Kinetic analysis of the interaction between plasminogen activator inhibitor-1 and tissue-type plasminogen activator

Catherine MASSON and Eduardo ANGLES-CANO*

I.N.S.E.R.M. U. 143, Institut de Pathologie Cellulaire, Hôpital de Bicêtre, 94275 Bicêtre Cedex, France

The kinetics of inhibition of tissue-type plasminogen activator (t-PA) by the fast-acting plasminogen activator inhibitor-1 (PAI-1) was investigated in homogeneous (plasma) and heterogeneous (solid-phase fibrin) systems by using radioisotopic and spectrophotometric analysis. It is demonstrated that fibrin-bound t-PA is protected from inhibition by PAI-1, whereas t-PA in soluble phase is rapidly inhibited ($K_i = 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) even in the presence of $2 \mu\text{M}$ -plasminogen. The inhibitor interferes with the binding of t-PA to fibrin in a competitive manner. As a consequence the K_d of t-PA for fibrin ($1.2 \pm 0.4 \text{ nM}$) increases and the maximal velocity of plasminogen activation by fibrin-bound t-PA is not modified. From the plot of the apparent K_d versus the concentration of PAI-1 a K_i value of $1.3 \pm 0.3 \text{ nM}$ was calculated. The quasi-similar values for the dissociation constants between fibrin and t-PA (K_d) and between PAI-1 and t-PA (K_i), as well as the competitive type of inhibition observed, indicate that the fibrinolytic activity of human plasma may be the result of an equilibrium distribution of t-PA between both the amount of fibrin generated and the concentration of circulating inhibitor.

INTRODUCTION

The endothelial tissue-type plasminogen activator (t-PA, EC 3.4.21.–) present in plasma is the serine proteinase responsible for physiological fibrinolysis (Astrup, 1956; Müllertz, 1987). The activity of this activator is highly stimulated by fibrin (Thorsen *et al.*, 1972) and specifically inhibited by PA inhibitors (Sprengers & Kluft, 1987). The fast-acting inhibitor of endothelial-cell origin (Dosne *et al.*, 1978; Loskutoff *et al.*, 1983) present in plasma (Thorsen & Philips, 1984; Kruithof *et al.*, 1984; Wiman *et al.*, 1984; Verheijen *et al.*, 1984), PAI-1, reacts with both single-chain t-PA (sc-t-PA) and two-chain t-PA (tc-t-PA) and with the two-chain urinary-type plasminogen activator (tc-u-PA, EC 3.4.21.31) with a second-order rate constant of above $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. PAI-1 forms SDS-stable complexes with these activators (Levin, 1983; Philips *et al.*, 1984). The inhibitor of placental origin (Kawano *et al.*, 1968; Astedt *et al.*, 1985), PAI-2, also found in plasma during pregnancy (Lecander *et al.*, 1984), can inhibit tc-t-PA and tc-u-PA with a second-order rate constant of $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. A recently described PA inhibitor of urinary origin (Stump *et al.*, 1986), PAI-3, is immunologically identical (Heeb *et al.*, 1987) with the inhibitor of activated protein C (Suzuki *et al.*, 1983) and reacts specifically with tc-u-PA with a second-order constant of above $8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. Non-specific inhibition of plasminogen activators by other serine proteinase inhibitors (α_2 -antiplasmin, α_2 -macroglobulin) has been described in purified systems (Korninger & Collen, 1981; Thorsen & Philips, 1984) but probably plays no role in a plasma milieu. Therefore, because of its plasma concentration (about 1.3 nM) and its second-order rate constant for the inhibition of t-PA, it is generally accepted that in

physiological conditions only PAI-1 (and PAI-2 during pregnancy) could play a significant role in fibrinolysis. Indeed, most of the t-PA in plasma circulates complexed to PAI-1 (Thorsen & Philips, 1984; Booth *et al.*, 1985), and a correlation has been reported between high concentrations of PAI-1 and thromboembolic diseases (Juhan-Vague *et al.*, 1987). However, its relevance in the regulation of physiological fibrinolysis is still a matter of debate (Sprengers & Kluft, 1987). Liquid-phase methods based on the measurement of residual t-PA activity have been developed (Verheijen *et al.*, 1984; Wiman *et al.*, 1984) to study this problem. Although useful, they are subject to several drawbacks: the use of fibrin substitutes instead of fibrin, the presence of sc-u-PA and α_2 -antiplasmin in plasma, and the particular conditions inherent to this type of assays, e.g. dilution or acidification of plasma, which can modify the equilibrium of t-PA–PAI-1 complexes. The aim of the present work was to investigate the interaction between PAI-1 and t-PA under conditions where the experimental complications mentioned above can be avoided. Our method employs a solid-phase fibrin network close to the physiological model and a spectrophotometric system that allows the study of t-PA-inhibition kinetics either in a purified system or in a plasma milieu.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals used were reagent grade and were obtained from either Prolabo (Paris, France) or Aldrich (Beerse, Belgium). Other products were obtained as indicated: EDTA, bovine serum albumin, Triton X-100 and Tween 20 were from Serva (Heidelberg, Germany);

