Specificity of Antigen Assays of Plasminogen Activator Inhibitor in Plasma: Innotest PAI-1 Immunoassay Evaluated

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We evaluate a new commercial enzyme immunoassay (EIA) of plasminogen activator inhibitor-1 (PAI-1) in plasma, the Innotest PAI-1. Because we wanted to measure PAI-1 in blood samples, we developed a procedure for evaluating the specificity of the assay for different PAI-1 forms in their natural environment. All molecular forms were prepared from a plasma that contained only active PAI-1. The recovery of the different molecular forms of PAI-1, relative to active PAI-1 (100%), was 99% ± 7% for PAI-1 complexed with recombinant tissue-type plasminogen activator (t-PA), 104% ± 4% for PAI-1 complexed with melanoma t-PA, 94% ± 11% for PAI-1 complexed with high-M₆ urokinase, and 113% ± 3% for latent PAI-1. The parallelism between the calibration curve of the EIA and the serial dilutions of the different PAI-1 forms was considered acceptable for clinical purposes. In selected clinical plasma samples, the PAI-1 values obtained with the Innotest PAI-1 EIA correlated well with those of the TintElize PAI-1 EIA (r = 0.913, n = 106); the observed correlation of the Innotest measurements with PAI activity was r = 0.795 (n = 79). The Innotest PAI-1 antigen assay appears to detect all molecular forms of PAI-1 to a similar degree, and comes close to being the so-called grand total assay for detecting the total molecular concentration of PAI-1 in plasma.

Indexing Terms: enzyme immunoassay/tissue-type plasminogen activator/fibrinolysis/urokinase

Plasminogen activators determine the fibrinolytic potential of blood; they are controlled in their activity by plasminogen activator inhibitors (PAI).⁴ PAI-1, a 48-kDa glycoprotein, is always present in plasma as well as in platelets; it is a very efficient inhibitor of both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).

Various observations indicate that the plasma content of PAI-1 is clinically significant. Most data refer to PAI activity, showing high PAI activity concentrations in plasma associated with coronary artery disease (3–8) and recurrent deep venous thrombosis (9–14). In young survivors of myocardial infarction, high plasma PAI activity is predictive of reinfarction (15). High concentrations of PAI-1 antigen have also been associated with coronary artery disease (8) and deep venous thrombosis (14). In deep venous thrombosis, measurement of PAI-1 antigen allows discrimination between good and poor responders in the venous occlusion test (11). Hyperinsulinemia, observed in patients with angina pectoris, might play a pathogenic role in the development of myocardial infarction through induction of a high concentration of plasma PAI-1 (16). Increases of PAI-1 antigen serve as a potent, and independent, prognostic factor for both relapse and overall survival in cancer patients. Its usefulness in breast cancer patients (17) is supported by studies of colon cancer, where analysis of the content of PAI-1 antigen in malignant tissues displays a good correlation with the malignant stage (18). Conversely, decreased functional activity of PAI-1 has been associated with bleeding diathesis (19). The PAI-1 concentration in plasma is, therefore, a useful marker for clinical diagnosis.

Several assays of PAI-1 activity in plasma have been developed (20–26), and various monoclonal antibodies against PAI-1 have been used to develop immunoassays of the antigen (27–31). The immunoassays must be able to deal with a complex analytical situation because PAI-1 can occur in various molecular forms in blood, including active PAI-1; PAI-1 complexed with vitronectin; inactive or latent PAI-1, mainly present in lysed platelets and dominant in serum; and PAI-1 complexed with its target proteases u-PA and (mainly) t-PA (1, 32).

In a previous study we showed that various commercially available assays of PAI-1 antigen vary in their specificity for different molecular forms of PAI-1 (33). None of the assays investigated was either specific for a particular molecular form or equally sensitive to all molecular forms of PAI-1 (33). This lack of defined specificity limits the applicability of these assays.

Here, we describe the evaluation of a new commercial PAI-1 immunoassay (Innotest PAI-1) that shows the broadest specificity of any assay so far examined and approaches the concept of a grand total assay. Our procedure for evaluating the specificity of the PAI-1 antigen assays can also be used to evaluate future PAI-1 immunoassays.

