The mechanism of activation of plasminogen at the fibrin surface by tissue-type plasminogen activator in a plasma milieu *in vitro*

Role of α_2 -antiplasmin

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The mechanism of activation of human Glu-plasminogen by fibrin-bound tissue-type plasminogen activator (t-PA) in a plasma environment or in a reconstituted system was characterized. A heterogeneous system was used, allowing the setting of experimental conditions as close as possible to the physiological fibrin/plasma interphase, and permitting the separate analysis of the products present in each of the phases as a function of time. The generation of plasmin was monitored both by spectrophotometric analysis and by radioisotopic analysis with a plasmin-selective chromogenic substrate and radiolabelled Glu-plasminogen respectively. Plasmin(ogen)-derived products were identified by SDS/PAGE followed by autoradiography and/or immunoblotting. When the activation was performed in a plasma environment, the products identified on the fibrin surface were Glu-plasmin (90%) and Glu-plasminogen (10%), whereas in the soluble phase only complexes between Glu-plasmin and its fast-acting inhibitor were detected. Identical results were obtained with a reconstituted system comprising solid-phase fibrin, t-PA, Glu-plasminogen and α_2 -antiplasmin. In contrast, when α_2 -antiplasmin was omitted from the solution, Lys-plasmin was progressively generated on to the fibrin surface (30%) and released to the soluble phase. In the presence of α_2 -antiplasmin or in plasma, the amount of active plasmin generated on the fibrin surface was lower than in the absence of the inhibitor: in a representative experiment the initial velocity of plasmin generation was 2.8×10^{-3} , 2.0×10^{-3} and 1.8×10^{-3} ($\Delta A_{405}/\text{min}$) for 200 nM-plasminogen, 200 nM-plasminogen plus 100 nM- α_2 -antiplasmin and native plasma respectively. Our results indicate that in plasma or in a reconstituted purified system containing plasminogen and α_2 -antiplasmin at a ratio similar to that found in plasma (1) the activation pathway of native Glu-plasminogen proceeds directly to the formation of Glu-plasmin, (2) Lys-plasminogen is not an intermediate of the reaction and therefore (3) Lys-plasmin is not the final active product. However, in the absence of the inhibitor, Lys-plasmin and probably Lys-plasminogen, which is more readily activated to plasmin than is Glu-plasminogen, are generated as well.

INTRODUCTION

Activation of Glu-plasminogen by tissue-type plasminogen activator (t-PA; EC 3.4.21.-) in a homogeneous system such as plasma is kinetically ineffective because in the absence of fibrin the K_m of Glu-plasminogen for t-PA is at least 30 times greater than the plasma plasminogen concentration (65 μM and 2 μM respectively [1]). In contrast, physiological fibrinolysis is a heterogeneous phase process localized at the plasma/fibrin interphase, where the kinetic parameters are efficiently improved and the specific interactions between the reactants t-PA, Glu-plasminogen, α_2 -antiplasmin and fibrin lead to adsorption phenomena that limit the generation of plasmin (EC 3.4.21.7) at the fibrin surface. Plasmin thus generated is protected from inhibition by α_2 -antiplasmin and may therefore efficiently remove the excess fibrin from the vascular bed [2].

Since Glu-plasminogen is sensitive to plasmin and the activation rates of Lys-plasminogen are higher than those of the Glu-form, it has been proposed [3–6] that activation of plasminogen yields Lys-plasmin and requires the formation of Lys-plasminogen as an intermediate. In most of the cases, however, a homogeneous purified system and urokinase (EC 3.4.21.31), an activator having no affinity for fibrin, have been used.

Specific protein assembly at the fibrin surface limits the activation of plasminogen by t-PA at the clot and involves interaction with plasma proteins that may modify the sequence of the activation mechanism. Two main plasma proteins with moderate affinity for plasminogen seem to be involved in such a mechanism, the histidine-rich glycoprotein ($K_d = 1 \mu\text{M}$) [7] and α_2 -antiplasmin ($K_d = 4.9 \mu\text{M}$) [8]. In purified systems data have been obtained indicating that these proteins may act by decreasing the available plasma plasminogen concentration and by interfering with its binding to fibrin [7–13]. However, no definitive data have been provided on the effect of these proteins on the generation of plasmin at the fibrin surface in a plasma milieu. This is in part due to the difficulties encountered in developing a model that efficiently resembles the fibrin/plasma interphase. In such a system the study of the activation of plasminogen by t-PA is complicated by the presence of plasmin inhibitors, and most authors have preferred the use of purified systems and fibrin substitutes to avoid methodological complications. In such a case, however, a correlation with physiological fibrinolysis is difficult to establish.