Abbreviations used: Pg, plasminogen; Fn, fibrin; PA, plasminogen activator; t-PA, tissue-type PA; sc-t-PA, single-chain t-PA; tc-t-PA, two-chain t-PA; u-PA, urinary-type PA; sc-u-PA, single-chain u-PA; PAI-1, PA inhibitor-1. The last seven are in accordance with the recommendations of the Subcommittee on Fibrinolysis of the International Society of Hemostasis and Thrombosis (1986).

* To whom correspondence should be addressed.

the bovine serum albumin used was proven to be free of plasmin and t-PA enzymic and inhibitory activities; glutaraldehyde in aqueous 25 % (v/v) solution was from TAAB Laboratories (Reading, Berks., U.K.); poly(vinyl chloride) U-shaped micro-titration plates and plate sealers were from Dynatech France (Marnes-La-Coquette, France); Ultrogel AcA 44 and DEAE-Trisacryl were from IBF (Villeneuve-la-Garenne, France); lysine-Sepharose 4B and gelatin-Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden); chromogenic substrates D-norleucylcyclohexylalanylarginine *p*-nitroanilide (CBS 3308) and methylmalonylhydroxypropylarginine *p*-nitroanilide (CBS 1065) were from Diagnostica Stago (Asnières, France); aprotinin (Trasylol) was from Bayer (Leverkusen, Germany); bovine thrombin was from Hoffmann-LaRoche (Basel, Switzerland); low- M_r markers were from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Plasma and PAI-depleted plasma

Human blood was collected from healthy volunteers (students and staff members, ages 20 to 40 years). Samples were drawn into a polypropylene tube containing 0.013 M-sodium citrate final concentration, kept on ice, and centrifuged immediately at 2000 *g* for 15 min at 4 °C to obtain platelet-poor plasma. A pool of plasma samples was prepared and a fraction was made deficient in PAI-1 by immunoabsorption of t-PA-PAI-1 complexes on an anti-t-PA monoclonal antibody-Sepharose 4 B column after addition of an excess of melanoma t-PA (200 i.u./ml, final concentration) to plasminogen-depleted plasma. Plasma thus prepared had a normal concentration of α_2 -antiplasmin (Teger-Nilsson *et al.*, 1977). The basal concentration of free t-PA in the pooled plasma was tested as indicated elsewhere (Angles-Cano, 1986). The content of PA inhibitor activity (20 units/ml) and its depletion from plasma were determined as described below under 'Assay procedures for the inhibition of t-PA'.

Purified proteins

Glu¹-plasminogen was purified from di-isopropyl phosphorofluoridate-treated fresh-frozen human plasma by affinity chromatography on lysine-Sepharose 4B (Deutsch & Mertz, 1970), ion-exchange chromatography on DEAE-Trisacryl and gel filtration on Ultrogel AcA 44 in the presence of Trasylol. The concentration of Glu¹-plasminogen was determined spectrophotometrically by using $A_{1\text{ cm}, 280}^{1\%} = 16.8$ (Wallén & Wiman, 1972), and 20 μM stock solutions in buffer A were stored at -80 °C. Fibrinogen was purified from human plasma by glycine precipitation at room temperature as previously described (Kazal *et al.*, 1963), and chromatographed successively on lysine-Sepharose 4B (Deutsch & Mertz, 1970) and gelatin-Sepharose 4B (Engvall & Ruoslahti, 1977) to remove plasminogen and fibronectin respectively. Fibrinogen concentration was determined spectrophotometrically by using $A_{1\text{ cm}, 280}^{1\%} = 15.1$ (Blombäck & Blombäck, 1956), and fractions containing 12 mg/ml in buffer A were stored at -80 °C. Tissue plasminogen activator (> 95 % single-chain t-PA) was purified from the conditioned medium of human Bowes-melanoma-cell cultures as previously described (Rijken & Collen, 1981) and was kindly given by Dr. H. R. Lijnen (Catholic University of Leuven, Leuven, Belgium) and Dr. M. Rånby (Biopool, Umeå, Sweden). The Inter-

national Standard for human t-PA, preparation coded 83/517, was kindly supplied by the National Institute for Biological Standards and Control, London, U.K.

