Measurement of Plasminogen Activator Inhibitor 1 In Biologic Fluids With a Murine Monoclonal Antibody–Based Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay for plasminogen activator inhibitor-1 (PAI-1) in biologic fluids was developed on the basis of two murine monoclonal antibodies raised against PAI-1 purified from HT-1080 fibrosarcoma cells. The lower limit of sensitivity of the assay in plasma is 2 ng/mL. The assay is 12 times less sensitive toward the PAI-1/human tissue-type plasminogen activator (t-PA) complex as compared with free PAI-1. The intraassay, interassay, and interdilution coefficients of variation are 5.2%, 8.0%, and 7.1%, respectively. The level of PAI-1 in platelet-poor plasma of healthy subjects is 18 ± 10 ng/mL (mean ± SD, n = 45). In platelet-rich plasma after freezing and thawing, 92% of PAI-1 antigen is released from platelets, whereas only 8% is found in the corresponding platelet-poor plasma. In platelet-poor plasma from healthy subjects, a linear correlation (r = 0.80) was found between PAI activity and PAI-1 antigen. In plasma approximately two thirds of the PAI-1 antigen was functionally active, whereas only 5% of the PAI-1 antigen released from platelets was active. During pregnancy a progressive increase of PAI-1 antigen levels up to three- to sixfold the control value was observed. In plasma of patients with recurrent deep vein thrombosis, PAI-1 levels were 44 ± 20 ng/mL (mean ± SD, n = 7), during a clinically silent phase. Four of these patients had a level above 38 ng/mL (mean ± 2 SD of normal). The present assay, based on stable and reproducible reagents, allows the specific determination of PAI-1 antigen in biologic fluids. It may facilitate interlaboratory comparisons and be useful for further investigations of the role of PAI-1 in clinical conditions associated with impaired fibrinolysis and/or a tendency to thrombosis and investigations of the role of PAI-1 in platelets.

PLASMINOGEN ACTIVATORS play a central role in the regulation of the fibrinolytic system in blood. Their activity is controlled by plasminogen activator inhibitors (PAI). The occurrence of these specific, fast-acting inhibitors in blood has only recently been demonstrated. Three different PAIs have been identified: in addition to protease nexin: the endothelial cell-type PAI (PAI-1), the placenta-type PAI (PAI-2), and the urinary-type PAI (PAI-3). PAI-1 appears to be the main physiological inhibitor of human tissue-type plasminogen activator (t-PA) in normal plasma.1 In earlier studies PAI levels in blood have mainly been determined with the use of functional assays.4-8 These methods were based on the neutralization of t-PA added to plasma samples by measurement of the residual t-PA activity on fibrin plates or with spectrophotometric methods. These assays have one or more of several shortcomings such as the requirement for euglobulin precipitation, interference by other protease inhibitors, and the occurrence of nonparallel standard curves of t-PA in buffer and in plasma. Furthermore, functional assays do not distinguish between the different PAIs. Consequently a significant variability in assay results by different laboratories has been reported,4,11 and results of current PAI activity assays are difficult to standardize between laboratories.12 More recently, immunoassays have been developed based on rabbit antisera.13-15 These assays suffer from the disadvantages typical of polyclonal antibodies, including problems with calibration, reproducibility, and availability of reagents.

Some clinical conditions including suffering from coronary artery disease, venous thrombosis, obesity or being in the postoperative phase are associated with increased levels of PAIs.6-8,16-19 The pathological significance of increased PAI levels remains, however, to be established in prospective studies.

To allow for more detailed studies of the pathophysiology of PAI-1, we have developed a simple, enzyme-linked immunosorbent assay (ELISA) for PAI-1 in plasma and biologic fluids that is based on two murine monoclonal antibodies. This assay was used to study the relationship between PAI-1 in plasma and in platelets and to compare the activity-antigen relationship in plasma, platelets, and cell culture fluids.

