase with avidin (Vector Laboratories, Inc, Burlingame, CA), washed three times for 15 minutes in TBS, and developed with a peroxidase staining reagent (Bio-Rad).

PAI activity assay. PAI activity in plasma was determined by the indirect chromogenic substrate assay as described by Verheijen et al. 4

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Preparation of PAI-1-deficient plasmas or platelet extracts. Five milliliters of a normal human plasma pool was passed over a 1-mL column of immobilized total anti-PAI-1 IgG (5 to 10 mg of IgG/mL gel) and the protein containing run-through fractions (except for the first milliliter) collected and stored at -70°C. As an alternative, small-scale procedure, PAI-1 or PAI-1/t-PA complexes were removed from plasma or platelet extracts by a one-hour incubation at room temperature with immunopurified or control IgG bound to immobilized antirabbit IgG antibodies (prepared as described earlier). The immunoprecipitant then was removed by centrifugation (five minutes, 1,000 g).

RESULTS

Characterization of the antibodies directed against the PAI purified from HT 1080 cells. HT 1080 cells are able to secrete both PAI-1 and PAI-2. To develop an RIA measuring PAI-1 in human plasma and in platelet extracts it was essential to determine whether the PAI purified by us from corticosteroid-stimulated HT 1080 cells is immunologically related to plasma PAI-1 and whether the antiserum raised reacts with PAI-1 in plasma and platelet extracts. To establish the specificity of the antiserum, we subjected an extract of washed platelets to SDS-PAGE followed by reverse fibrin autography as well as immunoblotting using biotinylated anti-PAI-1 IgG. A 51-kd immunoreactive band was observed in immunoblotting (Fig 1, lane 1). This band comigrated with the lysis inhibition band seen in reverse fibrin autography; the latter band could be removed by immunoprecipitation of the platelet extract with immunopurified anti-PAI-1 antibodies (Fig 1, lanes 2 and 3).

The addition of an excess of t-PA (5 U/mL, final concentration) to human plasma resulted in the formation of a 110-kDa t-PA/PAI-1 complex band; a minor amount of t-PA remained in its free form. Immunoprecipitation using immunopurified anti-PAI-1 antibodies completely removed the 110 kDa band, whereas the 67-kDa t-PA band still remained visible, thus demonstrating that the anti-PAI-1 antibodies also recognize PAI-1 complexed to t-PA (Fig 1).

Further proof of the immunologic relationship of the PAI purified by us from HT 1080 cells with PAI-1 was obtained by immunoprecipitation of radiolabeled PAI with antisera to bovine or human endothelial cell-derived PAI-1 (Fig 2). With our antiserum, half-maximal binding occurred at an antiserum dilution of 1:1,500. When using the antiserum against PAI-1 from human or bovine endothelial cells, half-maximal binding occurred at lesser dilutions, but the slopes of the three curves was comparable. Preincubation of our antiserum with different concentrations of the PAI purified by us from HT 1080 cell conditioned medium or with a PAI-1 preparation that, by aminoacid sequence analysis, was identical to the endothelial cell-derived PAI-1 (a kind gift of Dr. Andreasen) resulted in similar PAI concentration-dependent binding inhibition curves. Thus, the specificity and utility of these antigen and antiserum preparations for the development of an RIA for measuring PAI-1 has been established.

Determination of optimal plasma dilutions for the assay of PAI-1. The presence of a fairly high concentration of proteins in plasma may influence the binding of an antigen to antibodies. To validly use standard curves for PAI-1 in immunodilution buffer it was therefore essential to examine

![Fig 1. Analysis of the specificity of the anti-PAI-1 antiserum.](image)

![Fig 2. Binding of the PAI purified from HT 1080 cells to antibodies to human and bovine PAI-1.](image)
whether, at the plasma dilutions used, the binding of antigen is affected. To this end standard curves of PAI-1 in buffer and in different dilutions of PAI-1-depleted normal plasma were made by using the antiserum dilution (1:1,500) that resulted in half-maximal binding of labeled PAI-1 in the absence of added cold antigen. Standard curves of PAI-1 in plasma diluted five times or more were essentially identical to those made in immunodilution buffer (Fig 3). Furthermore, the dilution curve of pure PAI-1 added to plasma was parallel to that of plasma containing endogenous PAI-1. Therefore, fivefold-diluted plasma was used for all PAI-1 determinations. Figure 3 shows that the range of the RIA for PAI-1 was 1.6 to 50 ng/mL. This results in a factual range in plasma of 8 to 230 ng/mL and allows a direct assay of PAI-1 antigen in over 95% of all plasma samples.