Endothelial-cell cultures

The human vascular endothelial cells employed for the purification of the inhibitor were isolated from umbilical cord veins (Jaffe *et al.*, 1973) and cultured in minimal essential medium type alpha containing 15 % (v/v) foetal-calf serum (Rehatuin; Armour Biochemical Co., Kankakee, IL, U.S.A.), 20 μg of endothelial-cell growth factor (Collaborative Research, Lexington, MA, U.S.A.)/ml and 90 μg of heparin (Organon, Teknika, Fresnes, France)/ml as described previously (Thornton *et al.*, 1983). The culture medium thus prepared was determined to be free of PAI-1 inhibitory activity. Conditioned medium was collected at confluency, pooled, centrifuged at 2000 *g* for 5 min to remove cell debris, determined to be rich in PAI-1 by reverse fibrin autography (Loskutoff *et al.*, 1983) and stored at -70 °C until further use. Endothelial-cell cultures were obtained through the kind collaboration of Dr. L. Coulombel (Hôpital de Bicêtre).

Partial purification of PAI-1

The semi-purified inhibitor was obtained from conditioned medium by two $(\text{NH}_4)_2\text{SO}_4$ fractionations and DEAE-Trisacryl chromatography, as follows. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to 70 % saturation and stirred twice for 30 min, first at 22 °C and then at 4 °C. After centrifugation at 10000 *g* for 30 min at 4 °C the precipitate was resuspended in and dialysed against the equilibrium buffer of the DEAE-Trisacryl column. The second $(\text{NH}_4)_2\text{SO}_4$ fractionation was done at 50 % saturation under the same conditions. The final solution was passed through a DEAE-Trisacryl column (2.5 cm $\times$ 30 cm) pre-equilibrated with 0.025 M-sodium phosphate buffer, pH 6.5, containing 0.035 M-NaCl. Proteins in the flow-through were discarded and the retained PAI-1 was eluted by using a linear gradient of 0.035–0.5 M-NaCl in the same buffer. Fractions containing the t-PA-inhibitory activity (detected as indicated below) were pooled, concentrated in a dialysis bag against poly(ethylene glycol) (M_r 20000), dialysed against sodium phosphate buffer and stored at -70 °C until used. All procedures were performed at 4 °C.

Buffers

Buffer A was 0.05 M-sodium phosphate buffer, pH 7.4, containing 0.08 M-NaCl. Buffer B was 0.05 M-sodium phosphate buffer, pH 6.8, containing 0.08 M-NaCl. Assay buffer was buffer A containing 2 mg of bovine serum albumin/ml and 0.05 % Tween 20. Binding buffer was buffer B containing 4 mg of bovine serum albumin/ml, 0.01 % Tween 20 and 2 mM-EDTA. All other buffers were prepared as described in the text.

Preparation of solid-phase fibrin supports

The solid-phase fibrin supports for the specific assessment of t-PA activity were prepared as described previously (Angles-Cano, 1986). Briefly, poly(vinyl chloride)-bound stable polyglutaraldehyde derivatives were first produced by treating poly(vinyl chloride) plates with 2.5 % (v/v) glutaraldehyde in 0.1 M-sodium bicarbonate buffer, pH 9.5, for 2 h at 22 °C. Then fibrinogen (0.3 μM in 0.1 M-sodium phosphate buffer,

pH 7.4, containing 1 mM- CaCl_2) was covalently bound for 18 h at 4 °C. The excess fibrinogen was discarded, the plate was washed with assay buffer and a fibrin network was generated by thrombin treatment (1 N.I.H. unit/ml in assay buffer containing 1 mM- CaCl_2). The excess thrombin was discarded and fibrin-bound thrombin was eluted by three washes with a high-ionic-strength solution (0.5 M- NaCl /8 mM- CaCl_2 /0.05% Tween 20). A final wash with 5 mM-sodium phosphate buffer, pH 6.8, containing 0.05% Tween 20 was done, and 100 μl of buffer B containing 0.02 M-L-lysine, 0.2% bovine serum albumin and 0.01% NaN_3 was added. The plate was sealed and stored in this state at 4 °C until further use.

Assay procedures for the inhibition of t-PA

The t-PA-inhibitory capacity of plasma and purified PAI-1 was investigated by using two different inhibition approaches and was quantified by measuring the amount of residual fibrin-bound t-PA, as follows.

Inhibition of fibrin-bound t-PA. Solutions of melanoma t-PA containing 5 and 10 i.u./ml were prepared in binding buffer, and amounts of 100 μl per well were incubated in a solid-phase fibrin plate for 1 h at 37 °C. The wells were then washed with binding buffer, and amounts per well of 100 μl of binding buffer, plasma or PAI-depleted plasma, supplemented with 0–40 units of purified PAI-1/ml, were incubated at 37 °C on the fibrin-bound t-PA for 0–120 min. Unbound proteins were eliminated by extensive washing with binding buffer, and bound t-PA was determined by using the spectrophotometric method described below.

