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Tissue plasminogen activator (tPA) inhibits plasmin degradation of fibrin. A mechanism that slows tPA-mediated fibrinolysis but does not require alpha 2-antiplasmin or leakage of intrinsic plasminogen.

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Research Article

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Tissue Plasminogen Activator (tPA) Inhibits Plasmin Degradation of Fibrin

A Mechanism That Slows tPA-mediated Fibrinolysis but Does Not Require α_2-Antiplasmin or Leakage of Intrinsic Plasminogen

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Abstract

Thrombolysis is dramatically slower when high concentrations of lytic agent are used. This paradoxical observation, first described as "plasminogen steal," was originally believed to be due to depletion of extrinsic plasminogen and consequent leaching of clot-bound plasminogen. We report that administration of increasing concentrations of recombinant human tissue plasminogen activator (tPA) to fibrin gels resulted in lysis rates that displayed a maximum, with significantly slower rates found at higher tPA, regardless of whether plasminogen was supplied extrinsically or intrinsically. A similar maximum in lysis rates was observed in a system lacking an extrinsic phase when plasminogen was added to fibrin suspensions preincubated with increasing tPA. Thus, intrinsic plasminogen leakage and α_2-antiplasmin were not required for the decreased lysis at high tPA. No maximum was observed for increasing concentrations of urokinase. Using fibrin suspensions or gels preincubated with tPA before addition of plasmin, we report that tPA, but not urokinase, caused a dose-dependent inhibition of the fibrinolytic action of plasmin. With respect to optimal dosage schemes and the design of novel lytic agents, these findings indicate that (*a*) there exists a biochemical mechanism against minimizing reperfusion time with increasing tPA dosages and (*b*) the fibrin affinity of tPA may cause reduced fibrinolysis by plasmin. (*J. Clin. Invest.* 1995. 95:2483–2490.) Key words: fibrinogen • fluorescence • urokinase • diffusion • thrombolysis

Introduction

The observation that thrombolysis is dramatically slower when high concentrations of urokinase plasminogen activator (uPA)[1] or tissue plasminogen activator (tPA) are used (1–4) is completely unexpected and only partially understood. This paradoxical observation was first described as "plasminogen steal" (1) and was postulated to be due to depletion of extrinsic plasminogen and an associated leaching of clot-bound plasminogen out of the clot and into the plasminogen-depleted plasma (2). Within dissolving fibrin, reactions in the interstitial fluid of the clot combined with reactions on the fibrin fibers result in a continually evolving kinetic phenomena. The rates of transport of species across the boundary between the clot and the external plasma contribute to the overall kinetics of clot lysis (5–8). In general, the more plasminogen within the clot (intrinsic) or external to the clot (extrinsic) that is available for activation, the more rapidly lysis will occur (3, 9). When the local plasminogen concentration within a clot exceeds the local antiplasmin concentration, increasing the rate of plasminogen activation with increasing amounts of tPA or uPA is expected to produce plasmin activity in a more rapid manner. Although inhibition reactions mediated by α_2-antiplasmin or plasminogen activator inhibitor type 1 are expected to slow lysis, these inhibitors would not be expected to produce attenuated lysis rates as the concentration of thrombolytic agent is increased.

Given the relatively slow rate of diffusional processes to transport fibrin binding proteins over distances of millimeters (5–8), we hypothesized that the dramatically decreased lytic rate observed at high tPA concentration was a direct biochemical effect and did not require a "steal" of plasminogen from the clot. That the lysis rate of thrombi can display a bell-shaped dose-response curve (3) at increasingly high tPA concentrations is suggestive of competition between species for limited number of binding or cleavage sites. The present study was focused at determining the requirements in a purified system for a maximum to be observed in the lysis rate vs tPA dose-response curves. We have found that a maximum in lysis rates is observed for tPA-mediated lysis, but that this observed maximum does not require the presence of α_2-antiplasmin and is independent of intrinsic plasminogen leakage. Using fibrin gel lysis assays which have an extrinsic phase and assays using well mixed fibrin fiber suspensions which have no extrinsic phase, we have found that tPA can directly reduce the activity of plasmin on fibrin. These findings identify a mechanism that establishes optimal upper dosage during thrombolytic therapy via tPA administration. Continually increasing the concentration of tPA at the site of the thrombus will not lead to faster and faster reperfusion because high levels of tPA will eventually attenuate the action of plasmin on fibrin. High fibrin affinity plasminogen activator mutants may suffer a similar limitation of reducing plasmin degradation of fibrin.