Materials and Methods

Reagents

Innotest PAI-1 antigen kits were provided by Innogenetics, Ghent, Belgium. Recombinant human t-PA (Activase) was from Boehringer Ingelheim, Brussels, Belgium. Purified active PAI-1, added to PAI-depleted plasma (59 µg/L PAI-1), and PAI-1-t-PA complex in buffer (stock solution 10 mg/L) (27) were donated by F. J. Declerck, Leuven, Belgium. High-M₆ u-PA was...
obtained from Laboratoire Choay, Paris, France. Two-
chain melanoma t-PA was purified as described else-
where (34).

TintElizE PAI-1 (cat. no. 210220) and Imulyse TM5
(t-PA antigen) were obtained from Biopool, Umeå, Swe-
den. Monocyte PAI-1 EIA kit was obtained from
Monozyme, Virum, Denmark. All assays were car-
ried out according to the manufacturers’ instructions, ex-
cept that, for the t-PA antigen assay only, the range of
the calibration curve was lowered 10-fold, to 0.02–0.4 µg/L,
to check the content of the t-PA-depleted plasma.

Equipment

We used a Flow TiterTek Multiskan MCC/340 MKII
microplate reader (ICN, Zoetermeer, The Netherlands) con-
connected to a Tulip PC compact 2 personal computer
(Tulip, ’s-Hertogenbosch, The Netherlands); Flow Titer-
Tek DSG microplate shaker; a Type B6060 incubator
(Heraeus, Hanau, Germany); a Julabo Exatherm U3
electronic waterbath (Juchheim Labortechnik, Seelbach
über Lahr, Germany); an LKB 7000 Ultrorac fraction
collector (LKB, Bromma, Sweden); a Minipuls 2 peri-
static pump (Gilson, Villiers, France); and an SP-100
ultraviolet spectrophotometer (Pye Unicam, Cam-
bridge, UK).

Innotest PAI-1 Assay

The wells of a microplate were coated with mouse
monoclonal anti-PAI-1 antibodies (no. 15H12) (27). We
incubated for 60 min at 37°C 20 µL of test sample or
recombinant PAI-1 standard and 200 µL of sample di-
luent, containing phosphate buffer with stabilizing pro-
teins and EDTA. After a wash procedure, we added 200
µL of conjugate (mouse monoclonal anti-PAI-1 labeled
with horseradish peroxidase; no. 124A4) (27) and incu-
bated for 60 min at 37°C. The wells were washed again
and enzyme substrate (3,3’,5,5’-tetramethylbenzidine-
HCl) was added. After incubation for 30 min at 25°C,
the reaction was stopped with sulfuric acid. The amount
of color produced in the wells was proportional to the
amount of PAI-1 originally present in the sample or
standard solution. The absorbance at 450 nm was mea-
sured with a microplate reader. Using the absorbance
value of a sample, we established the PAI-1 concentra-
tion by determination from the calibration curve. Be-
cause no international standard for PAI-1 is available,
we calibrated the recombinant PAI-1 standard by amino
acid analyses.

Preparative Procedures

t-PA-depleted plasma. Citrated blood was collected
from three healthy volunteers with high PAI activity.
Blood collection and preparation of platelet-poor plasma
were carried out as described (35). The plasmas were
poled and then depleted of t-PA by affinity chromatog-
raphy on a homemade goat anti-t-PA IgG Sepharose
(Pharmacia, Uppsala, Sweden) column at 4°C. The t-PA
antigen concentrations were determined in each frac-
tion. Fractions with a t-PA antigen content <0.05 µg/L
were pooled. Total protein (A280), t-PA antigen, PAI-1
antigen, and PAI activity were determined both in the
initial plasma pools and in the t-PA-depleted plasma.
PAI-1 antigen was determined with the Innotest PAI-1;
PAI activity was measured as described, expressed in U/L
with 1 U being that amount neutralizing 1 IU of t-PA
(20). In the t-PA-depleted plasma, we measured
the u-PA–PAI-1 antigen complex by means of a sand-
wich enzyme-linked immunosorbent assay (G. Dooijew-
aard, unpublished), using the same principle as de-
scribed for the detection of urokinase antigen (36). In
brief, microtiter plates were coated with monoclonal
anti-PAI-1 IgG (MAI-12; Biopool). Monospecific goat an-
ti-u-PA IgG was used as the labeled antibody (36) and
was detected by reaction with donkey anti-goat IgG
antibodies conjugated with alkaline phosphatase (Jacks-
on Immunoresearch Labs., West Grove, PA). The de-
signation limit of this assay was 0.1 µg/L.