**Correlation of PAI activity and PAI-1 antigen.** In the plasma of 39 healthy individuals (19 males, 20 females) there was a significant correlation (Spearman’s rank correlation coefficient, 0.613; P < .001) between PAI-1 antigen measured by RIA and PAI activity (Fig 4). However, a threefold discrepancy between antigen and activity was noted. A concentration of 50 ng/mL active PAI-1 corresponds to approximately 30 U/mL (based upon a specific activity of t-PA of 0.5 U/ng and the respective mol wts of t-PA and PAI-1. Instead, the average PAI activity of plasma samples containing a mean PAI-1 concentration of 50 ng/mL was only 9 U/mL.

**Intraassay and interassay variability of the RIA.** To establish the intraassay variability of the RIA, four sets of 18 duplicate plasma samples were randomly distributed into four assay series and the RIA performed the same day. Interassay variability was determined by testing the same plasma samples on three different days. The intraassay variability was dependent on the PAI-1 concentration. At low concentrations (between 10 and 20 ng/mL) the average intraassay variability (SD/mean value x 100%) was 15%, whereas above 20 ng/mL it was 7%. The average interassay variability was approximately 10%. Because the majority of plasmas show PAI-1 concentrations above 20 ng/mL (see the following sections), the RIA permits the measurement of PAI-1 in clinical conditions with increased PAI-1 levels with a fairly good accuracy.

**Effect of complexation of PAI-1 to t-PA on the measurement of PAI-1 antigen.** Because PAI-1 exists in human plasma in a free and a t-PA-bound form, it was important to establish to what degree the RIA measures t-PA-complexed inhibitor. To this end PAI-1 antigen determinations were performed in ten normal plasmas before and after the addition of 10 U/mL exogenous t-PA. PAI-1 values in plasmas with or without added t-PA were not significantly different (P > .2; Wilcoxon signed rank test) and in general within the intraassay variability. The RIA thus effectively measures both free and t-PA-bound PAI-1.

**Influence of sex and age on PAI-1 antigen levels.** No significant difference was observed in plasma PAI-1 antigen levels in males (mean ± SD, 31 ± 18 ng/mL; median, 23 ng/mL; range, 6 to 65 ng/mL) and females (mean ± SD, 23 ± 12 ng/mL; median, 21 ng/mL; range, 6 to 57 ng/mL). A significant correlation of PAI-1 antigen levels with age was observed (r = .474, P < .005). Dividing the 39 blood donors into three equal groups based upon their age indicated that PAI-1 antigen levels were twofold higher in the oldest than in the youngest group (Table 1). The difference in PAI activity was less marked: the average PAI activity was 40% higher in the oldest age group (P > .05). The lesser difference in PAI activity between the two age groups probably is due to the 70% higher t-PA level in the oldest group (Table 1), which leads to a proportionately lower fraction of free PAI-1 in the older individuals.

**Release of PAI-1 by human platelets.** Collagen stimulation of ten PRPs adjusted to 200 × 10⁶ platelets/mL resulted

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**Fig 3.** Comparison of standard curves of PAI-1 in fivefold-diluted, PAI-1-depleted plasma and in immunodilution buffer. Different concentrations of PAI-1 in either immunodilution buffer (O) or fivefold-diluted, PAI-1-depleted plasma (Q) were incubated for two hours at 37°C with 1,500-fold-depleted antiserum to human PAI-1. Thereafter radiolabeled PAI-1 (0.2 ng, 8,000 cpm) was added and incubated overnight. Bound PAI-1 was separated from unbound by immunoprecipitation of the complexes with immobilized donkey antibodies to rabbit IgG (see Materials and Methods) and, after washing, counted in a gamma counter. The figure shows a logit-log plot of percent bound radioactivity vs added unlabeled PAI-1.