MATERIALS AND METHODS

Reagents. PAI-1 was purified from the conditioned medium of hydrocortisone-stimulated HT-1080 fibrosarcoma cells,13 t-PA was purified from melanoma cell culture fluid,20 or alternatively, recombinant t-PA was obtained from Genentech, Inc, South San Francisco, CA. Monoclonal antibodies against PAI-1 were produced essentially by the method of Galfre and Milstein.21 BALB/c mice were immunized by subcutaneous injection of 10 µg PAI-1 in complete Freund’s adjuvant, which was followed 2 weeks later by intraperitoneal injection of 10 µg PAI-1 in incomplete Freund’s adjuvant. After an interval of at least 6 weeks, the mice were boosted intraperitoneally with 10 µg PAI-1 in saline on days 4 and 2 before the cell fusion.
After washing of the plates, I 50-μL aliquots of a 0.1 mol/L applied
Then 180 μL of 7F5-HRP, diluted 1:4,000 in PBS-Tween, was emptied and washed (three cycles, Scatron microwash, Analis, once with PBS. Samples were diluted in PBS containing Tween 80 stored at -20°C. Immediately before use the plates were washed of mannitol and 20 g of saccharose the wells containing 10 g/L bovine serum albumin. Then obtained (5 mmol/L), and bovine serum albumin (1 mg/L (0.002%), EDTA mL) (dilution buffer), and 180 μL was added to the wells. After finally with a solution containing diluted (final concentrations I to 32 μg/mL) in 0.04 mol/L phos-
described previously.30 PAI-3 was purified from human urine as
with the positions of mol wts 1 10,000 and 70,000, were detected with the total t-PA ELISA. The fractions eluting with a 1 : 1 complex
G-200 column. Total t-PA27 monoclonal antibody against t-PA (MA-62E8) was added at a final concentration of 500 ng/mL to tion medium of endotoxin-stimulated human endothelial cells
with the method described by Verheijen et al. The results were expressed in units equivalent to the amount of international units of t-PA neutralized. The activity of t-PA was determined by comparison with the international reference preparation for t-PA (83/517) that was obtained from the National Institute for Biological Standards and Control, London. β-Thromboglobulin (β-TG) levels in plasma and in platelet lysates were measured by using a radioimmunoassay kit (Amersham, UK).

RESULTS

Development of an ELISA for PAI-1 in biologic fluids. The wells of polystyrene microtiter plates were coated with MA-7D4 diluted in PBS to final concentrations of 1 to 32 μg/mL. An optimal response was obtained at a concentration of 4 μg/mL, which was subsequently used. The IgG-HRP conjugate (7F5-HRP) was used at a final concentration of 0.4 μg/mL. Under those conditions PAI-1 concentrations between 0.2 and 4 ng/mL could accurately be measured (Fig 1). Figure 1 also illustrates dose-response curves of PAI-1 diluted in different mixtures of PAI-1-depleted, pooled normal human plasma and buffer. At plasma dilutions of 1:10 or more, interference of plasma proteins is no longer observed. Thus the lower limit of sensitivity of the assay in plasma is 2 ng/mL. When adding purified PAI-1 to PAI-1-depleted, pooled normal plasma to final concentrations of 90, 45, and 20 ng/mL, recoveries in the assay were 104% ± 6%, 107% ± 5%, and 99% ± 6%, respectively (mean ± SD, n = 3). The assay variability was assessed by using ten normal plasma samples, each assayed

Fig 1. Dose-response curve of PAI-1 after dilution in buffer (●) or in mixtures of pooled normal PAI-1-depleted human plasma and buffer in ratios of 1:5 (●), 1:10 (▲), and 1:20 (○).
six times and on six occasions. Intraassay and interassay coefficients of variation were 5.2% and 8.0%, respectively. The interdilution coefficient of variation was 7.1% (determined with three serial twofold dilutions of 25 different plasma samples).