Different forms of PAI-1 in plasma. t-PA-depleted
plasma, which contained about 20 kU/L PAI-1 activity,
was incubated overnight at 37°C with 150 kU/L recom-
binant t-PA, 150 kU/L melanoma t-PA, or 500 kU/L
high Mf, u-PA to obtain complexes of PAI-1 with each of
the three activators. Inactivated/latent PAI-1 was ob-
tained by incubating t-PA-depleted plasma overnight at
37°C.

These procedures were selected after preliminary in-
vestigations. Inactivation of PAI-1 at 37°C was moni-
tored by activity measurement (20) and showed an ap-
parent half-life for PAI-1 of 1.5–2 h at 37°C in various
plasmas. Overnight incubation at 37°C is clearly suf-
cient to inactivate PAI-1.

Complex formation of PAI-1 with u-PA or two-chain
melanoma t-PA was evaluated with the Monozyme
PAI-1 antigen assay. This assay shows a much stronger
response to these complexes than to active PAI-1 (33).
Comparison of PAI-1 reaction with 3-, 6-, or 10-fold
molar excess of either u-PA or t-PA for 5 h at 37°C
showed identical results of increased response. These
data indicate optimal conversion to the desired inhibitor
complexes in all cases. We used a sixfold excess in the
present study.

Dilution curves of each of these PAI-1 forms in plasma
were prepared in the sample diluent of the Innotest
PAI-1 kit. The plasmas were diluted 2, 4, 8, and 16-fold.

Platelet extracts. Platelet extracts were prepared from
platelet-rich citrated plasma from nine healthy volun-
teers as described elsewhere (37). Briefly, platelet-rich
plasma was prepared by centrifugation for 10 min at
120g at room temperature. Isolated platelets were ob-
tained by centrifuging the platelet-rich plasma for 10
min at 800g at room temperature. The pellet was resus-
pended in a glucose-containing phosphate buffer (per
liter: 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH2PO4, 1.44 g
of Na2HPO4, and 2 g of glucose, pH 7.35). After three
cycles of snap-freezing/thawing the platelet suspension,
PAI-1 antigen was measured with both the Innotest and
Monozyme PAI-1 assays.

Clinical Samples

To compare the Innotest and TintElizE PAI-1 antigen
assay, we used citrated clinical plasma samples: 27 sam-

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ples from obese patients, with and without type II diabetes (38), and 79 3-h samples collected to study the diurnal rhythm of three patients with generalized atherosclerosis, three elderly controls, and three young volunteers (39). PAI activity was also measured in these 79 samples. These studies were approved by the local medical ethics committees.

Results
Preparation of Molecular Forms of PAI-1 in Plasma

For evaluating specificity, we prepared active PAI-1 and subsequently converted it into various molecular forms. To obtain a model system representative for the natural environment of the PAI-1 forms, we decided to use plasma as the basic matrix. To obtain plasma with active PAI-1 only, we depleted pooled citrated plasma of t-PA by affinity chromatography, using goat anti-t-PA IgG immobilized on Sepharose; we started with undiluted plasma to limit dilution. As shown in Table 1, t-PA antigen was undetectable (<0.03 μg/L) in the depleted plasma, and ~40% of the PAI-1 antigen was apparently removed as t-PA–PAI-1 complex. Dilution was minimal and, as expected, losses in PAI activity were also minimal (Table 1). Immuno depletion with antibodies against u-PA proved unnecessary because the amount of u-PA–PAI-1 complexes was already below the detection limit (Table 1). Given that platelet-poor plasma prepared properly contains minimal amounts of latent PAI-1 (33), we considered that the PAI-1 in the pooled t-PA-depleted plasma consisted mainly of active PAI-1.

We converted the active PAI-1 in plasma into latent/in active PAI-1 by thermal inactivation, and into complexes with plasminogen activators by incubation with at least a sixfold molar excess of u-PA or t-PA.

Recovery of PAI-1 Molecular Forms in the Immunoassay

All molecular forms of PAI-1 prepared in plasma were measured in the immunoassay in five dilutions. The dilutions were prepared before adding the 20 μL of plasma sample to 200 μL of sample diluent. Table 2 shows the recovery (relative to plasma with active PAI-1) of the undiluted plasmas containing the different molecular forms. The recovery range of the five dilutions is also shown. We conclude that all forms of PAI-1 are recognized with about the same efficiency. However, the dilution curves of the different PAI-1 forms show a modest degree of nonparallelism (Fig. 1).

