Tissue-Type Plasminogen Activator Increases the Binding of Glu-Plasminogen to Clots

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Abstract. Porcine tissue-type plasminogen activator (t-PA) increases the binding of ^{125}I–glu-plasminogen to clots made from human plasma or purified fibrinogen in a time and t-PA concentration dependent fashion. The accumulation of plasminogen was faster and greater on noncrosslinked plasma clots than on clots which had been crosslinked by Factor XIIIa. Furthermore, the uptake of plasminogen to crosslinked fibrin clots occurred at a slower rate in the presence of α_2-plasmin inhibitor (α_2PI) than in its absence. The kinetics of the uptake of ^{125}I-plasminogen were analyzed using SDS-polyacrylamide gel electrophoresis and radioautography of solubilized plasma clots formed in the presence of t-PA. During the initial phase there was a decrease of clot-bound glu-plasminogen; simultaneously, there was a slight increase in clot-bound glu-plasmin and in plasmin complexed to α_2PI that was crosslinked to α-chain polymers of fibrin. This was followed by a marked increase in clot-bound plasminogen having glutamic acid as NH$_2$-terminal (glu-plasminogen) and glu-plasmin. t-PA-induced enhancement of glu-plasminogen uptake appears to be mediated by plasmin but does not require the conversion of glu-plasminogen to plasminogen having lysine or methionine as NH$_2$-terminal. The described mechanism assures an adequate supply of clot-bound plasmin, which is the enzyme ultimately involved in the degradation of fibrin.

Introduction

During blood coagulation, ~30% (0.2 μmol/l) of plasma α_2-plasmin inhibitor (α_2PI)[1] are crosslinked to α-chain polymers of fibrin (1); an equimolar amount of plasminogen having glutamic acid as NH$_2$-terminal (glu-plasminogen) is bound to fibrin (10% of plasma concentration or 0.2 μmol/l) (2). The amount of fibrin-bound α_2PI thus suffices to inhibit all plasmin generated from fibrin-bound plasminogen (2).

Tissue-type plasminogen activator (t-PA) mediates specific and efficient thrombolysis (3–8). This process is dependent on fibrin-bound plasmin which is protected against the inhibitory effect of circulating α_2PI (9–11). An excess of plasminogen or of plasmin is therefore needed at the fibrin surface to bring about fibrin degradation. The rapid complexation of circulating plasmin by α_2PI precludes accumulation of active plasmin on fibrin (9–11). Several authors postulated that the conversion of glu-plasminogen into plasminogen having lysine or methionine as NH$_2$-terminal (lys-plasminogen), which adsorbs to fibrin to a greater extent than the glu-form (12–18), would ensure an additional supply of plasminogen onto the clot.

In this report, we have investigated an alternative hypothesis, i.e., whether porcine t-PA promotes the uptake of glu-plasminogen onto fibrin.

Methods

Materials. Materials were supplied as follows: imidazole, epsilon-aminocaproic acid (EACA), L-lysine-HCl (puriss), ethylenediamine tetraacetic acid disodium salt (EDTA, puriss), diisopropylfluorophosphate (DFP), calcium chloride (purum), 2-mercaptoethanol (puriss) and, bovine serum albumin (BSA, fraction V) from Fluka, Buchs, Switzerland; sodium chloride, urea, acetic acid, trichloroacetic acid (20%), and hydrochloric acid (proanalysi) from Merck, Darmstadt,

1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; EACA, epsilon-aminocaproic acid; FSF, fibrin-stabilizing factor; glu-plasminogen, plasminogen having glutamic acid as NH-terminal; lys-plasminogen, plasminogen having lysine or methionine as NH-terminal; α_2PI, α_2-plasmin inhibitor; SDS-PAGE, SDS-polyacrylamide electrophoresis; t-PA, tissue-type plasminogen activator.

Federal Republic of Germany; Topostasin, a crude thrombin preparation which was found to be free of plasminogen activator when analyzed by the Granelli-Piperno method (8, 19), from Hoffmann-La Roche, Basel, Switzerland; ^{125}I-fibrinogen (FIBI-125C, 80–90% clottable) from Sorin Biomedica, Saluggia, Italy; Na ^{125}I (17.4 Ci/mg in 0.1 N sodium hydroxyde) from New England Nuclear, Boston, MA; β-D(+) glucose and aprotinin (Trasylol) from Bayer, Leverkusen, Federal Republic of Germany; affinity purified bovine lung aprotinin and Tween 80 from Sigma Chemical Co., St. Louis, MO; fibrinogen (human, grade L, 80–90% clottable) and H-D-Val-Leu-Lys-pNA (S-2251) from Kabi, Stockholm, Sweden; antisera against Factor XIII (fibrin-stabilizing factor [FSF]) and agarose grade L from Behringwerke, Marburg, Federal Republic of Germany; Biogel P-300, all reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immobilized lactoperoxidase/glucose oxidase (Enzymobeads) used for radioiodination from BioRad Laboratories, Richmond, CA; Sephadex G-100 and lysine-Sepharose 4B from Pharmacia, Uppsala, Sweden. The lys-form of plasminogen (NIBSC, code 66/234), the International Standard for human t-PA (NIBSC, code 83/517, 1,000 IU/ampoule), and the 2nd International Standard for plasmin (NIBSC, code 77/588, 10 IU/ampoule) were provided by Dr. P. J. Gaffney of the National Institute for Biological Standards and Control, London, United Kingdom. The amino-terminal of the lys-plasminogen preparation contained ~50% lysine and 50% methionine (kindly determined by Dr. E. Rickli, Institute for Biochemistry, Bern, Switzerland). Human α_2PI was donated by Drs. D. Collen and H. R. Lijnen from the Center for Thrombosis and Vascular Research, Leuven, Belgium. High M_r-urokinase (100,000 IU/mg) was generously supplied by Dr. D. Sauser, Serono Laboratories, Coinsins, Switzerland. All concentrations given in this paper are final. The imidazole buffer pH 7.35 used for many experiments contained 0.05 M imidazole and 0.14 M NaCl, pH 7.35.