Methods

Reagents. Purified human thrombin was obtained from Sigma Chemical Co. (St. Louis, MO) as a lyophilized powder (sp act: 3,000 NIH U/mg). Lyophilized human fibrinogen (Grade L; Kabi AB, Stockholm,

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1. *Abbreviations used in this paper:* sc-tPA, single chain tissue plasminogen activator; tc-tPA, two chain tissue plasminogen activator; tPA, tissue plasminogen activator; uPA, urokinase PA; v^{ss}, steady state lysis front velocity.

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Sweden) was dissolved in 0.05 M Tris-HCl (pH 7.4) and dialyzed at 4°C against 0.05 M Tris-HCl containing 0.1 M NaCl, centrifuged at 2,000 *g* for 20 min (4°C) and the supernatant was frozen in small aliquots at −75°C. Purified human Glu- and Lys-plasminogen and human plasmin (American Diagnostica Inc., Greenwich, CT) and human α_2-antiplasmin (Calbiochem-Novabiochem Corp., La Jolla, CA) were reconstituted, centrifuged at 2,000 *g* for 20 min (4°C), and stored at −75°C. Recombinant human tPA was obtained as a gift from Dr. W. Bennett (Genentech, Inc., South San Francisco, CA). Urokinase was obtained as a gift from Dr. A. Sasahara (Abbott Laboratories, Abbott Park, IL). For fluorescence labeling, fibrinogen (10 mg/ml) was incubated with 1 mg/ml FITC (Molecular Probes, Inc., Eugene, OR) with continuous stirring for 1 h at 22°C in 0.1 M sodium bicarbonate (pH 9.0). The reaction was stopped with hydroxylamine (0.15 M), dialyzed, and stored at −75°C. All concentrations are given as final concentrations in the reaction mixture.

Preparation of fibrin gels and gel lysis experiments. Gel lysis experiments with unmixed extrinsic phase were conducted as previously described (8) and carried out at 37°C in an environmental room. Purified fibrin gels (2-cm long) were formed by suction pipetting a rapidly mixed solution of fibrinogen (3 mg/ml) and thrombin (1 U/ml) up into 100-μl glass capillary tubes (1.5 mm inner diameter). The buffer for fibrin polymerization was 0.05 M Tris-HCl, 5 mM $CaCl_2$ (pH 7.4) with 0.1 M NaCl to obtain turbid, coarse gels (10). The gels were allowed to polymerize for over 10 h. In some experiments, Glu-plasminogen (2.2 μM) or α_2-antiplasmin (0.1–1 μM) were copolymerized with the fibrinogen. Purified fibrin clots were lysed with a 50-μl addition of lytic enzyme that was carefully loaded adjacent to the clot by backfilling the capillary. Changes in the position of fluid-gel interface were recorded with time as the clot lysed. Least squares fit ($r^2 > 0.99$) of front position with time over the first 90 min of the experiment allowed the determination of the steady state lysis front velocity (v^{ss}) (millimeters per minute).

For lysis experiments conducted with a well-mixed extrinsic phase, we used a fluorescence release assay using FITC-labeled coarse fibrin gels (11). A volume of 200 μl of polymerizing fibrinogen (3 mg/ml) containing 0.1 mg/ml FITC-fibrinogen was placed in the bottom of a cuvette and allowed to polymerize for over 90 min to form a 2-mm thick coarse fibrin gel. Gels were incubated in several changes of buffer to remove unincorporated fluorescence. In some experiments, plasminogen was incorporated into the polymerizing fibrin. At the time of the experiment, a 2.77-ml vol of solution containing tPA was added above the fibrin and allowed to incubate for 300 s under well mixed conditions with moderately strong stirring by a small, single-blade impeller (diameter 3 mm, 600 rpm). After incubation with tPA, plasmin was added to the extrinsic phase at a final concentration of 0.1 μM. Under these mixing conditions, the liquid in the cuvette achieved a well mixed state within 10 s as indicated by trypan blue dye mixing tests. For fibrin containing intrinsic plasminogen, lytic reactions were initiated by addition of tPA to the extrinsic phase. Release of fluorescent degradation products into the extrinsic phase was monitored in an LS-50 luminescence spectrometer (Perkin-Elmer Cetus Corp., Norwalk, CT) at room temperature.