Essentially the same results were obtained with purified PAI-1 forms (50 μg/L), showing a recovery (mean ± SD, n = 3) of 88% ± 2% for active PAI-1 and 92% ± 4% for the PAI-1–t-PA complex. In both cases the recovery was assessed relative to the protein content of the preparations and the PAI-1 standard of the commercial kit.

We considered the consequences of the observed nonparallelism of the dilution curves of the different PAI-1 forms. Because, we assumed, plasma could contain any mixture of the molecular forms, we calculated the mean absorbance of all different PAI-1 forms at a specific dilution. The curve of the mean absorbances is depicted in Fig. 2; the theoretical upper and lower 10% and 20% deviation of the mean absorbances is also shown. The highest and lowest absorbance values of the different PAI-1 forms at each dilution were also plotted; these values remained close together and deviated between 10% and 20% of the calculated mean. This analysis indicates that, with PAI-1 concentrations ranging over a fivefold dilution and with the possibility of all potential mixtures of molecular forms, the deviations in re-

| Table 1. Characteristics of the initial plasma and the t-PA-depleted plasma. |
|----------------------------------|---------------------|---------------------|
|                                  | Initial plasma      | t-PA-depleted plasma |
|                                  | Aₐ₄₀ (100-fold dilution) | 0.608               | 0.545               |
| t-PA Ag, μg/L                   | 11.2                | <0.03               |
| u-PA–PAI-1, μg/L                | NT                  | <0.1                |
| PAI-1 Ag, μg/L                  | 69.0                | 40.3                |
| PAI activity, × 10⁹ U/L         | 21.3                | 20.1                |

Ag, antigen; NT, not tested.
results are at most ~20%, which is minor, especially in relation to the biological variations of PAI-1.

In an approach using real plasma, we prepared plasma dilution curves from pooled plasma from healthy volunteers (n = 20) and from five individual plasmas of healthy volunteers (not shown). Good parallelism with the calibration curve of the assay was observed: The average recovery was 99% ± 12% compared with the undiluted plasma.

Correlations with Other Assays

In a set of 106 clinical plasma samples selected for studies of variation in plasma PAI-1 concentration due to diurnal fluctuation and related to increased insulin resistance, we compared the results of the new Innotest PAI-1 EIA with those of another commercially available EIA (TintElize PAI-1), which had previously shown the broadest specificity for this analyte (33). The correlation between methods was good (r = 0.913), with especially close agreement at the lower concentrations (Fig. 3). The measured values, which are expressed relative to the standard provided in each kit, appear to differ by a factor of 1.87. This is consistent with measurements of the standards of both commercial kits by both methods, which showed a relative value of 1.69.

The correlation is also quite good (r = 0.795) between the PAI-1 antigen value (Innotest) and the value for plasma PAI activity, measured by the method of Verheijen et al. (20).

In extracts of platelets, in which 95% of the PAI-1 is in the latent/inactive form, PAI-1 antigen concentration measured with the Innotest was 102 ± 33 ng per 10⁶ platelets in samples from nine individuals. The Monozyme EIA measured 211 ± 59 ng per 10⁶ platelets. The difference in the measured concentration of latent PAI-1 in platelet extracts is similar to the methods' proportional bias [232% for Monozyme (33) and 110% for Innotest] for latent PAI-1.

Discussion

The purpose of this study was to evaluate the specificity of a new commercially available assay of PAI-1 antigen. We had previously observed that the specificities of immune assays for PAI-1 vary and no method had yet been identified for measuring the various molecular forms of PAI-1 with equal efficiency (33). This limited the use of such methods in clinical studies and prevented the standardization of methods.