Assay of PAI-1 in plasma. In PPP samples obtained from 45 healthy individuals, PAI-1 levels were 18 ± 10 ng/mL (range, 4 to 43 ng/mL; median value, 16 ng/mL) with no significant difference between males (15 ± 7 ng/mL) and females (19 ± 10 ng/mL). One additional, apparently healthy subject had PAI-1 levels of 82 and 106 ng/mL measured at a 2-week interval. In 18 of these samples, both PAI activity and PAI-1 antigen levels were determined. A significant correlation \( r = 0.80, P < 0.001 \) was found (Fig 2). From the slope of this regression line, an equivalence of 1 unit PAI activity with 2.2 ng PAI-1 antigen was derived. A comparison of PAI activities and corresponding PAI-1 antigen levels in frozen and thawed platelet-rich plasma yielded a correlation coefficient of 0.88 (\( n = 9 \)), but with a ratio of 1 unit of PAI activity for 28 ng PAI-1 antigen (Table 1).

Figure 3 illustrates the differential reactivities of the ELISA toward free PAI-1 and the PAI-1/t-PA complex. The ELISA is 12 times less sensitive to the PAI-1/t-PA complex as compared with free PAI-1. This different reactivity is also demonstrated in Table 2 where it is shown that the addition of excess t-PA to plasma samples results in a decrease of the quantity of PAI-1 antigen recognized in the ELISA. The correlation \( r = 0.92, P < 0.01 \) between the decrease of PAI-1 antigen and the baseline PAI activity confirms that the decrease is caused by complex formation between t-PA and the active fraction of PAI-1 in plasma.

PAI-1 antigen levels were determined in samples of PPP and frozen and thawed platelet-rich plasma of 12 healthy subjects. The mean antigen level in PPP was 17 ± 14 ng/mL, and in the lyed platelet-rich plasma it was 221 ± 87 ng/mL. From measurements of the platelet number it was calculated that freezing and thawing resulted in the release of 0.7 ± 0.4 fg PAI-1/platelet. The baseline PAI-1 level in plasma was 8.4% ± 5.9% of that after lysis of the platelets. The \( \beta \)-thromboglobulin level, however, represented only 0.3% ± 0.2% of that after lysis of platelets, which suggested that the PAI-1 in plasma does not originate from platelets lysed either in vivo or during collection or processing of the samples in vitro.

PAI-1 antigen levels were measured in 29 women with normal pregnancies. A significant increase (three- to sixfold at term) was observed in the second half of pregnancy (Fig 4). In pregnant patients with insulin-dependent diabetes mellitus (\( n = 6 \)), intrauterine growth retardation (\( n = 7 \)), lupus erythematosus disseminatus (\( n = 4 \)), or preeclampsia

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**Table 1. PAI Activity and PAI-1 Antigen Levels in Lysed Platelet-Rich Plasma (L-PRP) and in PPP**

<table>
<thead>
<tr>
<th>No.</th>
<th>Activity (U/mL)</th>
<th>Antigen (ng/mL)</th>
<th>Activity (U/mL)</th>
<th>Antigen (ng/mL)</th>
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<td>200</td>
<td>14</td>
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<td>3</td>
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</table>

Group values represent mean ± SD.

Abbreviation: \( \Delta \), difference between L-PRP and PPP.
(n = 7), PAI-1 levels were not significantly different from those found during normal pregnancy.

PAI-1 antigen levels in seven patients with recurrent deep vein thrombosis during a clinically silent period of at least 3 months were 44 ± 20 ng/mL. Four of these patients had a level above 38 ng/mL (mean ± 2 SD of normal).

Assay of PAI-1 in cell culture media. PAI-1 antigen levels in 24-hour conditioned medium of human endothelial cells (30 mL for a 175-cm² confluent cell surface) were 2.3 ± 0.2 μg/mL, whereas PAI activities were only around 146 ± 13 U/mL. A similar discrepancy was observed with conditioned medium of the melanoma cell line MJZJ (0.45 ± 0.06 μg/mL and 33 ± 18 U/mL).