Inhibition of t-PA in solution. Solutions of binding buffer containing 100–10000 units of melanoma t-PA/ml were diluted (1:10, v/v) with binding buffer, plasma or PAI-depleted plasma supplemented with 0–100 units of purified PAI-1/ml. After 15 min incubation at room temperature 100 μl of each mixture per well was incubated in a solid-phase fibrin plate for 1 h at 37 °C. The plate was then extensively washed with binding buffer, and the residual t-PA activity was determined spectrophotometrically as described below.

Determination of the activity of fibrin-bound t-PA

The amount of t-PA quenched by the inhibitor was indirectly determined by measuring the activity of residual t-PA bound to fibrin as previously described (Angles-Cano, 1986). Briefly, amounts of 100 μl of assay buffer containing 0.2 μM -Glu^t-plasminogen and 0.6 mM-CBS 3308 were added per well. The plate was sealed and absorbance readings at 405 and 490 nm were performed in a microplate counter (MR 610; Dynatech) equipped with a thermostatic device to maintain constant 37 °C. Complete automation was obtained by coupling the MR 610 apparatus to an Apple IIe microcomputer through an RS232 serial interface by using a computer program developed in our laboratory (Cinenzymic-log INSERM). Initial velocities of each reaction were calculated as indicated below. The residual t-PA activity was determined by reference to a standard curve made in binding buffer or PAI-depleted plasma and obtained by plotting $\Delta A_{405}/\text{min}$ versus the concentration of t-PA. The amount of t-PA inhibited was considered to be equivalent to the PAI-1 activity content of the sample tested. It was calculated by subtraction of the residual t-PA activity

from the t-PA concentration originally present in the mixture. Under these conditions 1 unit of PAI-1 was defined by the inhibition of 1 i.u. of t-PA.

Data analysis

Initial rates for each kinetic were calculated by using an appropriate statistical analysis of data in its original co-ordinate system, i.e. ΔA_{405} versus t . Since the progress curve thus obtained is described by a parabolic relationship (Kosow, 1975; Christensen & Müllertz, 1977; Petersen *et al.*, 1985) and the acceleration of the reaction is proportional to the activation of plasminogen, the initial rates were calculated by determining the equation of second degree for linear motion with constant acceleration, $x = \frac{1}{2}at^2 + bt + c$, as follows. The progress curve was represented by 50 experimental measurements evenly distributed in the initial parabolic period of the trace, where the acceleration of product formation is constant. To obtain the slope that corresponds better to the experimental points, the data were fitted to five polynomials (orders 1 and 3 to 6) by using non-linear least-squares analysis. The variance of each fit with respect to the experimental points minimized by the degree of the polynomial was calculated and the one with the least estimated standard deviation was taken to express the initial velocity from the slope of its maximal tangent. This was proportional to the concentration of $[\text{t-PA}]_{\text{FN}}$ in the interval corresponding to the initial phase of the fibrin binding curve. This approach was used to avoid errors inherent to a graphical transformation of the parabolic curve into straight lines. It requires computer treatment, which is included in a program of the package used for the monitoring of the assay and the collection of the data.

Plasminogen-t-PA-PAI-1 interaction in the absence of fibrin

Reactions were performed in a fibrin-free system with poly(vinyl chloride) U-shaped micro-titration plates and a plasmin substrate (CBS 1065) with a high catalytic-centre activity. Mixtures containing (final concentrations) purified t-PA (10 i.u./ml) and PAI-1 (2.5–40 units/ml) were equilibrated at room temperature for 15 min or were immediately mixed with plasminogen (50 nM–2 μM). The rates of activation in the presence and in the absence of PAI-1 were determined with 1.5 mM-CBS 1065 in a final volume of 50 μl . Absorbance readings and initial velocities were determined in triplicate as indicated before.

Formation of complex between semi-purified PAI-1 with t-PA

To monitor the formation of complexes, radiolabelled t-PA (3 $\mu\text{Ci}/\text{ml}$) and PAI-1 (0–320 units/ml) were mixed (1:1, v/v) and incubated for 15 min at 22 °C. The mixtures were then diluted (1:1, v/v) with sample buffer containing 0.125 M-Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 0.02% Bromophenol Blue. After SDS/polyacrylamide-gel electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 (2.2 g/l) in acetic acid/methanol/water (1:4:4, by vol.) and dried on a slab-gel-drying apparatus. Autoradiography was done for 18 h on Kodak XS-1 film, with a Kodak X-Omatic intensifying screen. The mobilities of t-PA or t-PA-PAI-1 complexes were compared with those of M_r -marker proteins.