Because our interest was in applying the assay to blood samples, we selected plasma as the medium in which the different molecular forms of PAI-1 were to be studied. Compared with the use of purified components, this might be especially relevant, in view of the PAI-1–vitronectin complex in plasma (32). Our approach was to start with active PAI-1 in plasma and to convert this “known” and constant amount into the various other molecular forms of PAI-1 such as inactive PAI-1 and complexes with plasminogen activators. In a previous study (33), we used plasma as such; consequently, the already existing t-PA–PAI-1 and u-PA–PAI-1 com-
plexes were included in the analysis, appearing as background and reducing the sensitivity of the analysis. In this study, we started with pooled plasma that had been carefully separated from platelets (35); the increased PAI-1 antigen concentrations allowed us to make dilution curves of up to 16-fold dilutions. The plasma was further depleted of t-PA by immunoaffinity chromatography with immobilized antibodies to t-PA. Much (40%) of the antigenic response was lost by the depletion, indicating removal of 40% of PAI-1 in complex with t-PA. This finding concurs closely with other reports (27, 40). Analysis of the plasma with a sandwich assay for u-PA–PAI-1 complexes revealed an absence of this complex in our material, confirming the observation that this complex is usually present in very low quantities only (G. Dooijewaard, unpublished results). Platelet-poor plasma from healthy volunteers, properly prepared, also contains minimal amounts of latent PAI-1 (33). We conclude that the depletion of t-PA of normal citrated plasma results in plasma with mainly active PAI-1.

Analysis of the specificity of PAI-1 forms in plasma shows a close correlation with the results of purified active PAI-1 added to PAI-1-depleted plasma, and purified PAI-1–t-PA complex in buffer. This supports the validity of the improved procedure followed in this study, and suggests that vitronectin is not influencing the plasma results to any large extent.

The observed moderate nonparallelism of dilution curves of the various PAI-1 forms illustrates once more the problem of obtaining an immunoassay of PAI-1 that is equally reactive to all PAI-1 molecular forms. We evaluated the consequences of the observed nonparallelism for practical use of the assay, and concluded that estimation of PAI-1 would be, at the most, 20% inaccurate. In view of the much lesser variability in composition of plasma PAI-1, which probably is the case in clinical practice, and in view of the known broad interindividual variation in PAI-1 (0–80 μg/L) (13, 27, 41), we consider this acceptable for clinical practice.

Correlations of results obtained with the present method and those obtained with the TintElize assay and the activity assay of PAI (Fig. 3) were rather close, despite the less broad specificity of the TintElize assay (33) and the fact that the activity assay recorded only a specific fraction of PAI-1. This close correlation with the TintElize assay can be explained by the facts that the TintElize assay showed a reduced specificity for latent or inactive PAI-1, and this form is not usually present in plasma in significant amounts. We would expect Innotest and TintElize to show a clear divergence of results when analyses of serum, cell-conditioned media, and platelet extracts are also included, because these contain significant amounts of the latent PAI-1 form.

When considering the good correlation of antigen results with PAI activity, one should keep in mind the possibility of a biological association between t-PA and PAI-1 levels in blood, which might contribute to the correlation. Several reports indicate such a correlation between t-PA and PAI-1 in blood (40, 42, 43); consequently, the ratio of PAI-1 antigen and PAI activity shows only a moderate variability (42). We expect that in other pathological situations this t-PA/PAI-1 association might dissociate (44), leading to a much poorer correlation.

The significance of the specificity of PAI-1 antigen methods depends on their application. Methods for specific molecular fractions of PAI-1 or complexes of PAI-1, and a method to measure all PAI-1 (grand total), both have a place. In general, however, as discussed previously (33), methods with a so-called mixed specificity for various molecular forms are less desirable: With such methods, conversions from one molecular form to the other might be recorded and misinterpreted as changes in the molecular concentration of PAI-1. In fibrinolysis, such possibilities occur, e.g., during tests such as the venous occlusion test and in the performance of exercise or desamino-D-arginine vasopressin tests, where the t-PA in circulation is increased and an increase in the conversion of active PAI-1 to the t-PA–PAI-1 complex is likely. In such cases, a specific method is preferable when one wants to monitor the concentrations of t-PA–PAI-1 complexes. When the molar concentration of PAI-1 is the focus of interest and a decision is required as to whether or not PAI-1 is increased, then a method that is not sensitive to the conversion of PAI-1 forms is preferable. Similar considerations apply to thrombolytic treatments involving plasminogen activators, in which the question is whether PAI-1 concentrations are changing and, if so, how.

In conclusion, in addition to specific assays for specific molecular forms of PAI-1, it is also desirable to have a broad-specificity, or grand total, assay that can determine the total molar concentration of PAI-1 in body fluids. Such a method can be used to compare plasma and serum results and different pathological states; it is also desirable in terms of the standardization of methods, because it allows the comparison of various standards. The assay we evaluated here approaches the closest to a grand total assay among commercially available assays of PAI-1.

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