With the use of various types of fibrin mimics it has been recently proposed that the initial generation of Glu-plasmin by t-PA may proceed to the formation of Lys-plasmin by auto-

Abbreviations used: Glu-plasminogen, native human plasminogen with *N*-terminal glutamic acid; Lys-plasminogen, plasmin-modified forms of Glu-plasminogen with *N*-terminal lysine, valine or methionine (mostly lysine) obtained by hydrolysis of the Arg-68–Met-69, Lys-76–Lys-77 or Lys-77–Val-78 peptide bonds; Glu-plasmin and Lys-plasmin, activator-modified forms of Glu-plasminogen and Lys-plasminogen respectively; t-PA, tissue-type plasminogen activator; CBS 3308, chromogenic substrate D-norleucylcyclohexylalanylarginine *p*-nitroanilide; GGACK, dansyl-L-glutamyl-glycylarginylchloromethane.

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catalysis [6,14] or through the formation of Lys-plasminogen as an intermediate [4,5,15]. Nevertheless, Holvoet *et al.* [16] have recently suggested, on the basis of the absence of Lys-derived forms of plasminogen in the circulation, that activation of native Glu-plasminogen *in vivo* appears to occur by direct cleavage of the Arg-560-Val-561 bond in Glu-plasminogen and not via formation of Lys-plasminogen. The present work was aimed at testing this hypothesis. We aimed to determine the pathway of activation of plasminogen at the fibrin/plasma interphase under conditions as close as possible to the physiological model, i.e. using human plasma and a solid-phase fibrin surface that has been proved to be a useful tool for the study of the interaction of fibrinolytic proteins [17,18]. The spectrophotometric and radioisotopic analysis of the generation of plasmin would provide qualitative and quantitative data on plasma, indicating that Glu-plasminogen in plasma is directly transformed into Glu-plasmin by fibrin-bound t-PA. The absence of Lys-derivatives would indicate that fibrin-bound Glu-plasmin has no autocatalytic properties and that Glu-plasminogen is not a substrate of Glu-plasmin present at the plasma/fibrin interphase.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals used were of the best reagent grade commercially available. Other products were purchased from the following sources: EDTA, BSA and Tween 20 from Serva (Heidelberg, Germany); Ultrogel AcA 44 and DEAE-Trisacryl from I.B.F. (Villeneuve-la-Garenne, France); chromogenic substrate D-norleucylcyclohexylalanylarginine *p*-nitroanilide (CBS 3308) from Diagnostica Stago (Asnières, France); lysine-Sepharose 4B, gelatin-Sepharose 4B and PD-10 Sephadex G-25 (medium grade) columns from Pharmacia (Uppsala, Sweden); poly(vinyl chloride) U-shaped micro-titration plates and plate sealers from Dynatech (Marne-la-Coquette, France); glutaraldehyde [aq. 25% (v/v) solution] from TAAB Laboratories (Reading, Berks., U.K.); SDS, acrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate, *NNN'*-tetramethylethylenediamine, *M_r* markers and silver staining kit from Bio-Rad Laboratories (Richmond, CA, U.S.A.); Na¹²⁵I from Amersham International (Amersham, Bucks., U.K.); nitrocellulose from O.S.I. (Paris, France); benzamidine and 6-aminohexanoic acid from Aldrich (Beerse, Belgium); dansyl-L-glutamylglycylarginylchloromethane (GGACK) from France Biochem (Meudon, France); non-fat dry milk from Gloria (Courbevoie, France); M-Partigen plates from Behringwerke (Marburg, Germany); Protein A from Cappel (Flobio, Courbevoie, France); rabbit antiserum against human plasminogen from Diagnostica Stago; human plasmin from Boehringer (Mannheim, Germany).

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 BSA/ml and 0.01% (v/v) Tween 20. Binding buffer was buffer B containing 4 mg of BSA/ml, 0.01% (v/v) Tween 20 and 2 mM-EDTA. All other buffers were prepared as described in the text.

Preparation of plasma

Human venous blood obtained from healthy volunteers was drawn into a polypropylene tube containing 0.013 M-sodium citrate (final concentration) and centrifuged immediately at 2000 *g* for 15 min at 4 °C. The platelet-poor plasma was then separated and stored at -70 °C until used.