**Fig 4.** Correlation between PAI-1 antigen levels and PAI activity in normal human plasma. PAI-1 antigen levels and PAI activity were measured in the plasma of 39 healthy volunteers aged 22 to 56.
in a significant increase of PAI-1 antigen levels \((P < .002;\) Wilcoxon signed rank test) from 22 ± 13 ng/mL (mean ± SD, median, 21 ng/mL; range, 8 to 45 ng/mL) in platelet-poor plasma (PPP) to 79 ± 30 ng/mL (median, 78 ng/mL; range, 40 to 137) in the PRPs after platelet aggregation. From the increase in PAI-1 antigen levels after platelet aggregation (57 ng/200 × 10^6 platelets) the amount of platelet-associated PAI-1 was calculated to be 0.3 fg/platelet (or approximately 4,000 molecules of PAI-1/platelet). No correlation \((r = -.05)\) was observed between plasma PAI-1 levels and platelet-associated PAI-1, which indicated that platelets and plasma constitute two independent compartments for PAI-1. The mean, total platelet-associated PAI-1 calculated from the amount of PAI-1 released by 200 × 10^6 platelets and corrected for each individual platelet count was 139 ± 55 ng/mL, a value sixfold higher than that of PPP.

**PAI-1 and t-PA antigen concentrations before and after venous occlusion.** During venous occlusion plasma antigen concentrations of both PAI-1 and t-PA antigen augmented. PAI-1 antigen levels increased from 27 ± 16 ng/mL (median, 22 ng/mL) to 43 ± 33 ng/mL (median, 38 ng/mL) after ten minutes and to 59 ± 53 ng/mL (median, 44 ng/mL) after 20 minutes of venous occlusion (Fig 5). Concomitantly, t-PA antigen levels increased from 3.4 ng/mL to 12 and 26 ng/mL after ten and 20 minutes, respectively, of venous occlusion (Fig 5). Several mechanisms may be responsible for the increase in PAI-1 concentrations: (a) hemocoagulation induced by the venous occlusion, (b) release of PAI-1 by partially activated platelets, and (c) release of PAI-1 by local sources, eg, endothelial cells. To evaluate the effect of hemocoagulation, the total protein level of each individual plasma was determined; mean plasma protein concentrations increased 27% after ten minutes and 38% \((P < .001,\) Wilcoxon signed rank test; Table 2) after 20 minutes of venous occlusion. After correction of each PAI-1 value for the corresponding hemocoagulation, PAI-1 concentrations after a 20-minute venostasis still were significantly \((P < .001)\) increased with respect to those before stasis. To assess the contribution of platelets to stasis-induced augmentations of PAI-1, the extent of the platelet release reaction was estimated from β-thromboglobulin (β-TG) levels. Concentrations of β-TG increased significantly from 320 to 581 ng/mL, but after correction for hemocoagulation this increase was not significant anymore. Because platelets release β-TG and PAI-1 simultaneously, it is obvious that part of the PAI-1 in the plasma before and after venous occlusion stems from platelets. Linear regression analysis of PAI-1 and β-TG levels before venous occlusion yielded a regression coefficient of .312 (not significant). The increases in PAI-1 and β-TG levels after correction for hemocoagulation were correlated in a borderline manner \((r = .333, P = .04)\). From the slopes of the latter regression curve, it was estimated that an increase of 100 ng/mL of β-TG corresponds to an increase of 2.2 ng/mL of PAI-1 (ratio of PAI-1/β-TG, 0.022). These values are slightly higher than those arrived at by determining β-TG and PAI-1 concentrations in PRP before and after induction of platelet aggregation (β-TG, 12 µg/mL; PAI-1, 140 ng/mL; PAI-1/β-TG ratio, 0.012; average determination for ten plasmas). To avoid underestimating the contribution of platelets to increases in PAI-1 concentrations before and after venous occlusion, we have chosen the larger of the two ratios for our calculations.