Human plasma. Blood was obtained from healthy donors. It was anticoagulated with sodium citrate-phosphate-dextrose-adenine (Travenol, Caslehar, Ireland). Platelet-poor plasma was obtained by centrifugation for 30 min at 3,600 g and 4°C. In some experiments, 10 mM EDTA was added to plasma. The fibrinogen concentration of the three plasma pools (nine donors for each pool) used in the experiments, as measured by the Clauss method, was between 1.8 and 2 g/l.

FSF-deficient plasma was kindly provided by Dr. F. Duckert of the Coagulation Laboratory, Kantonsspital, Basel, Switzerland, who found it to contain <1% FSF compared with normal plasma by a method measuring the incorporation of dansyl cadaverine.

Glu-plasminogen. Glu-plasminogen was prepared from fresh-frozen plasma by affinity chromatography on lysine-Biogel by the method of Deutsch and Mertz (20). All purification steps were performed in the presence of 10 mM DFP and 150 KIU/ml aprotinin, except for the final dialysis against imidazole buffer. The amino-terminal was glutamic acid, as characterized by the dansyl chloride method (21). Its specific activity was 14 IU plasmin/mg. Plasminogen was determined after its conversion into plasmin by urokinase (22). This was accomplished by incubating the purified plasminogen (0.5 mg/ml) with various urokinase concentrations (1,000, 2,000, and 4,000 IU/ml) in 50 mM phosphate-10 mM EACA, pH 8.2 at 37°C. Aliquots were removed after 2, 5, 10, and 15 min, diluted 250-fold, and added to the chromogenic substrate S-2251 (0.6 mM) in 50 mM Tris HCl-12 mM NaCl, pH 7.4, containing 0.01% Tween 80. The initial rate of substrate conversion was measured in a recording spectrophotometer at a wavelength of 405 nm. The highest values were selected for comparison with the initial rate of S-2251 conversion by the International plasmin reference preparation. The glu-plasminogen was radioiodinated by the lactoperoxidase/glucose

oxidase method (12, 23). One-fifth of a milliliter of glu-plasminogen (1 mg/ml) was mixed with 10 μl of carrier free Na ^{125}I (1 mCi), 75 μl of β-D(+) glucose (1%), and 75 μl of Enzymobeads, and incubated at room temperature for 15 min. The beads were removed by centrifugation at 3,600 g for 5 min. Radioiodinated plasminogen was separated from unbound ^{125}I on a 25 ml Sephadex G-100 column using imidazole buffer that contained 0.1% BSA and 0.05% sodium azide. The incorporation of ^{125}I into the plasminogen molecules varied between 7 and 20% of the Na ^{125}I added, and the specific radioactivity between 0.3 mCi and 2 mCi/mg plasminogen (one atom to eight atoms of ^{125}I per 100 molecules of plasminogen). The ^{125}I-plasminogen had the same electrophoretic mobility as unlabeled glu-plasminogen in SDS-PAGE and in urea/acetic acid electrophoresis. Labeled glu-plasmin and lys-plasmin were made as follows: ^{125}I-plasminogen was added to a solution of unlabeled plasminogen (3.25 mg/ml) and incubated at 37°C for 1 h with 0.1 M lysine monochloride and 150 IU/ml of urokinase in the presence or absence of aprotinin. In the absence of aprotinin, ^{125}I-plasminogen was converted into lys-plasmin, whereas, in the presence of 10,000 KIU/ml aprotinin, glu-plasmin was formed. After reduction by 2-mercaptoethanol, these samples were subjected to SDS-PAGE in a 12.5% separating gel followed by staining in Coomassie Blue and radioautography as described in the "Electrophoretic procedure and radioautography" section. This allowed the comparison of the conversion of unlabeled and labeled glu-plasminogen into plasmin by urokinase and the determination of the relative radioactivities related to the different domains of the ^{125}I-plasminogen molecule. In the presence of aprotinin, 67±2% (mean±SD of quadruplicate experiments) of ^{125}I-glu-plasminogen was converted into ^{125}I-glu-plasmin (heavy chain 67%, light chain 33%) by urokinase. In the absence of aprotinin, 93±4% of ^{125}I-glu-plasminogen was activated into ^{125}I-lys-plasmin (heavy chain 73%, light chain 20%, and preactivation peptide 7%). The conversion of unlabeled glu-plasminogen into the glu-form, respectively, lys-form of plasmin, as judged from stained gels, was similar to that of ^{125}I-glu-plasminogen.

