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

By Paul J. Declerck, Marie-Christine Alessi, Maria Verstreken, Egbert K.O. Kruithof, Irène Juhan-Vague, and Désiré Collen

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 plasminogen activator 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- and plasminogen activator (t-PA) in normal plasma. In earlier studies PAI levels in blood have mainly been determined with the use of functional assays. These methods were based on the neutralization of t-PA added to plasma samples by measurement of the residual t-PA activity in 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, and results of current PAI activity assays are difficult to standardize between laboratories. 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. The pathological significance of increased PAI levels remains, however, to be established in prospective studies.

To allow 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. t-PA was purified from melanoma cell culture fluid, or alternatively, recombiant t-PA was obtained from Genentech, Inc, South San Francisco, CA. Monoclonal antibodies against PAI-1 were produced essentially by the method of Galfré and Milstein. 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.

From the Center for Thrombosis and Vascular Research, University of Leuven, Belgium; Laboratoire d'Hémato logie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; and Laboratoire d'Hémato logie, Hôpital Universitaire Timone, Marseille, France.

Submitted June 16, 1987; accepted September 15, 1987.

Supported by the Geconcerteerde Onderzoeksacties (Project No. 85-00/3). Dr Declerck is a Senior Research Assistant of the National Fund for Scientific Research, Belgium. Dr Alessi was the recipient of a fellowship from the "Fondation pour la Recherche Médicale." Dr Collen is the recipient of a Research Professorship from the "Fondation Louis Jeanet de Médecine," Switzerland.

Address reprints requests to Désiré Collen, MD, Center for Thrombosis and Vascular Research, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.

0006-4971/88/7101-0039$3.00/0

ELISA FOR PAI-1 IN PLASMA

Spleen cells were isolated and fused with P3X63-Ag8.6-5.3 myeloma cells (obtained from Dr O. Schönerr, Organon, Oss, The Netherlands) according to Fazekas de St Groth and Scheidegger.2 After selection in hypoxanthine, aminopterine, thymidine medium, the supernatants were screened for specific antibody production with a one-site noncompetitive micro-ELISA using microtiter plates coated with PAI-1. The bound immunoglobulins were detected with horseradish peroxidase (HRP)-conjugated rabbit antimouse IgG. Positive clones were used for the production of ascitic fluid in pristane-primed BALB/c mice.2 The IgG fraction of the monoclonal antibodies was purified by affinity chromatography on Protein A-Sepharose.2

HRP-conjugated monoclonal antibody MA-7F5 (7F5-HRP) was produced as described by Nakane and Kawaoi.2 The final IgG concentration in the stock solution of conjugate used for analysis was 1.6 mg/mL.

The PAI-1/t-PA complex was prepared as follows. Single-chain t-PA was added at a final concentration of 500 ng/mL to conditioned medium of endotoxin-stimulated human endothelial cells as described.10 This medium was then applied on a Sepharose 4B column to which a monoclonal antibody against t-PA (MA-62E8) was coupled. Bound antigen was eluted with 3 mol/L KSCN. The t-PA-containing fractions were pooled and applied on a Sephadex G-200 column. Total t-PA and free t-PA were measured in the fractions. One peak was detected in the free t-PA ELISA at a position with molecular weight (mol wt) 70,000, and two peaks, corresponding with the positions of mol wts 110,000 and 70,000, were detected with the total t-PA ELISA. The fractions eluting with an apparent mol wt of 110,000 were pooled and used as complex. The quantity of PAI-1 present as a complex was calculated from the amount of t-PA present, assuming a 1:1 complex formation.

Platelet-rich plasma was obtained by collecting plasma on citrate, pH 7.5 (0.1 mol/L, 0.1 vol), and centrifugation at 200 g for 10 minutes. Platelet-poor plasma (PPP) was obtained by collecting plasma on citrate, pH 4.5 (0.1 mol/L, 0.1 vol), immediately cooling on ice, and centrifugation at 2,000 g for at least 15 minutes at 4°C.

PAI-1-depleted plasma was prepared by adsorption of pooled normal plasma on a monoclonal antibody against PAI-1 (MA-12A4) that was coupled to Sepharose 4B.

PAI-2 was purified from the histiocytic lymphoma cell line U-937 as described previously.22 PAI-3 was purified from human urine as described previously.22 The human melanoma cell line MJZJ was kindly provided by Dr Vetterlein (University of Vienna, Austria).