Table 2 shows that both before and after venous occlusion approximately 25% of PAI-1 antigen was derived from platelets. After correction for the contribution of platelets and hemostasis a significant increase in PAI-1 levels still occurred after ten and 20 minutes of venous occlusion \((P < .02, P < .001,\) respectively, Wilcoxon signed rank test).

The effect of the increases in both t-PA and its inhibitor on the fibrinolytic activity of the euglobulins was measured on fibrin plates. It increased from an average of 0.3 U/mL before to 1.7 and 5.4 U/mL after ten and 20 minutes of venous occlusion, respectively. Before venous occlusion fibrinolytic activity was not correlated to t-PA or to PAI-1 levels (Table 3). After ten minutes of venous occlusion, fibrinolytic activity was positively correlated with t-PA levels and negatively with PAI-1 levels. After 20 minutes of venous occlusion, fibrinolytic activity was even more strongly correlated with t-PA levels but no longer with PAI-1 levels. Interesting-
They are sensitive to interference by other, nonspecific systems have been developed that estimate PAI activity in antigen levels (Table 3). A striking positive correlation was before venous occlusion concentrations in each plasma was estimated from fl-TG (C) concentrations in that plasma, by assuming a PAI-1/fl-TG ratio of 0.022. All protein assay.

Furthermore, stimuli such as exercise that induce the release of t-PA reduce PAI activity. Assays can be rendered more specific for PAI-1 by using antibodies to PAI-1 in conjunction with a PAI activity determination. To understand the contribution of platelets to PAI-1 (C) concentrations in each plasma was estimated from fl-TG (C) concentrations in that plasma, by assuming a PAI-1/fl-TG ratio of 0.022. All protein concentrations in nanograms per milliliter. Results are given as means ± SD; between parentheses are given range and median values.

### Table 2. Effect of Hemoconcentration and Platelet Activation on Plasma Antigen Concentration of PAI-1

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<th>20-min VO</th>
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<td>PAI-1</td>
<td>27 × 16</td>
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<td>PAI-1 (C)</td>
<td>27 × 16</td>
<td>34 ± 25*</td>
<td>40 ± 36*</td>
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<td>(6-58; 22)</td>
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<td>β-TG</td>
<td>320 ± 215</td>
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<td>Plasma PAI-1 (C)</td>
<td>20 ± 15</td>
<td>26 ± 23†</td>
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<td>(0-189; 23)</td>
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<td>Platelet PAI-1 (C)</td>
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<td>8 ± 6</td>
<td>9 ± 6</td>
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<td>(2-22; 6)</td>
<td>(2-35; 7)</td>
<td>(2-29; 7)</td>
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Plasma concentrations of PAI-1 and β-TG are given before and after correction (C) for hemoconcentration. The contribution of platelets to PAI-1 (C) concentrations in each plasma was estimated from β-TG (C) concentrations in that plasma, by assuming a PAI-1/β-TG ratio of 0.022. All protein concentrations in nanograms per milliliter. Results are given as means ± SD; between parentheses are given range and median values.

Abbreviation: VO, venous occlusion.

*P < .001.
†P < .05.
‡P < .02.

We have used cortisol-stimulated HT 1080 fibrosarcoma cells as a source of PAI-1. Because these cells are able to secrete both PAI-1 and PAI-2, it was essential to establish that the purified PAI indeed corresponds to PAI-1. Its immunologic relationship with PAI-1 and the specificity of the antiserum raised against this protein were established by the following criteria: (a) in platelet extracts one 51-kDa immunoreactive band comigrated with the platelet PAI-1 band, which was demonstrable by reverse fibrin autografting; (b) the latter was immunoprecipitable with antibodies raised against the HT 1080 PAI. The addition of t-PA to human plasma resulted in the formation of a 110-kDa complex with PAI-1. The complex but not free t-PA reacted with our immunopurified antibodies. (c) Radiolabeled HT 1080-derived PAI was immunoprecipitated by antibodies to human or bovine endothelial cell-derived PAI-1.