Fibrinogen. Fibrinogen was rendered plasminogen poor by passage over lysine-Sepharose 4B. About 2 μg of plasminogen were detectable per milligram of fibrinogen by the chromogenic substrate assay. Rocket immunoelectrophoresis in 1% agarose containing an antiserum against human Factor XIII demonstrated that the plasminogen-poor fibrinogen (2 mg/ml) contained 5–10% of Factor XIII a subunit and b subunit as compared with normal plasma.

Plasminogen activator. Pig heart t-PA was purified as previously described (24). It moved as a single band on reduced and nonreduced SDS-PAGE (M_r of 68,000). In analogy to highly pure high M_r urokinase, we assigned arbitrarily a specific activity of 100,000 U/mg to highly purified pig heart t-PA. Therefore, in this work, 1 U equals 10 ng of pig heart t-PA. Comparison with the International Standard for t-PA which was established in June 1984 by the expert committee of biological standardization of the World Health Organization revealed that in the fibrin plate assay, 1 U of pig heart t-PA equals 3.4 IU of t-PA. DFP-inactivated t-PA (1 h incubation at 37°C with 10 mM DFP) did not exhibit any activity in clot lysis or fibrin plate assays.

Binding studies of plasminogen to clots. Plasma clots were formed by the addition of 1 U/ml thrombin to plasma, with or without added CaCl₂ (10 mM for citrated plasma and 20 mM for citrated-EDTA plasma). 0.46 ml of plasma, containing ^{125}I-plasminogen (100,000 cpm/ml), were rapidly mixed with 10 μl of 500 mM CaCl₂, 10 μl of 50 U/ml thrombin, and 20 μl of various concentrations of t-PA. After incubation at 37°C for various periods of time, clots were compacted with a plastic spatula, washed in 2 ml of imidazole buffer containing

1 mM EDTA, and dried on absorbant paper. The amount of plasminogen bound to the clots was expressed in percentage of added radioactivity. In one experiment, FSF-deficient plasma alone or supplemented with 20% normal plasma was used to study the effect of crosslinking on the t-PA induced uptake of plasminogen onto clots. For the study of the effect of aprotinin on the binding of plasminogen to plasma clots, 10 mg of lyophilized aprotinin were added per milliliter of plasma pool (117,000 KIU/ml), which was then serially diluted in the same plasma.

Fibrin clots were made the same way as plasma clots from a fibrinogen solution (2 mg/ml imidazole buffer) containing 1% BSA, glu-plasminogen (0.2 mg/ml), and ^{125}I-plasminogen (100,000 cpm/ml) with or without α_2PI (0.07 mg/ml).

Clot lysis assays. The relationship between t-PA concentration and clot lysis time was studied as follows: plasma clots were made as described above in the presence of various t-PA concentrations. After thorough mixing the tubes were incubated at 37°C until complete solubilization of the clots had occurred. A straight line was obtained when the inverse of lysis time was plotted against the t-PA concentration.

To determine whether t-PA-induced accumulation of plasminogen preceded clot lysis, ^{125}I-fibrinogen (100,000 cpm/ml, radiolabel 80–90% clottable, 90–95% precipitable in 10% trichloroacetic acid) was added to plasma before the addition of $CaCl_2$, thrombin, and t-PA or buffer. The clots were incubated at 37°C up to 360 min, squeezed against the tube wall, washed, and dried on filter paper. The radioactivity was then determined. In the absence of t-PA, recovery of ^{125}I in the clots remained 80–90% over the entire incubation period. Lysis of t-PA containing clots was expressed as percent reduction of clot-bound ^{125}I in comparison with clots made in the absence of t-PA (8).

Electrophoretic procedure and radioautography. The nature of ^{125}I-plasmin(ogen) forms bound to crosslinked clots was studied by SDS-PAGE of the solubilized clot followed by radioautography. SDS-PAGE was performed as described by Laemmli (25), using a 10% polyacrylamide separating gel of 12.7 × 14 × 0.15 cm and a 4% polyacrylamide stacking gel. Crosslinked clots were solubilized by boiling the washed and dried clots at 100°C for 10 min in 0.5 ml of 10 M urea, 10% SDS, and 10% 2-mercaptoethanol. The solubilized samples were mixed with 0.125 ml of 50% glycerol and 0.64 ml of sample buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.02% bromophenol blue. 0.1 ml of the mixture was subjected to SDS-PAGE. After electrophoresis the gels were stained in a 0.25% solution of Coomassie Brilliant Blue R-250 in H_2O, methanol, acetic acid (4:4:1), and destained in the same solution without Coomassie Brilliant Blue. Radioautography was performed by exposing the dried gels to Kodak RPO2 X-ray film (Eastman Kodak Co., Rochester, NY) in the presence of intensifying screens (CAWO, SE 2) at −70°C for 2–5 d. After radioautography the gels were cut into 2-mm strips. The radioactivity of each strip was determined to estimate the relative amount of the different forms of plasminogen and plasmin bound to clots.