DISCUSSION

We have developed an ELISA using murine monoclonal antibodies that allows measurement of PAI-1 antigen levels in biologic fluids. Levels of PAI-1 in PPP from healthy individuals were found to be 18 ± 10 ng/mL. Comparison of those levels with corresponding PAI activities yielded a good correlation with a ratio of 1 unit activity to 2.2 ng antigen. This ratio varied, however, between 1:1 and 1:7 in individual subjects (Tables 1 and 2). Assuming that neutralization of t-PA is the result of a 1:1 complex formation, one would expect a ratio of 1 unit activity to 2.2 ng antigen. This would suggest that on average about two thirds of PAI-1 antigen in plasma represents active PAI. The remaining part of the PAI-1 antigen probably represents predominantly free but inactive PAI-1 because the ELISA is 12 times less sensitive to complexes between t-PA and PAI-1 than to free PAI-1. The variability between individuals may be due to a variable release of inactive PAI-1 from platelets, to intrinsic functional instability of the inhibitor with variable inactivation during processing of plasma, or to inaccuracy of available functional assays. In view of these sources of variability, our finding of a ratio of active to total PAI-1 of 2/3 is in good agreement with a value of 1/3 that was reported by Kruithof et al.13 These data do not even exclude the possibility that most or all PAI-1 in circulating blood is active and that all apparently inactive material demonstrated in vitro might result from artifacts occurring after blood collection.

Similar measurements of PAI activity and PAI-1 antigen in platelet-rich plasma after freezing and thawing also revealed a linear correlation between activity and antigen. The PAI activity in the lysed platelet-rich plasma only increased twofold, whereas the PAI-1 antigen level increased 13-fold, which suggested that the bulk of the PAI-1 released from platelets is inactive. Whether this inactive material is already present in platelets or whether platelet PAI-1 is inactivated during release in plasma remains to be established. Previous studies31,32 based on activity measurements had suggested that 25% to 50% of the total PAI-1 after lysis of platelets is already present in plasma. On the basis of our present activity determinations, we would reach the same conclusion. Antigen measurements, however, indicate that only 8% of PAI-1 is found in plasma and 92% is released from lysed platelets. This observation stresses the necessity of careful blood collection, ie, on tubes containing inhibitors of the platelet release reaction such as prostaglandin E1 and theophylline33 or on acidified citrate (pH 4.5),31 and standardized sample processing. We observed no differences between PAI-1 levels in plasma collected on the antiplatelet reagents or on acidified citrate.

It has been suggested in previous studies31,32 that PAI activity in plasma is not derived from platelets. Our present results indicate that 8% of the PAI-1 antigen after lysis of platelets is present in PPP, whereas this ratio is only 0.3% for β-TG. This observation together with the known difference in half-life (a few minutes for PAI-126 and about 100 minutes for β-TG4) seems to exclude platelets as the source of PAI-1 in plasma, which may originate from endothelial cells or liver cells.35

In plasma about two thirds of the PAI-1 antigen is active, whereas in conditioned media of cultured human endothelial cells only 9% is active (146 units activity for 2,300 ng antigen). It has been suggested previously that endothelial cells synthesize and store PAI-1 in its active form36,37 and that inactivation occurs, in vitro, after release from the cell. Our results obtained in plasma demonstrate that such an inactivation is much less important in vivo. This may be due to the short half-life of PAI-1 in plasma, thus preventing the accumulation of inactive PAI-1.

Apparently, endothelial cells are not suitable for purification of active PAI-1 material. Because it has been observed that a melanoma cell line MJZJ produces PAI-1 that could not be reactivated by treatments such as were used to activate PAI-1 obtained from endothelial cells, the authors concluded that this cell line produces mainly active material.31 Our present data, however, show that also only 10% of the material in the conditioned medium of MJZJ cells is active.

Table 2. PAI Activity and PAI-1 Antigen Levels in Normal PPP

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Baseline PAI-1 Antigen Levels (ng/mL)</th>
<th>After Addition of 100 ng t-PA/mL Δ</th>
<th>Baseline PAI Activity (U/mL)</th>
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</table>

The correlation coefficient of the Δ antigen levels with the baseline PAI activity, determined by linear regression analysis, is 0.92.
PAI-1 levels in the third trimester of pregnancy were three- to sixfold higher than those in nonpregnant women, thus confirming previous results. In patients with recurrent deep vein thrombosis during a clinically silent period, PAI-1 antigen levels above the mean value ± 2 SD of normals were observed in four of seven patients.

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Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay

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