Miscellaneous

The human melanoma t-PA was used as the internal standard. Its activity was expressed in i.u. by reference to the First International Standard for t-PA (preparation 83/517). The basal concentration of free t-PA in the pooled plasma was tested as indicated above and was less than 0.01 i.u./ml. Protein was determined by using the method of Bradford (1976). Melanoma t-PA (Biopool) was radioiodinated by the Iodogen method (Fraker & Speck, 1978) with a labelling time of 4 min at 4 °C; the specific radioactivity obtained was 40 nCi/ng and the specific activity was 0.7 i.u./ng.

RESULTS AND DISCUSSION

Characteristics of the PAI-1-enriched fraction

A semi-purified fraction with a high content of PAI activity (3000 units/ml) was obtained from endothelial-cell culture supernatants. Although several protein bands were observed by SDS/polyacrylamide-gel electrophoresis, a main band in the 54000- M_r zone was present and identified as PA inhibitor by reverse fibrin autography (Loskutoff *et al.*, 1983). The semi-purified product was immunologically related to PAI-1 by using specific monoclonal antibodies and was able to form a complex of M_r 110000 with sc-t-PA. The PAI-1 activity of this preparation was decreased by prolonged incubation at 37 °C but was not modified by treatment with denaturants (results not shown). The PAI-1-enriched fraction contained 5 mg of protein/ml, and from this a specific activity of 600 units/mg was calculated. A 40-fold purification was thus obtained by reference to the initial source.

Inhibition of fibrin-bound t-PA

No significant inhibition of fibrin-bound t-PA by its fast-acting inhibitor present in plasma or in the semi-purified source could be demonstrated. As shown in Fig. 1, the results obtained with different t-PA/fibrin molar ratios and different incubation times were similar for both plasma and buffer. In the semi-purified system prolonged incubation periods (over 60 min) or a very low t-PA/PAI-1 ratio were necessary to observe a decrease in t-PA activity. This was correlated with a similar decrease in the amount of bound radiolabelled t-PA (results not shown). The possible interaction of other plasma serine proteinase inhibitors, such as α_2 -antiplasmin and α_2 -macroglobulin present in high concentration in plasma, can be eliminated since they are not fixed by the solid-phase fibrin surface, as demonstrated with immunological methods (Angles-Cano *et al.*, 1987). The slight decrease in bound t-PA suggests that small amounts of t-PA are desorbed from the fibrin surface to attain a new equilibrium binding. A similar phenomenon was observed with semi-purified PAI-1, but in that case the amount of t-PA desorbed is simultaneously quenched by PAI-1 and does not any more participate in the equilibrium. As a result, a slightly lower fibrin-bound t-PA activity was obtained.

Inhibition of t-PA in solution

In contrast with the solid-phase t-PA-inhibition experiments, t-PA in solution was efficiently quenched by PAI-1 present in plasma or in the semi-purified source. The curves shown in Fig. 2(a) represent the binding isotherm

Fig. 1. Interaction of PAI-1 with fibrin-bound t-PA

Solutions containing (a) 10 or (b) 5 i.u. of t-PA/ml were incubated with solid-phase fibrin. Excess t-PA was discarded and fibrin-bound t-PA was incubated with plasma (■) or binding buffer containing 0 (□), 20 (◆) and 40 (▲) units of PAI-1/ml for the indicated times. After washing, residual t-PA activity was measured by addition of 200 nM-Pg and 0.6 mM-CBS 3308 in assay buffer. The initial velocity of residual t-PA is given in $\Delta A_{405}/\text{min}$.

of t-PA to fibrin in the absence and in the presence of PAI-1. They were obtained by plotting the initial velocity of residual fibrin-bound t-PA versus the initial t-PA concentration input. The t-PA-binding curve obtained with buffer is identical with the one obtained with PAI-depleted plasma. This indicates that the main factor able to modify the binding of t-PA to fibrin is the presence of PAI-1 through the formation of t-PA-PAI-1 complexes, which were identified by SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 3). As a consequence, in the presence of PAI-1, e.g. normal plasma, the binding curve is shifted to the right, producing three different zones (Straus & Goldstein, 1943) of t-PA activity, which correspond to the presence of free and/or complexed t-PA as determined by SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 2b). These experimentally observed zones can be represented by the inhibition equation governing the t-PA-PAI-1 interaction under equilibrium conditions (see below). Zone A corresponds to the quenching of small amounts of t-PA by a relative excess of PAI-1. Therefore the amount of free t-PA can be neglected and no or very low residual t-PA activity can be detected. In zone B three forms of t-PA can be distinguished: free, complexed to PAI-1 and bound to fibrin. The last is the molecular form detected by our assay. This zone corresponds to a region around the half-saturation boundary of the binding curve. In zone C the amount of t-PA exceeds PAI-1 and upon saturation of fibrin the binding asymptote approaches the one obtained in the absence of PAI-1.