Results

Influence of t-PA on the binding of plasminogen to clots. Fig. 1 shows that t-PA induced a progressive uptake of radioiodinated plasminogen to fibrin clots. The rate of plasminogen accumulation on the clot was t-PA concentration dependent but maximum binding never exceeded 50%, regardless of the t-PA concentration. In the absence of t-PA, the amount of
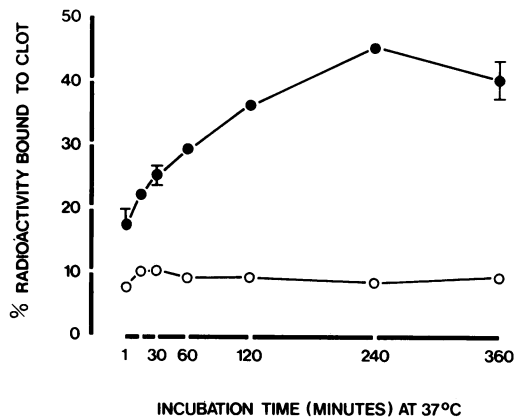

INCUBATION TIME (MINUTES) AT 37°C
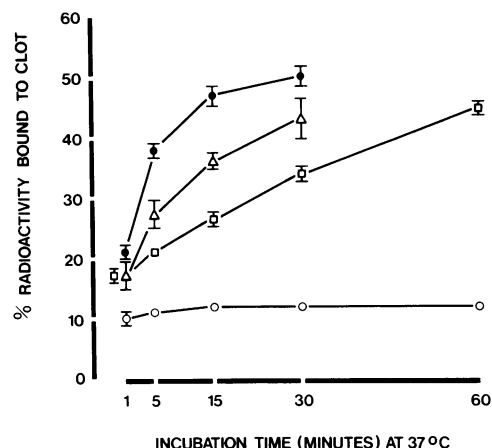
Figure 1. Effect of t-PA on the binding of plasminogen to fibrin clots. Clots were formed by the addition of 10 μl of $CaCl_2$ (500 mM), 10 μl of thrombin (50 U/ml), and 20 μl of buffer or a t-PA containing solution to 0.46 ml of a solution of fibrinogen (2 mg/ml imidazole buffer) containing 1% BSA, 0.2 mg/ml of glu-plasminogen, and ^{125}I-glu-plasminogen (100,000 cpm/ml). Clots were made in the absence (○) or in the presence of 3 U/ml (●), 1.5 U/ml (△), and 0.75 U/ml (□) of t-PA. After incubation at 37°C, clots were squeezed with a plastic spatula, washed in 2 ml of imidazole buffer containing 1 mM EDTA, and dried on absorbant paper. Clot-bound plasminogen is expressed as percentage of total radioactivity added. The mean±SD of quadruplicate experiments are given. At t-PA concentrations ≥1.5 U/ml clot lysis occurred within 60 min.

clot-bound plasminogen remained at ~10% regardless of the incubation time.

A similar t-PA-induced accumulation of plasminogen was observed on plasma clots (Fig. 2). The maximum amount of clot-bound plasminogen, in the presence of 3 U/ml t-PA, was comparable with that observed on fibrin clots, but the maximum was only attained after 240 min instead of 30 min incubation time. At 360 min there was a slight decrease in clot-bound radioactivity due to partial lysis of the clot. Effectively, when clots made under the same conditions were labeled with ^{125}I-fibrin(ogen), no lysis occurred up to 120 min incubation, whereas, respectively, 5 and 10% lysis was measured at 240 and 360 min incubation. No increase in plasminogen binding to plasma clots was observed in the absence of t-PA. There was good correlation ($r > 0.9$) between the amount of ^{125}I-plasminogen bound to plasma clots made in the presence of various t-PA concentrations after 30 min at 37°C, and the reciprocal of the lysis time of clots made under the same conditions but incubated until complete solubilization had occurred (Fig. 3).

Aprotinin inhibited the t-PA-induced accumulation of plasminogen to plasma clots in a concentration dependent manner but had no influence on plasminogen uptake in the absence of t-PA (Fig. 4).
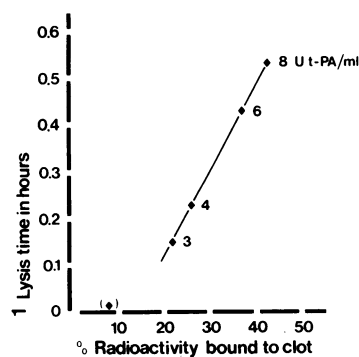
DFP-inactivated t-PA did not produce any increase in clot-

Figure 2. Effect of t-PA on the binding of plasminogen to plasma clots. Clots were made by the addition of 10 μl of $CaCl_2$ (500 mM), 10 μl of thrombin (50 U/ml), and 20 μl of buffer or a t-PA containing solution to 0.46 ml citrated plasma containing ^{125}I-plasminogen (100,000 cpm/ml). Clots were made in the absence of t-PA (o) or in the presence of 3 U/ml of t-PA (●), and incubated up to 360 min at 37°C. Otherwise as described in Fig. 1.