**DISCUSSION**

Only recently was the key role of PAIs on fibrinolytic activity in human plasma established. A number of assay systems have been developed that estimate PAI activity in human plasma. These assay systems were based on the inhibition of t-PA and measure PAI levels only indirectly. They are sensitive to interference by other, nonspecific inhibitors of t-PA and do not distinguish between different PAIs. Furthermore, stimuli such as exercise that induce the release of t-PA reduce PAI activity. Assays can be rendered more specific for PAI-1 by using antibodies to PAI-1 in conjunction with a PAI activity determination. To better understand the contribution of PAI-1 to overall activity of the fibrinolytic system we have developed a PAI-1 antigen assay.

### Table 3. Correlation Between PAI-1 Antigen, PAI Activity, t-PA Antigen, and Fibrinolytic Activity Before and After Ten Minutes of Venous Occlusion

<table>
<thead>
<tr>
<th></th>
<th>Before VO</th>
<th>10-min VO</th>
<th>20-min VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA v t-PA antigen</td>
<td>.037</td>
<td>.457*</td>
<td>.671*</td>
</tr>
<tr>
<td>FA v PAI antigen</td>
<td>-.176</td>
<td>- .329†</td>
<td>- .059</td>
</tr>
<tr>
<td>PAI activity v t-PA antigen</td>
<td>.479‡</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PAI activity v PA-1 antigen</td>
<td>.613*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>t-PA antigen v PA-1 antigen</td>
<td>.602*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The table gives Spearman’s rank correlation coefficients. Abbreviations: FA, fibrinolytic activity; ND, not done.

*P < .001.
†P < .05.
‡P < .005.

Standard curves of PAI-1 in PAI-1-depleted plasma were identical to those in immunodilution buffer, provided the plasma had been diluted at least fivefold. At this plasma dilution, the RIA has a range of 8 to 250 ng/mL and allows, therefore, the direct determination of PAI-1 levels in resting (range, 6 to 57 ng/mL) and postocclusion plasma (10 to 275 ng/mL) (Fig 3). No difference was observed between free and t-PA–bound PAI-1. The assay thus measures the total PAI-1 concentration.

A threefold discrepancy between PAI-1 antigen and PAI activity was noted. Part of this discrepancy is due to the measurement of complexes of PAI-1 with t-PA that are...
obviously inactive. A further explanation for the lower PAI activity levels may be ascribed to the presence, in plasma, of an inactive, "latent" form of PAI-1 that can be (re-)activated by chaotropic agents. In endothelial cell conditioned medium, the active form is converted into the latent form with a half-life of a few hours, and after four hours of culture the ratio of latent to active PAI-1 was more than 12. In human serum a ratio of over 3 was observed. Because in these studies the completeness of activation of the latent form of PAI-1 was not established, actual ratios of latent to active PAI-1 may even have been higher.

As has been observed before for PAI activity in plasma, PAI-1 antigen levels likewise were extremely variable: in normal individuals there was a more than tenfold difference between the lowest and highest values. PAI-1 antigen levels were the same in males and females but increased with age. Average PAI-1 antigen levels were twofold higher in the oldest group of volunteers, aged 53 ± 4 years, as compared with the youngest group, aged 29 ± 3 years. This twofold increase did not match entirely a nonsignificant 40% increase in PAI activity. The lesser increase in PAI activity than t-PA antigen concentration may be due, at least in part, to an increased t-PA level in the higher age groups. Our results are in accordance with those of Aillaud et al who recently found that PAI activity and t-PA antigen levels were 60% and 120% higher, respectively, in a group of elderly individuals (average age of 80 years) as compared with a young control group. Whether the increased PAI-1 and t-PA levels are truly an age-related phenomenon or merely reflect an increased occurrence of subclinical pathologies at a higher age remains to be established.