Determination of the dissociation constant (K_d) of the t-PA-Fn interaction in the presence of PAI-1

Under the conditions described, the semi-logarithmic transformation of the binding isotherm (Fig. 2a inset) provided a sigmoidal curve from which an apparent dissociation constant ($K_d = 1.2 \pm 0.4$ nM) for the fibrin-t-PA interaction was estimated by determining the abscissa of the point where the derivative is maximal

Fig. 2. Interaction of PAI-1 with t-PA in soluble phase

(a) Isotherms of the binding of t-PA to fibrin in the presence and in the absence of PAI-1. Solutions containing various concentrations of t-PA were equilibrated for 15 min at 22 °C in binding buffer (□), PAI-depleted plasma (◆) or plasma (■) containing 20 units of PAI-1/ml. Then, 100 μl of each solution/well was incubated for 60 min at 37 °C in a solid-phase fibrin plate. After washing, fibrin-bound t-PA was measured by addition of 200 nM-Pg and 0.6 mM-CBS 3308. Initial velocities are given in $\Delta A_{405}/\text{min}$ and were calculated as described in the Materials and methods section. A, B and C represent the zones of behaviour of the enzyme-inhibitor system, t-PA-PAI-1, described in the text. The inset represents the semi-logarithmic replot of data; the arrows indicate the inflexion points of the curve in the absence of PAI-1 (K_d) and in its presence (K'_d). (b) Autoradiograph of a polyacrylamide gel from a similar experiment with ^{125}I -labelled t-PA. Solutions containing various concentrations of radiolabelled t-PA (6.25–300 i.u./ml) were equilibrated (1:1, v/v) for 15 min at 22 °C in binding buffer containing 50 units of PAI-1/ml. A sample of each mixture was electrophoresed in a 10% polyacrylamide slab gel, and free and complexed ^{125}I -labelled t-PA were detected by autoradiography. A, B and C correspond to the zones of enzyme behaviour indicated in (a); c represents the migration of t-PA (50 i.u./ml) in the absence of PAI-1. In zone A most of the t-PA has been complexed. Zone B represents the zone of equilibrium. In zone C all the PAI-1 is complexed and an excess of free t-PA is observed. The M_r values of protein standards are shown at the left of the autoradiograph. The concentrations of t-PA used are indicated at the bottom.

(inflexion point). The presence of a constant amount of PAI-1 induces the formation of t-PA-PAI-1 complexes and a concomitant decrease in the amount of t-PA able to bind to fibrin. Thus in zone B the abscissa of the inflexion point of the binding isotherm (K_d) is displaced to the right (K'_d) and under these conditions the difference in value between K'_d and K_d gives the amount of active PAI-1 in the tested plasma. Therefore, in the absence of any other factor interfering with the binding of t-PA to fibrin, the degree of this displacement is a measure of the content of active PAI-1 in a given plasma. Twelve different plasmas obtained from healthy volunteers were tested for their PAI-1 activity following this principle. We obtained a mean value of 26 ± 5.1 units/ml.

Kinetics of inhibition

The activation of Pg by Fn-bound t-PA can be schematically represented as follows:

In this scheme, both Fn and Pg interact with t-PA, making the activation process analogous to a two-substrate enzyme system. It has been shown (Hoylaerts *et al.*, 1982) that the activation follows a sequential mechanism. The formation of a Fn-t-PA complex:

is the preliminary step that induces a modification of the quaternary structure of t-PA and enhances the binding and cleavage of native Pg:

The affinity of the t-PA-Fn interaction (eqn. 2) is defined by:

$$K_d = \frac{[\text{t-PA}] \cdot [\text{Fn}]}{[\text{t-PA}]_{\text{Fn}}} \quad (4)$$

and the velocity (v) of Pg breakdown by:

$$v = k_{+2} \cdot [\text{t-PA}]_0 \quad (5)$$

If both substrates are present in excess the reaction will follow zero-order kinetics and virtually all the t-PA will form a ternary complex with Fn and Pg, so:

$$V = k_{+2} \cdot [\text{t-PA}]_0 \quad (6)$$

In the presence of t-PA inhibitor, e.g. PAI-1, a fraction of $[\text{t-PA}]_0$ will combine with it to form inactive complexes:

where the inhibition constant is given by:

$$K_i = \frac{[\text{t-PA}] \cdot [\text{PAI-1}]}{[\text{t-PA-PAI-1}]} \quad (8)$$

Fig. 3. Autoradiographic analysis of the formation of t-PA-PAI-1 complexes

A constant amount of ^{125}I -radiolabelled t-PA was incubated with binding buffer containing 0–160 units of PAI-1/ml (final concentration), for 15 min at 22 °C. A sample of the mixture was electrophoresed on a 10% polyacrylamide slab gel, and the complexes were detected by autoradiography. The M_r values of protein standards are shown at the left. The concentrations of PAI-1 used are indicated at the bottom.