Figure 4. Effect of aprotinin on the plasminogen uptake by plasma clots. Clots were made from citrated plasma containing various concentrations of aprotinin in the absence (o) or presence (●) of 3 U/ml of t-PA and incubated at 37°C for 4 h. Otherwise as described in Fig. 2.
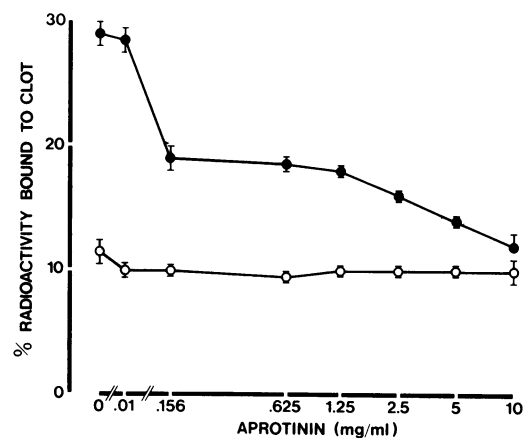
bound plasminogen. When plasma clots were formed in the presence of 100 mM EACA the amount of clot-bound radioactivity was <3%, both in the presence and absence of t-PA (Fig. 5).

Identification of the forms of plasmin(ogen) bound to plasma clots. SDS-PAGE analysis and radioautography of solubilized clots allowed the identification of different forms of plasminogen and plasmin bound to clots. The upper part of Fig. 6 shows that, in the presence of 3 U/ml of t-PA, mainly glu-plasminogen and glu-plasmin accumulated on plasma clots. Comparing the positions of lys-plasminogen, lane 1, and of lys-plasmin, lane 5, in Fig. 6, top, with those of the two major bands of lane 6 and 7, and taking into account the comparable labeling efficiency of the heavy chain regions of glu- and lys-plas-

min(ogen) (see Methods), it appears that a minimal amount of lys-plasminogen bound to fibrin. The lower part of Fig. 6 demonstrates the time course of glu-plasminogen and glu-plasmin uptake. Over a period of 6 h, a band of weak intensity appeared progressively at the top of the gel consisting of plasmin-α_2PI complexes crosslinked to α-chain polymers of fibrin (see also Fig. 10). After 120 min incubation, very weak bands appeared in the position of the lys-plasmin heavy chain and in the 50,000–55,000 mol wt range. In the absence of t-PA, only a faint band of plasmin-α_2PI crosslinked to the α-chain polymers of fibrin, and small amounts of glu-plasminogen were apparent on the radioautography (last lane on the right, lower part of Fig. 6, and Fig. 7).

Quantitative analysis of the different forms of plasmin(ogen)

Figure 3. Correlation between the ^{125}I-plasminogen uptake on plasma clots made in the presence of various t-PA concentrations and the reciprocal of the lysis time of clots made under the same conditions. Clots were made as described in Fig. 2 in the absence or in the presence of 8, 6, 4, and 3 U/ml of t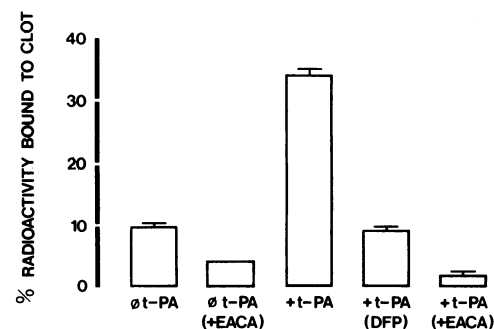-PA. After 1 h incubation at 37°C, the percentage of ^{125}I-plasminogen uptake on the clots was determined as described in Fig. 1. The lysis time of clots was determined at the time of their complete solubilization. (♦): in the absence of t-PA, clots did not lyse within 48 hours of observation.