Two-site ELISA for PAI-1. The IgG fraction of MA-7D4 was diluted (final concentrations 1 to 32 μg/mL) in 0.04 mol/L phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl (PBS). Two hundred–microtiter samples of this solution were incubated for 48 hours at 4°C in the wells of polystyrene microtiter plates (Costar, Cambridge, MA). The plates were emptied, and the wells were treated for two hours at room temperature with 200 μL PBS containing 10 g/L bovine serum albumin. Then the wells were washed with 200 μL PBS and finally with a solution containing 10 g of mannitol and 20 g of saccharose per liter. The plates were then stored at -20°C. Immediately before use the plates were washed once with PBS. Samples were diluted in PBS containing Tween 80 (0.002%), EDTA (5 mmol/L), and bovine serum albumin (1 mg/mL) (dilution buffer), and 180 μL was added to the wells. After incubation for 18 hours at 4°C in a moist chamber, the wells were emptied and washed (three cycles, Scatron microwash, Analis, Belgium) with PBS containing 0.002% Tween 80 (PBS-Tween). Then 180 μL of 7F5-HRP, diluted 1:4,000 in PBS-Tween, was applied to the wells and incubated for two hours at room temperature. After washing of the plates, 150-μL aliquots of a 0.1 mol/L citrate–0.2 mol/L sodium phosphate buffer, pH 5.0, containing 300 μg/mL o-phenylenediamine and 0.003% hydrogen peroxide were added. After 30 minutes at room temperature the peroxidase reaction was stopped with 50 μL of 4 mol/L H2SO4. The absorbance was measured at 492 nm with a multiscan spectrophotometer (Titertek, Flow Laboratories, Irvine, Scotland). Standard curves were constructed by dilution of purified PAI-1 in dilution buffer. The PAI-1 content in the purified PAI-1 preparation was determined by amino acid analysis.

Other assays. PAI-1 activities were determined by using a modification of the method described by Verheijen et al.24 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...
six times and on six occasions. Intrassay 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 β-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

---

**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>
<th>Activity (U/mL)</th>
<th>Antigen (ng/mL)</th>
<th>Δ Ratio</th>
<th>Antigen (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>170</td>
<td>11</td>
<td>19</td>
<td>8</td>
<td>150</td>
<td>1:14</td>
<td>1:28</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>350</td>
<td>7.5</td>
<td>8</td>
<td>13</td>
<td>340</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>300</td>
<td>12</td>
<td>20</td>
<td>10</td>
<td>280</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>280</td>
<td>28</td>
<td>47</td>
<td>5</td>
<td>230</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>270</td>
<td>9.5</td>
<td>10</td>
<td>8</td>
<td>260</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td>370</td>
<td>16</td>
<td>35</td>
<td>14</td>
<td>330</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>170</td>
<td>8</td>
<td>12</td>
<td>4.5</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.5</td>
<td>120</td>
<td>3.5</td>
<td>3</td>
<td>3</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>200</td>
<td>14</td>
<td>19</td>
<td>3</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>18 ± 6.5</td>
<td>250 ± 87</td>
<td>10 ± 4</td>
<td>19 ± 14</td>
<td>8 ± 4</td>
<td>230 ± 83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Group values represent mean ± SD.

Abbreviation: Δ, difference between L-PRP and PPP.

---
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),34 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-TG26) seems to exclude platelets as the source of PAI-1 in plasma, which may originate from endothelial cells35 or liver cells.36

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.38 Our present data, however, show that also only 10% of the material in the conditioned medium of MJZJ cells is active.
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.

REFERENCES

10. van Mourik JA, Lawrence DA, Loskutoff DJ: Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J Biol Chem 259:14914, 1984
15. Schleef RR, Sinha M, Loskutoff DJ: Immunoandrometric assay to measure the binding of a specific inhibitor to tissue-type plasminogen activator. J Lab Clin Med 106:408, 1985
35. Sprengers ED, Princen HMG, Kooistra T, van Hinsbergh
VWM: Inhibition of plasminogen activators by conditioned medium of human hepatocytes and hepatoma cell line Hep G2. J Lab Clin Med 105:751, 1985
Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay

PJ Declerck, MC Alessi, M Verstreken, EK Kruithof, I Juhan-Vague and D Collen