The formation of such complexes will modify the initial rate, by interfering with the activity of t-PA at the level where each substrate participates in the reaction sequence. Since in the absence of Fn the K_m of Pg for t-PA (62 μM) is at least 30-fold higher than its plasma concentration (approx. 2 μM) the rate of plasminogen activation will be very low even in the absence of PAI-1. This is indeed the case, as indicated in Fig. 4. As shown, high concentrations of Pg were unable to modify the t-PA-PAI-1 interaction. Therefore PAI-1 cannot be considered to be an inhibitor of t-PA at the Pg-t-PA interaction level in the absence of Fn. However, if we consider that Fn behaves in a substrate-like manner in the reaction sequence of activation, then the possibility that PAI-1 could be a modulator of the t-PA-Fn interaction should be considered. In such a case, the residual t-PA, i.e. $[\text{t-PA}]_R$ in eqn. (7), will be a function of the inhibition constant (eqn. 8) and of the affinity of t-PA for Fn (eqn. 4), and can be represented by:

$$[\text{t-PA}]_R = [\text{t-PA}]_0 - [\text{t-PA-PAI-1}] \quad (9)$$

In the heterogeneous model described here, $[\text{t-PA}]_R$ behaves as $[\text{t-PA}]_0$ vis-à-vis Fn and Pg (eqns. 2 and 3), leading to the formation of active $[\text{t-PA}]_{\text{Fn}}^{\text{Pg}}$ complexes.

Since the v values obtained are lower than those of $[\text{t-PA}]_0$, an apparent increase in the K_d or K'_d is observed. The fractional inhibition, i , of this process is represented by:

$$i = \frac{[\text{t-PA-PAI-1}]}{[\text{t-PA}]_0} \quad (10)$$

and the fractional activity, α , which is the relation between v in the presence of inhibitor and V in its absence, by:

$$\alpha = \frac{[\text{t-PA}]_R}{[\text{t-PA}]_0} = \frac{v}{V} \quad (11)$$

By substitution from eqns. (5), (6) and (9):

$$\frac{v}{V} = \frac{k_{+2} \cdot [\text{t-PA}]_0 - [\text{t-PA-PAI-1}]}{k_{+2} \cdot [\text{t-PA}]_0} = 1 - \frac{[\text{t-PA-PAI-1}]}{[\text{t-PA}]_0} \quad (12)$$

and combining with eqns. (10) and (11):

$$\alpha = 1 - i \quad \text{and} \quad i = 1 - \frac{v}{V} \quad (13)$$

The fractional inhibition can thus be measured experimentally by determining the observed velocity in the presence of PAI-1 (v), i.e. $[\text{t-PA}]_R$, and the maximal velocity (V) in its absence, i.e. $[\text{t-PA}]_0$.

From the analysis of the binding isotherms of t-PA in the presence and in the absence of PAI-1 (Fig. 2a) it is clear that the best zone for the calculation of the fractional inhibition is zone B. This zone corresponds to a state of equilibrium determined by the affinity constant of both the t-PA-Fn (eqn. 4) and the t-PA-PAI-1 (eqn. 8) interactions. This is in agreement with our previously published data and with results shown in Fig. 5, indicating that PAI-1 behaves as a reversible and competitive inhibitor of t-PA. Thus both equilibria (eqns. 2 and 7) should be taken into account, and as a consequence the absolute concentration of t-PA, $[\text{t-PA}]_0$ in zone B is distributed in three forms:

$$[\text{t-PA}]_0 = [\text{t-PA}]_{\text{Fn}} + [\text{t-PA-PAI-1}] + [\text{t-PA}]_f \quad (14)$$

Since $[\text{t-PA}]_f$, i.e. t-PA not bound either to Fn or to PAI-1, can be neglected, the fractional inhibition can be defined by the fractional activity of $[\text{t-PA}]_{\text{Fn}}$, as indicated by eqns. (12) and (13). Then, from eqns. (9) and (12)–(14):

$$[\text{t-PA-PAI-1}] = [\text{t-PA}]_0 - [\text{t-PA}]_{\text{Fn}} \quad (15)$$

which is the transformed form of eqn. (13), and from eqn. (7):

$$[\text{PAI-1}]_0 = ([\text{t-PA}]_0 - [\text{t-PA}]_{\text{Fn}}) + [\text{PAI-1}]_f \quad (16)$$

Finally, since the equilibrium of the $[\text{t-PA}]_0$ -Fn interaction is defined by the K_d , and the equilibrium observed in zone B corresponds to an apparent K_d or K'_d , then:

$$[\text{PAI-1}]_0 = (K_d - K'_d) + [\text{PAI-1}]_f \quad (17)$$

Fig. 4. Interaction of plasminogen with the inhibition of t-PA by PAI-1 in the absence of fibrin

Mixtures containing 10 i.u. of purified t-PA/ml and 40 units of PAI-1/ml (final concentrations) were incubated at room temperature for 15 min (◆) or were immediately (■) mixed with 50 nM–2 μM -plasminogen (final concentrations). The rate of activation in the presence (■ and ◆) or in the absence (□) of PAI-1 was determined with 1.5 mM-CBS 1065. Initial velocities were determined as described in the Materials and methods section.