Figure 5. Effect of EACA and DFP-inactivated t-PA on the binding of plasminogen to plasma clots. Clots were made from citrated plasma as described in Fig. 2, in the presence or absence of 3 U/ml of active t-PA or DFP-inactivated t-PA. +EACA: clots were made from plasma containing 100 mM of EACA. All clots were incubated at 37°C for 2 h. Otherwise as described in Fig. 2.
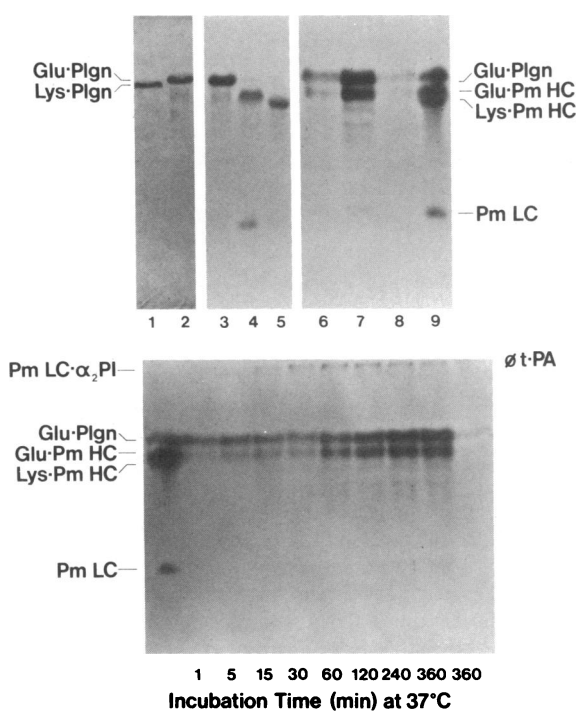
Figure 6. Identification of molecular species of clot-bound plasmin(ogen). Clots, made in the absence or presence of t-PA as described in Fig. 2, were solubilized and subjected to SDS-PAGE under reducing conditions followed by radioautography. *Top:* 1 and 2 Coomassie Blue stains, 3–9 radioautographies. 1: lys-plasminogen; 2: glu-plasminogen; 3: ^{125}I-glu-plasminogen diluted in unlabeled glu-plasminogen; 4 and 5: ^{125}I-glu-plasmin and ^{125}I-lys-plasmin prepared as described in Methods; 6 and 7: solubilized plasma clots made in the presence of 3 U/ml of t-PA after 1 and 360 min incubation at 37°C; 8: solubilized plasma clot made in the absence of t-PA after 360 min incubation at 37°C; 9: mixture of 3, 4, and 5. *Bottom:* First lane on the left: see lane 9, *Top:* 1–360: solubilized plasma clots made in the presence of 3 U/ml of t-PA, as described in Fig. 2, and incubated at 37°C for the indicated periods of time. Last lane on the right: solubilized plasma clot made in the absence of t-PA after 360 min incubation at 37°C. Plgn, plasminogen; Pm, plasmin; HC, heavy chain; LC, light chain.

bound to plasma clots. Fig. 7 illustrates the quantitative determination of various forms of ^{125}I-plasmin(ogen) bound to plasma clots which had been incubated for 360 min at 37°C in the presence or absence of t-PA. The same bands as described in Fig. 6 were detectable by radioautography. In the presence of t-PA the percentage of radioactivity bound for each major band was: 8% for the plasmin-α_2PI complex (Fig. 7:1), 25% for glu-plasminogen (a clear distinction between this band and small amounts of lys-plasminogen was not possible) (Fig. 7:2), 36% for the different forms of the heavy chain of plasmin (Fig. 7:3, 23% for the glu-plasmin heavy chain, 5% for the lys-plasmin heavy chain, 8% for the iodinated (degradation ?) products in the 50,000–55,000 molecular weight
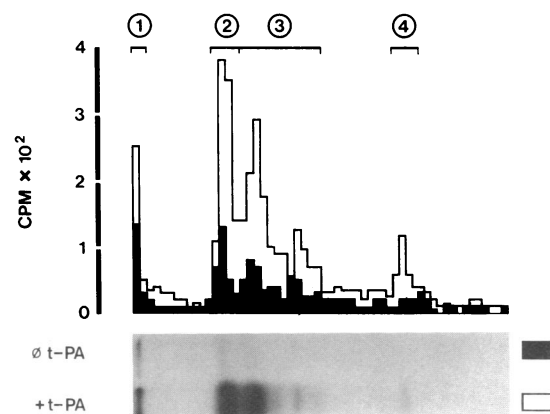
Figure 7. Procedure for the quantitative estimation of plasminogen and plasmin accumulated onto clots. Plasma clots were made in the presence of 3 U/ml of t-PA (open bars) or in the absence of t-PA (closed bars) and incubated at 37°C for 360 min. After solubilization of the clots, SDS-PAGE and radioautography were performed. The dried gel was cut into 2-mm strips, and each strip was counted for radioactivity. The following forms of plasmin(ogen) were identified: 1: plasmin-α_2PI complex crosslinked to the α-chain polymers of fibrin; 2: glu-plasminogen; 3: heavy chain of glu- and lys-plasmin and degradation products of M_r of 50,000–55,000; 4: plasmin light chain.

range), and 7% for the plasmin light chain (Fig. 7:4); 24% of the total radioactivity could not be attributed to distinct peaks. Total radioactivity bound was three times lower in the absence of t-PA and distributed mainly into the plasmin-α_2PI complex (12%), glu-plasminogen (18%), glu-plasmin heavy chain (16%), lys-plasmin heavy chain (4%), and the 50,000–55,000 molecular weight range species (10%). Using this methodology, the quantity of each of the various molecular forms of fibrin-bound ^{125}I-plasmin(ogen) was determined at different time intervals (Fig. 8). There was an initial phase of up to 30 min in which the fraction of glu-plasminogen that initially constituted 35% of the total clot-bound radioactivity decreased to 20% while the plasmin-α_2PI complex increased progressively and, after 60 min, reached a constant level of ~10% of the clot-bound radioactivity. ^{125}I-plasmin remained constant at ~40% of clot-bound radioactivity during the first 30 min. After this initial lag phase there was progressive increase in glu-plasminogen and particularly in glu-plasmin uptake to, respectively, 25 and 55% of the total clot-bound radioactivity.