Proteins

Glu-plasminogen was purified by affinity chromatography on lysine-Sepharose 4B [19], ion-exchange chromatography on DEAE-Trisacryl and gel-filtration chromatography on Ultrogel AcA 44, in the presence of Trasylol. Fibrinogen was purified from fresh-frozen human plasma by the method of Kazal *et al.* [20] and was depleted in plasminogen and fibrinogenectin as described previously [19,21]. The concentrations of plasminogen and fibrinogen were determined by measuring the absorbance at 280 nm using $A_{1\text{cm}}^{1\%} = 16.8$ [22] and 15.1 [23] respectively. Human t-PA (> 95% single-chain) was generously given by Dr. M. Rånby (Biopool, Umeå, Sweden). The purified α_2 -antiplasmin was kindly provided by Dr. H. R. Lijnen (University of Leuven, Leuven, Belgium).

Preparation of solid-phase fibrin supports

The solid-phase fibrin supports were prepared as previously described [17,18]. Briefly, poly(vinyl chloride)-bound stable poly(glutaraldehyde) derivatives were first produced by treating U-shaped micro-titration 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 μ M in 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-CaCl₂) was covalently fixed for 18 h at 4 °C. After washing, a fibrin network was generated by thrombin (EC 3.4.21.5) treatment (1 N.I.H. unit/ml in assay buffer containing 1 mM-CaCl₂). The excess thrombin was eluted by three washes with a high-ionic-strength solution (0.5 M-NaCl/8 mM-CaCl₂/0.05% Tween 20). A final wash with 5 mM-sodium phosphate buffer, pH 6.8, containing 0.05% Tween 20 was done, and 100 μ l per well of buffer B containing 0.02 M-lysine, 0.2% BSA and 0.01% NaN₃ was added. The plate was sealed and stored at 4 °C until further use. The ability of thrombin to cleave fibrinogen was monitored with a horseradish-peroxidase-labelled mouse monoclonal antibody (Y18-HRP) kindly provided by Organon Teknika (Fresnes, France). The immunoreactivity of this antibody with the A α stretch 1-51 of human fibrinogen disappears upon treatment with thrombin [24].

Activation of plasminogen

Plasminogen was activated by t-PA in the presence of fibrin as follows. Amounts of 100 μ l of binding buffer containing 20 i.u. of t-PA/ml per well were incubated in a solid-phase fibrin plate for 1 h at 37 °C. The plate was washed twice with the same buffer to eliminate unbound proteins and the reaction was started by adding 100 μ l of the activation mixture per well. This mixture was either normal human plasma or assay buffer containing various amounts of Glu-plasminogen and α_2 -antiplasmin at ratios similar to those found in normal human plasma. When indicated, a trace amount (4-8 nM final concentration) of ¹²⁵I-Glu-plasminogen was added. The activation was performed in series of three wells and was monitored for 4 or 7 h. Every 5-15 min the reaction was stopped by removing the fluid phase and washing the wells with the assay buffer containing 1 mM-benzamidine and 10 μ M-GGACK when indicated. Products present in the fluid phase and the solid phase were analysed by spectrophotometry, radioisotopic counting and SDS/PAGE followed by autoradiography or specific immunoblotting. Plasmin present in the fluid phase was detected by mixing it with an equal volume of 1.2 mM-CBS 3308 and measuring the change in absorbance ($\Delta A_{405}/\text{min}$) at double-wavelength absorbance ratio (A_{405}/A_{490}). The kinetics of the reaction was followed as previously described [18]. Plasmin generated at the fibrin surface was detected by adding 100 μ l of 0.6 mM-CBS 3308 per well and measuring the change in absorbance as previously indicated [25]. Since fibrin-

bound plasmin was measured at the end of the activation in the absence of plasminogen and t-PA, and the amount of fibrin was maintained at a very low level [17] while the concentration of the chromogen was 5 times its K_m for plasmin, it is very unlikely that plasmin activity was underestimated by competition with alternative substrates.

The molecular species bound to the fibrin surface were eluted by incubating 100 μ l of SDS/PAGE sample buffer containing 0.2 M-6-aminohexanoic acid for 18 h at 22 °C, and were identified as follows. SDS/PAGE was performed with 7.5 or 10 % resolving gels and 4 % stacking gels according to the method of Laemmli [26]. After electrophoresis, the gels were either blotted or dried and autoradiographed on Kodak XS-1 films for 24–48 h at –70 °C, with a Kodak X-Omatic intensifying screen. The corresponding amount of each of the components of the total products bound to fibrin or present in the soluble phase was calculated by counting the radioactivity of each band after electrophoresis.