Fibrinolysis assays of fibrin suspensions. To continuously monitor lysis in dilute suspensions of fibrin, we used a fluorescence dequenching assay (12). To produce suspensions of fibrin fibers, thrombin (final concentration 1 U/ml) was mixed (for 5 s) into dilute FITC-fibrinogen (80 nM) solutions at 0.1 M NaCl and 5 mM $CaCl_2$ after which the polymerization was monitored until fluorescence quenching was complete and stable for 1 h. The daily made fiber suspensions yielded a highly repeatable extent of quenching due to neighbor–neighbor interactions of protofibrils in the fiber, and were stable for several hours as indicated by the stability of the fluorescence signal. Small volumes of the fiber suspension were then pipetted into 2.4 ml of the reaction buffer, mixed, and monitored for 200–400 s to establish the fluorescence baseline before addition of other reagents. The increase in intensity of fluorescence emission (516 nm) during plasmin degradation of the fibrin due to generation of dequenched fragments was measured in a luminescence spectrometer at room temperature.

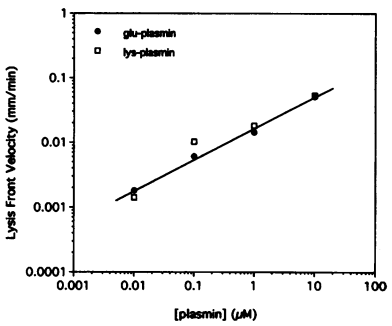

Figure 1. Plasmin-mediated lysis of fibrin under conditions of diffusion-mediated transport of extrinsic Glu-plasmin (●) or Lys-plasmin (□) into preformed coarse fibrin (3 mg/ml). The fluid/fibrin interface moved at a steady state lysis front velocity for over 100 min. Lys-plasmin was generated from Lys-plasminogen by 30-min incubation with uPA before the placement of the solution next to the fibrin.

For SDS-PAGE analysis, 1.5 μM fibrinogen was polymerized with 1 U/ml thrombin, sonicated for 5 min, and subjected to lytic regimes. Small samples were removed from fibrinolytic reactions at various times and promptly heated at 95°C for 5 min in SDS running buffer (Tris-EDTA buffer [pH 8.0], 2% [vol/vol] SDS, 8 M urea (13, 14). The samples were run on 0.5-mm thick, 4–15% polyacrylamide gels (PhastGel System; Pharmacia LKB Biotechnology Inc., Piscataway, NJ), silver stained, and imaged with a CCD camera (Dage-MTI Inc., Michigan City, IN).

Results

Attenuation of fibrinolysis by tPA, but not uPA, under conditions of diffusion from unmixed extrinsic phase. Placement of Glu-plasmin or Lys-plasmin adjacent to coarse fibrin (3 mg/ml) led to a reaction front, the velocity of which was governed by diffusion and the amount of plasmin at the front (Fig. 1). As the plasmin concentration was increased to 10 μM, v^{ss} continually increased. In this reaction front system, we found that Glu-plasmin and Lys-plasmin were nearly equally effective in dissolving fibrin. This similarity may be due to autocatalytic conversion of Glu-plasmin to Lys-plasmin which was expected to occur during the experiment. We conclude that at very robust levels of fibrinolysis, plasmin does not fall off the fibrin surface in some unusual manner so as to impair lysis at these very high levels of plasmin. During solubilization of fibrin, bound plasmin was returned to solution, but fibrin rebinding by plasmin was evidently fast. No optimal plasmin concentration was observed beyond which lysis was impaired.

Placement of a solution of tPA adjacent to coarse fibrin (3 mg/ml) that contained plasminogen (2.2 μM) caused a lysis front to move across the fibrin. As the tPA diffused into the gel, it activated the intrinsic plasminogen. The lysis front moved faster as the concentration of tPA used for dissolving each gel increased from 0.01 to 10 μM (Fig. 2 *A*). However, at the highest concentration of 80 μM tPA, the front moved significantly slower with a steady state velocity that was only 68% of the maximum velocity measured at 20 μM tPA. This bell-shaped dose-response curve for tPA-mediated fibrinolysis occurred in the absence of antiplasmin and in the absence of extrinsic plasminogen. The presence of 0.1 and 1.0 μM antiplasmin in the fibrin slowed the lysis rates at all concentrations of tPA used. The optimal concentration of tPA at which the maximum v^{ss} was obtained was not affected by the presence of