Influence of crosslinking on the t-PA-induced uptake of plasminogen to fibrin. During coagulation, in the presence of calcium ions, FSF brings about the crosslinking of fibrin and of α_2PI to the α-chains of fibrin (1, 26–28). Since crosslinked α_2PI still preserves its capacity to inhibit plasmin (2), we studied the effect of crosslinking of fibrin on the t-PA induced plasminogen binding to fibrin. In a FSF-deficient plasma, no difference was observed in the t-PA-induced ^{125}I-plasminogen uptake onto clots formed in the presence or absence of calcium ions. In clots made from FSF-deficient plasma supplemented
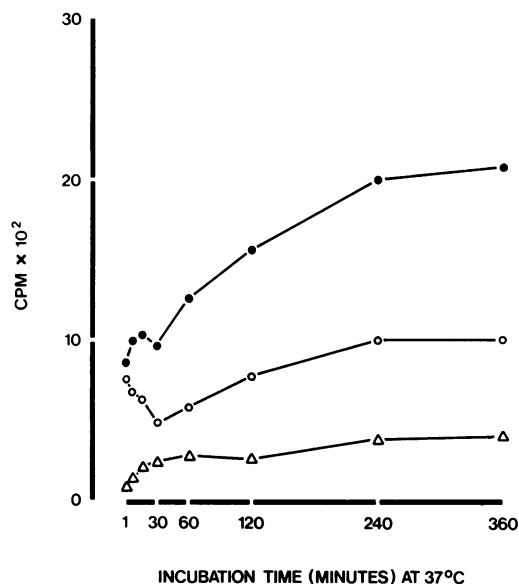
Figure 8. Kinetic analysis of the three major forms of clot-bound plasmin(ogen). Procedure as described in Fig. 7. The radioactivity of the three major plasmin(ogen) species is illustrated at each time point: (●) heavy and light chains of plasmin; (○) glu-plasminogen; (△) plasmin-α_2PI complex.

with 20% normal plasma, the uptake was higher in the absence than in the presence of calcium ions as was observed in normal plasma clots (Fig. 9). When crosslinked fibrin clots were formed at a t-PA concentration of 1.5 U/ml and in the presence of a physiological concentration of α_2PI, 13% of ^{125}I-plasminogen bound to clots after 30 min incubation at 37°C, whereas 45% binding occurred in the absence of α_2PI.

The right hand side of Fig. 10 illustrates an SDS-PAGE of a redissolved crosslinked ^{125}I-fibrin clot. The high molecular weight band at the top corresponds to the α-chain polymers of ^{125}I-fibrin (29) and had the same mobility as the ^{125}I-plasminogen-derived high molecular weight band observed in crosslinked plasma clots and in α_2PI containing fibrin clots.
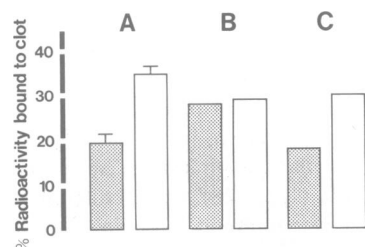
Figure 9. Binding of plasminogen to clots made from (A) normal plasma; (B) FSF-deficient plasma; (C) 1 vol of normal plasma mixed with 4 vol of FSF-deficient plasma. Clots were made from citrated (10 mM)-EDTA (10 mM) plasma containing 1.5 U/ml of t-PA, with (closed columns) or without (open columns) 20 mM Ca^{++} added. Clots were incubated at 37°C for 30 min. Otherwise as described in Figs. 1 and 2.
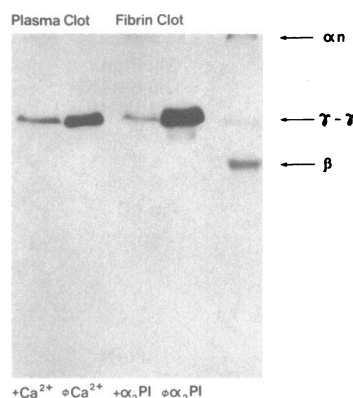
Figure 10. Identification of plasmin-α_2PI complex crosslinked to the α-chain polymers of fibrin. Plasma clots were made from citrated (10 mM)-EDTA (10 mM) plasma by the addition of 1 U/ml of thrombin, with or without CaCl$_2$ (20 mM), and in the presence of 1.5 U/ml of t-PA and ^{125}I-glu-plasminogen (100,000 cpm/ml). Fibrin clots were made in the presence of 1.5 U/ml of t-PA from a plasminogen-poor fibrinogen solution (2 mg/ml) to which were added 10 mM EDTA, 20 mM CaCl$_2$, 0.2 mg/ml of glu-plasminogen, and ^{125}I-glu-plasminogen (100,000 cpm/ml) with or without 0.07 mg/ml of α_2PI. Clots were incubated at 37°C for 30 min, solubilized, and subjected to SDS-PAGE and radioautography. The last lane depicts the autoradiography of a redissolved fibrin clot, made as the α_2PI containing fibrin clot described above, to which ^{125}I-fibrinogen (100,000 cpm/ml) had been added instead of the ^{125}I-plasminogen. αn, α-chain polymers of crosslinked fibrin; γ-γ, γ-chain dimers of fibrin; β, β-chains of fibrin.