Immunoblotting with specific antibodies to human plasminogen and α_2 -antiplasmin was performed as described by Towbin *et al.* [27], with the following modifications. The blocking buffer contained 5 % (w/v) non-fat dry milk instead of BSA. Radiolabelled Protein A (350 nCi/ml) was used to detect the antibodies bound to nitrocellulose, and autoradiography was done for 8 h as indicated above.

The plasminogen products bound to the fibrin surface were quantified by counting the radioactivity in the wells in a γ -radiation counter. The total amount of plasmin(ogen) derivatives bound to fibrin was calculated by dividing the radioactivity of each well by the molar radioactivity of plasminogen in each of the solutions (plasma or buffer) containing the radioactive probe. The latter was calculated from the total concentration of plasminogen and the radioactivity added.

Miscellaneous

Glu-plasminogen and Protein A were radioiodinated with 125 I by using the Iodogen method of Fraker & Speck [28] with the following modifications: an iodination time of 4 min at 4 °C and removal of free 125 I- by molecular sieving on a PD-10 Sephadex G-25 (medium grade) column. The specific radioactivities obtained were 9–11 nCi/ng of protein for the plasminogen and 12 nCi/ng of protein for Protein A.

The concentration of plasminogen in human plasma was determined by using M-Partigen plates according to the instructions of the manufacturer.

RESULTS

Characteristics of the fibrin surface

The formation of fibrin by thrombin-catalysed fibrinopeptide cleavage from solid-phase fibrinogen was verified by the disappearance of immunoreactivity of the monoclonal antibody Y18-HRP with solid-phase fibrinogen upon treatment with thrombin (results not shown). The amino acid residue A α -16, i.e. the thrombin cleavage site, is essential for the conformational antigenic determinant recognized by this antibody in the A α stretch 1–51 of human fibrinogen [24]. After fibrin formation, thrombin was efficiently eliminated from the fibrin surface, as indicated by the disappearance of surface-bound clotting and amidolytic activities after addition of fibrinogen and the thrombin-selective substrate S-2366 (1 mM) respectively. Under the conditions described, the maximal fibrin surface density that can be obtained is 410 ± 4 fmol/cm².

Kinetics of activation

The kinetic parameters for the activation of plasminogen were determined as previously indicated [29]. The concentrations of

Fig. 1. Plasminogen activation by fibrin-bound t-PA: analysis of solid-phase bound proteins

A solution containing 20 i.u. of t-PA/ml was incubated with solid-phase fibrin. Excess t-PA was discarded and the reaction was started by incubating at 37 °C for the indicated times activation mixtures containing a trace amount of 125 I-Glu-plasminogen. The activation mixture was (A) assay buffer containing 200 nM-Glu-plasminogen (●), (B) assay buffer containing 200 nM-Glu-plasminogen plus 100 nM- α_2 -antiplasmin (▲) or (C) normal human plasma containing 1.8 μ M native plasminogen (■). To stop the reaction the fluid phase was removed and the wells were washed three times with assay buffer containing 1 mM-benzamidine and 10 μ M-GGACK and analysed as follows. (a) After being washed, the wells were dried, cut off and their radioactivities counted in a γ -radiation counter. The bound radioactivity was converted into fmol of plasmin(ogen) bound per well as calculated from the molar radioactivity of the plasminogen input. The number of fmol (mean $\pm$ s.d.) of plasmin(ogen) bound/min during the initial phase of the activation was: (A) 11.3 ± 1.9 ; (B) 6.66 ± 1.4 ; (C) 4.33 ± 1.5 . At 4 h the total amount of bound products (fmol/well) was: (A) 909 ± 56 ; (B) 590 ± 50 ; (C) 511 ± 37 . The result of a representative experiment is shown. (b) After the wells had been washed, bound proteins were eluted with SDS electrophoresis sample buffer containing 0.2 M-6-aminohexanoic acid and electrophoresed under reducing conditions. The gels were dried and exposed to autoradiography (parts A and B). The activation mixtures were as in (a) except that in part C native conditions were ensured by excluding the radioactive probe, and the products eluted from the fibrin surface were identified by immunoblotting with specific antibodies to human plasminogen and autoradiography with 125 I-protein. The M_r values of protein standards (phosphorylase b, 92000; BSA, 66000) are shown at the left. Glu-Pg, Glu-Pn and Lys-Pn represent the mobility of purified Glu-plasminogen, Glu-plasmin and Lys-plasmin respectively. Some degree of variability in the elution of bound plasmin(ogen) may account for the decreased Glu-plasmin observed at the 1 h time point in part B.