Fig. 5. Effect of PAI-1 on the binding of t-PA: determination of the dissociation constant (K_d) of the t-PA–PAI-1 interaction

Double-reciprocal plots of initial velocity versus t-PA concentration were obtained by measuring fibrin-bound t-PA by using the activity assay described in the Materials and methods section. The PAI-1 concentrations were 0 ($\square$), 25 ($\blacklozenge$), 50 ($\blacksquare$), 75 ($\blacktriangle$) and 100 ($\triangle$) units/ml. The inset represents the replot of the slopes versus PAI-1 concentrations. The value of K_i was calculated at $-1/[\text{PAI-1}]$ from the equation of the curve.

Determination of the inhibition constant (K_i) of PAI-1 for t-PA

The plot of initial velocities (v) versus t-PA concentrations (Fig. 2a), represented by an hyperbola, can be conveniently transformed into a straight line by using a double-reciprocal plot of data (Fig. 5). The intercept on the abscissa axis corresponds to $-1/K_d$ or $-1/K'_d$ if PAI-1 is present in the reaction mixture. For different concentrations of PAI-1 present in plasma or in buffer the curves of the double-reciprocal plot intercept at the same value of y , indicating an identical V and thus a competitive type of inhibition by PAI-1. Since the K'_d obtained are a linear function of the inhibitor concentration, a plot of K'_d versus PAI-1 concentrations (Fig. 5 inset) provides the t-PA–PAI-1 dissociation constant, K_i , at $-1/K_i$. This value of K_i was obtained in units/ml, but, as a stoichiometric association is assumed between t-PA and PAI-1, the molarity can be obtained by using the specific activity of t-PA and the M_r of the inhibitor. Thus a value of 1.3 ± 0.3 nM was determined for both plasma and semi-purified PAI-1.

CONCLUSION

We report here the results of a study on the inhibition of t-PA by its fast-acting inhibitor, PAI-1, present in plasma and secreted by endothelial cells. Whereas in other studies a method in liquid phase with fibrin substitutes or CNBr-cleavage fragments of fibrinogen has been employed to measure residual t-PA activity, our approach was to study t-PA inhibition under conditions as close as possible to the physiological situation, i.e. with fibrin in solid phase. An interesting advantage of this approach is that our method physically removes the many variable components of biological samples that may influence activator activity. Thus both the inhibition of t-PA by PAI-1 and the activation of plasminogen by residual t-PA can be conveniently analysed. The double-reciprocal plot (Fig. 5) of the inhibition data revealed

that PAI-1 behaves as a competitive inhibitor of the binding of t-PA to fibrin. Since the inhibition constant (K_i) of PAI-1 for t-PA is of the same order of magnitude (1.3 ± 0.3 nM) as the dissociation constant of the t-PA–Fn interaction (1.2 ± 0.4 nM), the formation of t-PA–PAI-1 complexes resulting in impaired fibrinolysis or of Fn–t-PA complexes leading to the lysis of clots will be a function of the relative concentrations of PAI-1 and fibrin. Thus, in an early stage, t-PA–PAI-1 complexes will be able to dissociate in the presence of a thrombus equivalent to a massive amount of fibrin. That this is probably the case is strongly supported by our experiments showing that PAI-1 is an inefficient inhibitor of fibrin-bound t-PA in spite of the active site being exposed. In other words, no binding of PAI-1 to the Fn–t-PA complex and therefore no modification of the kinetics of the activation were observed. This is probably due to steric hindrance of the PAI-1-binding site and suggests that it involves a sequence in or close to the fibrin-binding domain of t-PA. An analogous protective effect has been described for the inhibition of fibrin-bound plasmin by α_2 -antiplasmin (Wiman & Collen, 1978). In parallel experiments we have shown (Fig. 4) that plasminogen at plasma concentration does not modify the equilibrium of the t-PA–PAI-1 interaction in the absence of fibrin, i.e. plasminogen does not displace the inhibitor from the t-PA–PAI-1 complexes. Moreover, since the activation of plasminogen by t-PA in the absence of fibrin is an unproductive process, we cannot consider PAI-1 to be an inhibitor of the activation at this level. The interference of PAI-1 with the activation of plasminogen will be therefore localized at the fibrin surface by interfering with the binding of t-PA.

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