This band was not observed when the presence of EDTA in plasma clots did not allow crosslinking of α_2PI to the α-chain polymers or when α_2PI was absent in crosslinked fibrin clots. This observation suggests that the radiolabeled plasmin light chain complexes with the α_2PI crosslinked to the α-chain polymers of fibrin.

Discussion

Clot lysis results from the dissolution of the fibrin network brought about by plasmin. The simultaneous absorption of plasminogen and t-PA onto fibrin during clotting facilitates the degradation of fibrin (3, 4, 11). Several studies including this one have shown that ∼5–10% of plasmatic glu-plasminogen (equivalent to 0.2 μM) binds to a plasma clot (2, 12–15). It is interesting to note that an equimolar amount of α_2PI crosslinks to plasma clots (2). This explains why normal plasma clots do not lyse spontaneously (2, 8). The cleavage by plasmin of a 76 residue peptide from the amino-terminal end of glu-plasminogen results in lys-plasminogen (30). The latter binds more strongly to fibrin (31) and is more rapidly activated into plasmin than glu-plasminogen (3, 4, 32, 33). Some authors have postulated that the conversion of the glu- into the lys-form is a prerequisite for an increased uptake of plasminogen onto fibrin. Such a mechanism would enhance the formation of fibrin-bound plasmin and thus the dissolution of fibrin (18, 34–36). However our study demonstrates that fibrinolysis induced by porcine t-PA is conditioned by a progressive

accumulation onto fibrin of mainly glu-plasmin(ogen) and not of lys-plasmin(ogen). The described mechanism was also actuated in clots made from plasma rich in plasminogen activator which was obtained from human volunteers after infusion of deamino-D-arginine-vasopressine, or from normal plasma to which human melanoma cell line derived t-PA had been added (data not shown). Our methodology did not allow detection of the eventual presence of small amounts of clot-bound lys-plasminogen. Attempts to distinguish glu- and lys-plasminogen by urea/acetic acid electrophoresis were unsuccessful, since fibrin interferes with the electrophoretic separation (12). The accumulation of glu-plasminogen took place before any significant lysis of fibrin had occurred and appeared to be specific. In the presence of EACA, only 3% of plasminogen was recovered in a clot, regardless of whether t-PA had been added or not. This was probably due to nonspecific trapping of plasminogen, since an equivalent amount of ^{125}I-albumin is incorporated in clots (31, 37). The t-PA-induced uptake of plasminogen requires active t-PA; DFP-inactivated t-PA did not enhance the binding of plasminogen to fibrin.

The crosslinking process effectuated by activated FSF inhibited the plasminogen uptake induced by t-PA. Furthermore, the presence of α_2PI in fibrin clots and/or in the surrounding milieu had the same effect. When plasma or fibrinogen was clotted under conditions allowing crosslinking of α_2PI to fibrin, radiolabeled plasmin(ogen), presumably plasmin light chain covalently linked to α_2PI, was found to be associated with the α-chain polymers of fibrin. These three observations taken together suggest that the crosslinking of α_2PI to fibrin modulates the t-PA induced uptake of plasminogen to fibrin. Further studies will be needed, however, to determine whether free α_2PI contributes or not to this regulatory mechanism.

Aprotinin, a powerful plasmin inhibitor, decreased the t-PA-induced accumulation of plasminogen to fibrin at low concentrations which are known not to inhibit t-PA activity (38). Suenson et al. (23) have recently reported that fibrin prepared from fibrinogen which had been partially degraded by plasmin bound higher amounts of glu-plasminogen than nondegraded fibrin. Preliminary experiments in our own laboratory indicate that preformed fibrin which had been treated with plasmin for various lengths of time also takes up higher amounts of glu-plasminogen than native preformed fibrin (manuscript in preparation). These observations lend support to the hypothesis that t-PA induced accumulation of glu-plasminogen is plasmin-mediated.

In conclusion, fibrinolysis induced by physiological concentrations of t-PA is preceded by an accumulation of glu-plasminogen onto fibrin. On a molar basis, the amount of clot-bound plasmin(ogen) was up to five times that of α_2PI crosslinked to fibrin. The accumulation of plasminogen does not require its conversion into the lys-form and is probably mediated by limited plasmin degradation of fibrin. Finally, the amount of clot-bound plasmin(ogen) determines the rate of clot lysis induced by t-PA.

Acknowledgments

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