Fig. 2. Plasminogen activation by fibrin-bound t-PA: spectrophotometric detection of plasmin on the fibrin surface and in the fluid phase

Conditions for the activation of plasminogen were as indicated in Fig. 1 legend. The activation mixture was (A) assay buffer containing 200 nM-Glu-plasminogen (●), (B) assay buffer containing 200 nM-Glu-plasminogen plus 100 nM- α_2 -antiplasmin (▲) or (C) normal human plasma containing 1.8 μM native plasminogen (■). The reaction was stopped by removing the fluid phase at the indicated times, and plasmin generated on the fibrin surface or released to the fluid phase was detected as follows. (a) The wells were washed three times and conserved with assay buffer until the end of the activation experiment. Then the plate was washed again with assay buffer, and fibrin-bound plasmin was measured by addition of 0.6 mM-CBS 3308. The release of *p*-nitroaniline from the substrate was monitored at 37 °C in a micro-plate counter at double-wavelength absorbance ratio (A_{405}/A_{490}). Initial velocities for each time point are given in $10^{-3} \times v (\Delta A_{405}/\text{min})$ and were calculated as described elsewhere [18]. Rates of plasmin generation for the representative experiment shown were (A) 2.8, (B) 2.0 and (C) 1.8 in $10^{-3} \times v (\Delta A_{405}/\text{min})$. The values obtained from eight different experiments were (A) 3.36 ± 0.75 , (B) 1.67 ± 0.9 and (C) 1.72 ± 0.8 . (b) The fluid phase was removed at the indicated times and mixed with an equal volume of 1.2 mM-CBS 3308 in a micro-titration plate. The initial velocities of *p*-nitroaniline release from the chromogenic substrate were determined as indicated above.

plasminogen used in purified systems were chosen taking as the upper limit a concentration of 200 nM, which was previously determined to be saturating for the system [30] and corresponds to 10-fold the K_m (25 ± 6 nM) of human Glu-plasminogen for t-PA [29]. Each experiment was performed at least eight times to ensure the reproducibility of results. Intra-assay and inter-assay coefficients of variations derived from measurements of plasmin activity were 2.5 ± 0.1 and 8.1 ± 0.2 respectively.

Plasminogen activation in purified systems

Plasminogen in assay buffer at various concentrations, 50 to 200 nM, containing a trace amount of ^{125}I -plasminogen, was activated by fibrin-bound t-PA in the presence and in the absence of 100 nM- α_2 -antiplasmin. Activation products present in the fluid phase or bound to fibrin were analysed at the end of the activation experiment (4 or 7 h). As shown in Fig. 1(a), the amount of plasminogen derivatives bound to fibrin increased constantly as a function of time throughout the activation period. However, the amount of functionally active plasmin bound to fibrin reached a plateau by 1 h and was 50% of the total in 10 min, as demonstrated by kinetic analysis with a plasmin-selective chromogenic substrate (Fig. 2a). The increasing binding of radioactive material until the end of the activation (Fig. 1a) suggested the presence of additional inactive products (mainly Glu-plasminogen; see below). At 4 h the total amount of plasmin(ogen) bound per well (mean $\pm$ S.D. for eight different experiments) was 909 ± 56 fmol in the absence of α_2 -antiplasmin, and decreased to 590 ± 51 fmol and 511 ± 37 fmol in the presence of the inhibitor and in plasma respectively. The corresponding amounts of active fibrin-bound plasmin in the presence of α_2 -antiplasmin reached even lower values (approx. 50%), as indicated by the activity of plasmin detected at the end of the activation: $v (\Delta A_{405}/\text{min}) \times 10^3 = 8.66 \pm 2.44$ in the absence of α_2 -antiplasmin, 4.34 ± 1.7 in the presence of the inhibitor and 3.10 ± 0.9 in plasma. Fig. 2(a) represents a typical experiment where the decrease in plasmin generation by α_2 -antiplasmin at 1 h was 25%.