During venous occlusion, antigen levels of both t-PA and PAI-1 increased linearly with time. After 20 minutes of venous occlusion PAI-1 levels had increased twofold and t-PA levels eightfold. Correction for hemoconcentration according to total plasma protein concentrations indicated that half of the increase in PAI-1 levels was due to hemoconcentration. Measurement of β-TG in these plasmas suggested that both before and after venous occlusion approximately one quarter of PAI-1 was derived from platelets that had been partially activated. After correction for platelet-derived PAI-1, a significant increase in PAI-1 was still observed after 20 minutes of venous occlusion. This suggests that the local vascular bed contributes to PAI-1 levels, most likely by release of PAI-1 from endothelial cells. The corrected increases in t-PA levels (fivefold) are much greater than those of PAI-1 (50%), thereby resulting in general in a net increase in fibrinolytic activity.

In resting plasma no correlation was observed between t-PA or PAI-1 levels and fibrinolytic activity as measured in twofold-concentrated euglobulins. Apparently, in resting plasma the larger part of t-PA exists in an inactive form, and "resting" fibrinolytic activity is little influenced by PAI-1. After ten minutes of occlusion, the average PAI-1 levels were 43 ng/mL, and average t-PA levels were 12 ng/mL. Assuming that only one third of PAI-1 is in an active form, the average concentrations of active PAI-1 and t-PA are roughly equimolar. As expected in such a situation, a positive correlation of fibrinolytic activity with t-PA levels and a negative one with PAI-1 levels was observed. This suggests that, after ten minutes of venous occlusion, fibrinolytic activity reflects a balance between activator and inhibitor and implies that a deficient fibrinolytic activity may be due to a high concentration of PAI-1 or a deficient release of t-PA. At longer occlusion times (20 minutes), the amount of t-PA secreted is in such an excess over active PAI-1 that fluctuations of PAI-1 contribute much less to fluctuations of fibrinolytic activity.

In the resting plasma of healthy individuals a striking correlation was observed between t-PA/PAI-1 antigen concentrations on the one hand and PAI-1 antigen levels and PAI activity on the other. This correlation could be explained if the PAI/PAI-1 complexes had a longer in vivo half-life than free PAI-1 or t-PA or if there was a coordinate regulation of the release and/or clearance of PAI-1 and t-PA. In rabbits, the half-life of complexes of t-PA with PAI-1 and of free t-PA was only a few minutes and shorter than that of PAI activity (seven minutes). Therefore it seems more likely that a common mechanism regulates plasma levels of PAI-1 and t-PA.

Direct measurement of PAI-1 antigen levels before and after platelet aggregation confirms previous results of activity assays and immunologic studies that platelets release PAI-1 The amount of PAI-1 per platelet (0.3 fg or 4,000 molecules) is quite small. Nevertheless, quantitation of PAI-1 released by platelets suggests that in human blood the larger part of PAI-1 (approximately 85%) is associated with platelets, as has been estimated previously from PAI activity assays. In the present study in which plasma was prepared as recommended for fibrinolytic tests, approximately one quarter of PAI-1 was derived from platelets. The lack of correlation, however, between plasma and platelet-associated PAI-1 concentrations and the poor correlation between β-TG and PAI-1 levels in resting and poststasis plasma confirms previous suggestions that plasma and platelets constitute two independent compartments of PAI-1. However, the presence of β-TG in the citrated plasmas indicated that approximately 25% of PAI-1 in plasma actually was derived from platelets. By collecting the blood on anticoagulants that prevent platelet release, e.g., those recommended for measuring β-TG in plasma, a more precise determination of the plasma concentration of PAI-1 could be achieved.

The development of a specific RIA for PAI-1 now permits studies on the fluctuations of PAI-1 antigen in health and disease. As exemplified by the modifications of PAI-1 antigen and PAI activity with age, antigen measurements may be more sensitive in detecting increases in PAI-1 levels than are activity assays.

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Plasminogen activator inhibitor 1: development of a radioimmunoassay and observations on its plasma concentration during venous occlusion and after platelet aggregation

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