Bound products were eluted from the fibrin surface and further characterized. All radiolabelled plasmin(ogen)-derived molecular species eluted were decreased by 25–30% in the presence of 100 nM- α_2 -antiplasmin (Fig. 1b part B). Some degree of variability in the elution of bound plasmin(ogen) may account for the enhanced decrease in plasmin observed at the 1 h time point in this Figure. The products eluted were identified as indicated in the Materials and methods section. In the absence of α_2 -antiplasmin (Fig. 1b part A), only Glu-plasmin was present in the initial phase of the activation, but a significant amount of the radioactivity present in the latest phase of the activation was identified as Lys-plasmin (30%) and Glu-plasminogen (10%). The addition of 100 nM- α_2 -antiplasmin to the reaction mixture not only induced a decrease (30%) in the amount of plasmin generated on the fibrin surface but also prevented the formation of Lys-derivatives (Fig. 1b part B). However, it did not appear to influence the amount of Glu-plasminogen bound to fibrin.

In the fluid phase (Fig. 2b), in the absence of α_2 -antiplasmin, increasing amounts of free plasmin liberated from the fibrin surface were detected after a short period of time (10 min). The kinetic analysis demonstrated time correlation between this and the 50% saturation of the fibrin surface by plasmin. After 1 h of activation, the amount bound was more than 90% the saturation, and the increase in free plasmin was linear with time until a maximum conditioned by the consumption of plasminogen was reached at 7 h. The decay with time in fluid-phase Glu-plasminogen can be observed in Fig. 3(a) part A (without α_2 -antiplasmin) and Fig. 3(a) part B (with α_2 -antiplasmin). These autoradiographs were obtained from non-reduced gels and it is therefore difficult to observe the Lys-derivatives in Fig. 3(a) part A. In the corresponding reduced gel (Fig. 3b) Lys-plasmin, however, whose activity was shown in Fig. 2(b) curve A, was clearly identified. In contrast, in the presence of α_2 -antiplasmin, no plasmin activity was detected in the soluble phase (Fig. 2b), and a radioactive band of M_r approx. 140000, which was not observed in the absence of the inhibitor (Fig. 3a part A), was present in non-reduced gels (Fig. 3a part B). This band was

Fig. 3. Plasminogen activation by fibrin-bound t-PA: analysis of plasmin(ogen)-derived products in the fluid phase

Conditions for the activation of plasminogen by fibrin-bound t-PA and the activation mixtures were as indicated in Fig. 1 legend. During the activation the fluid phase was removed at the indicated times, mixed with an equal amount of SDS sample buffer and electrophoresed in a 10% polyacrylamide slab gel. The gel was dried and exposed to autoradiography. (a) Electrophoresis under non-reducing conditions: autoradiography of ^{125}I -plasminogen activated in a reconstituted system in the absence (A) or in the presence (B) of α_2 -antiplasmin, and in plasma (C). (b) Reduced gel electrophoresis of sample A. The M_r values of protein standards (lactate dehydrogenase, 140 000; phosphorylase b, 92 000; BSA, 66 000) are shown at the left of the autoradiographs. Pn- α_2 AP represents the mobility of Glu-plasmin- α_2 -antiplasmin complexes as identified by immunoblotting with antibodies to both α_2 -antiplasmin and plasminogen; Glu-Pg, Glu-Pn and Lys-Pn indicate the mobility of purified Glu-plasminogen, Glu-plasmin and Lys-plasmin respectively.

identified as complexes between plasmin and α_2 -antiplasmin by immunoblotting with specific antibodies against plasminogen and the fast-acting inhibitor of plasmin (results not shown).

Plasminogen activation in a plasma environment

Normal human plasma was supplemented with a constant amount of radiolabelled plasminogen and the activation was performed as indicated above. The total amount of fibrin-bound plasminogen derivatives in plasma (Fig. 1a) was 40% less than that observed in a purified system in the absence of inhibitors. A similar decrease in plasmin activity was detected at the fibrin surface (Fig. 2a). As shown in the same Figure, the initial rate of generation of fibrin-bound plasmin was lower than that observed in the purified system in the presence of α_2 -antiplasmin [v ($\Delta A_{405}/\text{min}$) = 2.8×10^{-3} , 2.0×10^{-3} and 1.8×10^{-3} for plasminogen alone, plasminogen plus α_2 -antiplasmin and plasma respectively]. The time necessary to obtain 50% of the maximal amount of plasmin bound to the fibrin surface was 25 min and the plateau was reached by 2 h. The corresponding values in the purified system in the absence of inhibitor were 10 min and 1 h. As in the purified system, the plasmin activity curve reached a plateau, but the amount of total plasminogen-related species was still increasing after 4 h of activation (Fig. 1a). Bound products were identified by specific immunoblotting (Fig. 1b part C): only Glu-plasmin and Glu-plasminogen were detected. The increasing amount in fibrin-bound products (Fig. 1a) was related to the binding of a substantial amount of Glu-plasminogen (Fig. 1b part C) over the amount of Glu-plasmin already bound to the fibrin surface. The plasma phase was analysed at each time point for the presence of plasmin(ogen)-derived products. As expected, no plasmin activity was detected (Fig. 2b), and the analysis of radioactive products by SDS/PAGE and autoradiography revealed the presence of increasing amounts of plasmin- α_2 -antiplasmin complexes (Fig. 3a part C). This indicates that plasmin released from the fibrin surface was immediately quenched by its specific inhibitor. In contrast, no traces of such complexes were detected at the fibrin surface (results not shown). Since the concentration of plasminogen in the plasma phase was about $1.8 \mu\text{M}$, its consumption during the activation was negligible (less than 2% as calculated from data in Figs. 1a and 2a) and therefore difficult to detect in the autoradiographs (Fig. 3a part C). In contrast, when the concentration of plasminogen was

only 200 nM, consumption of the radioactive probe was easily detected in the corresponding autoradiographs (Fig. 3a parts A and B).

DISCUSSION

In the present study we have investigated the pathway of plasminogen activation by t-PA *in vitro* in both a purified system and a plasma milieu using a solid-phase fibrin heterogeneous system. The value of this two-dimensional fibrin monolayer as a model for a fibrin deposit is reflected both by its absence of immunoreactivity with a monoclonal antibody to fibrinopeptide A and by its capacity to bind t-PA specifically ($K_d = 1.4 \pm 0.2 \text{ nM}$) and stimulate its plasminogen-activating activity [17]. This system makes it feasible (1) to analyse separately the products present either in the solid phase or in the soluble phase, and (2) to study the mechanism and regulation of plasminogen activation in a physiological environment, i.e. the plasma/fibrin interphase [18].

The results obtained in this study show that the activation of native Glu-plasminogen by fibrin-bound t-PA in normal plasma environment proceeds via the direct cleavage of the Arg-560-Val-561 bond of the proenzyme as indicated by the absence of Lys-derivatives either in the solid phase or in the fluid phase. This leads to the formation of (1) Glu-plasmin at the fibrin surface and (2) Glu-plasmin- α_2 -antiplasmin complexes in the fluid phase. Identical results were obtained in a reconstituted system comprising solid-phase fibrin, t-PA, purified Glu-plasminogen and α_2 -antiplasmin. This indicates, as previously shown by others [31], that N-terminal peptide fragments are not released from Glu-plasminogen in the presence of an excess of α_2 -antiplasmin, and is compatible with the existence of constant rates of activation (Fig. 2a) that could otherwise be altered if Lys-plasminogen forms, which are more readily activated by t-PA [1,32], were present. Our results are in accordance with previous data indicating that in plasma plasminogen is in the Glu-form [5] and that the Glu-plasmin generated by activators from it form complexes with α_2 -antiplasmin [4]. In agreement with this, Holvoet *et al.* [16] have shown that in normal plasma the concentrations of Lys-plasminogen, as measured in an antibody neutralization assay, were below the detection limit of 1 nM. Moreover, during activation of the fibrinolytic system *in vitro* in plasma as well as *in vivo* in patients undergoing thrombolytic

therapy, these authors failed to demonstrate Lys-plasmin- α_2 -antiplasmin complexes. Also, Norrman *et al.* [33] were unable to detect Lys-plasminogen *in vitro* during the acceleration phase of fibrinolysis induced by t-PA. In contrast, Thorsen *et al.* [4] reported that in clotted plasma a considerable amount of Lys-plasminogen and α_2 -antiplasmin-Lys-plasmin complexes were formed. This was most probably related to the fact that at high concentrations of activator a possible consumption of α_2 -antiplasmin may lead to autocatalytic conversion of Glu-plasmin into Lys-plasmin and to plasmin-catalysed Lys-plasminogen formation.

Further, the results show that the activation of plasminogen by fibrin-bound t-PA in a reconstituted system excluding α_2 -antiplasmin proceeds to the formation of Lys-plasmin. The late appearance of Lys-plasmin on to the fibrin surface and the absence of Lys-plasminogen either in the solid phase or in the fluid phase suggest that Lys-plasmin was generated by autocatalysis in our particular model, where the covalently bound fibrinogen is transformed to fibrin on the solid phase. However, others [4,5,15] have shown using fibrin(ogen) derivatives that in the absence of α_2 -antiplasmin important amounts of Lys-plasminogen can be generated and that the high rate of Lys-plasminogen formation may depend on the presence of X-fragment polymers [15]. It has been suggested thereafter that the transformation of Glu-plasminogen into Lys-plasminogen may represent a potential stimulatory mechanism of fibrinolysis. In this study we have started the activation of plasminogen by using t-PA bound to intact fibrin, a condition that may be equivalent to the incorporation of t-PA into a plasma clot during clotting [34], and determined the appearance of plasmin(ogen) derivatives as the fibrin was degraded. Since in our solid-phase fibrin system the products generated by plasmin during the activation are covalently bound to the plate and cannot be released from it, their presence in the solid phase was detected with monospecific antibodies (results not shown) kindly provided by Dr. W. Nieuwenhuizen [24,35]. This indicates that, in the early phase of degradation, fibrin was converted into X-fragments during the activation of Glu-plasminogen by t-PA. The experiments performed in the presence of purified or native α_2 -antiplasmin (plasma) failed, however, to show any trace of Lys-plasmin(ogen) either in the early phase of degradation or in the latest phase corresponding to the appearance of Glu-plasminogen (Fig. 1b), which has been shown to have affinity to fragment D [36]. This indicates that the absence of Lys-forms during the activation of plasminogen in a plasma environment is most probably related to the inhibitory effect of α_2 -antiplasmin. Indeed, in the absence of this inhibitor, formation of Lys-plasmin was observed in the latest phase of the activation (Fig. 3b).

Glu-plasminogen was continuously bound to the fibrin surface, as indicated by its transformation into Glu-plasmin and by the simultaneous detection of both forms at the end of the activation experiment (Fig. 1b). This binding was probably related to the unveiling of new C-terminal lysine binding sites [37–39], as indicated by the ability of 6-aminoheptanoic acid to elute fibrin-bound plasmin(ogen) derivatives.

As shown in Fig. 2(a), the total amount of plasmin activity generated at the fibrin surface was about 30% lower when plasma was used instead of the reconstituted activation mixture. Under the conditions of our assay we can reasonably exclude that plasmin might have been underestimated by competition with alternative substrates. However, plasma proteins other than α_2 -antiplasmin might interfere specifically (histidine-rich glycoprotein [8,11]) or by a mass-action effect (albumin) with the binding of plasminogen on to the fibrin surface, and be responsible for the decreased plasmin generation observed.

Plasmin continuously formed on the surface was released from

the degraded fibrin when the amount bound to it attained half-saturation of the binding sites. In the reconstituted system plasmin thus released formed complexes with added α_2 -antiplasmin (Fig. 3a part B). In plasma, similar complexes (Fig. 3a part C) were identified by immunological methods. We were unable to detect complexes between plasmin and α_2 -macroglobulin. This is most probably related to the fact that under the conditions of our assay α_2 -antiplasmin was in excess over the plasmin generated by the limited amounts of fibrin-bound t-PA. In accordance with this, complexes between α_2 -antiplasmin and plasmin present in plasma were only of the Glu-type, as indicated by the absence of Lys-type heavy chains in reduced gels (results not shown). Moreover, the reaction rate between plasmin and α_2 -macroglobulin being considerably lower ($K_1 = 1.3 \times 10^5 \pm 0.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) [40] than that between plasmin and its primary inhibitor ($K_1 = 3.8 \times 10^7 \pm 0.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) [31], the complexes were preferentially formed with the latter.

All these data taken together suggest that the transformation of Glu-plasmin(ogen) into Lys-plasmin(ogen) does not constitute, under the conditions of our assay, a significant mechanism of amplification in fibrinolysis. Similar results were obtained by Holvoet *et al.* [16] *in vivo* and during thrombolytic therapy. However, the transformation of Glu-plasmin(ogen) into Lys-plasmin(ogen) could be of significance in cases of congenital deficiency of α_2 -antiplasmin [41] or in pathological states [42] with a sustained hyperfibrinolytic state leading to its consumption.

In conclusion, our findings indicate that Glu-plasminogen is directly transformed into Glu-plasmin by fibrin-bound t-PA and that Lys-plasminogen is unlikely to be an intermediate of the activation of Glu-plasminogen.

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