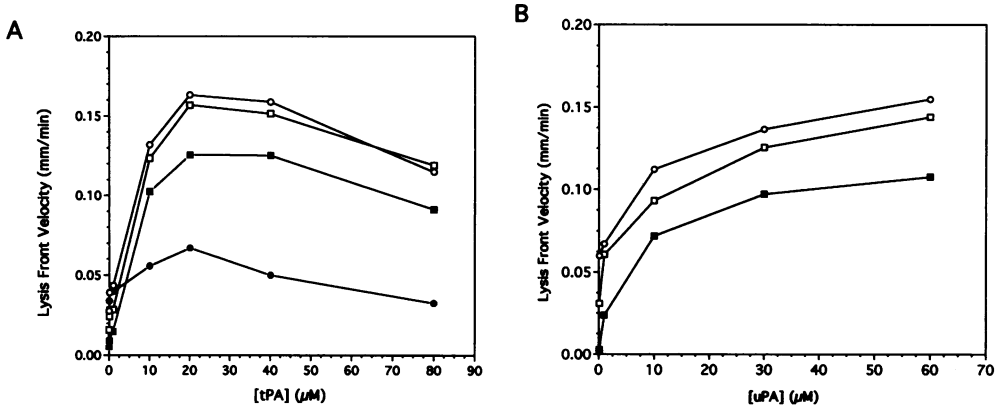

Figure 2. Decreased lysis rates at high tPA concentration, but not uPA, under conditions of diffusion-mediated transport. Steady state lysis front velocity when tPA solutions were placed adjacent to fibrin-containing intrinsic Glu-plasminogen (2.2 μM) and 0.0 (○), 0.1 (□), or 1.0 μM α_2-antiplasmin (■), or when tPA solutions containing extrinsic plasminogen (2.2 μM) were placed adjacent to fibrin containing no intrinsic plasminogen (●) (*A*). Steady state velocity of the fluid/gel interface when uPA solutions of various concentrations were placed adjacent to fibrin containing intrinsic Glu-plasminogen (2.2 μM) and 0.0 (○), 0.1 (□), or 1.0 μM antiplasmin (■) (*B*).

antiplasmin although the antiplasmin did reduce the magnitude of v^{ss}.

To explore the requirement for intrinsic plasminogen, we created coarse fibrin gels that contained no intrinsic plasminogen. A solution with a given concentration of tPA (0.01–80 μM) was rapidly mixed with plasminogen (2.2 μM final concentration) and immediately placed adjacent to each fibrin gel. Some fluid phase activation of plasminogen by the tPA may occur and the resulting plasmin would be part of the lytic cocktail. As expected (3), the lysis rates were considerably slower than those observed with intrinsic plasminogen in the fibrin (Fig. 2 *A*). This is due to the fact that both tPA and plasminogen must penetrate and adsorb to the fibrin, whereas in the previous experiments with intrinsic plasminogen, the plasminogen was equilibrated with the fibrin. Also, plasminogen levels within the boundary layer at the moving front are considerably lower than the bulk concentration. In the absence of intrinsic plasminogen, a maximum in v^{ss} = 0.067 mm/min was observed at 20 μM tPA. At 80 μM tPA, the measured v^{ss} was 51.4% slower than the maximum velocity measured at 20 μM tPA. In this experiment which contained no intrinsic plasminogen, the concentration gradients for plasminogen diffusion could only create fluxes of soluble plasminogen species into the fibrin, not in the reverse direction. From these experiments, we concluded that unusually high concentrations of tPA at the reaction front reduced the efficiency of fibrinolysis, regardless of the source of plasminogen (intrinsic or extrinsic).

We found no maximum in the lysis velocity when solutions with various concentrations of urokinase (0.1–60 μM) were placed adjacent to coarse fibrin (3 mg/ml) containing 2.2 μM of intrinsic plasminogen (Fig. 2 *B*). Similar to the results with tPA (and plasmin), the fastest lysis front velocity that was achievable under conditions of diffusive transport of the species into fibrin was about 0.15 mm/min. Similar to the results with tPA, the presence of 0.1 and 1.0 μM antiplasmin slowed the lysis, particularly at low uPA concentrations and high antiplasmin concentrations where the slow generation of plasmin may not locally deplete the antiplasmin at the front. However, even in the presence of antiplasmin, no maximum in lysis was observed as the urokinase concentration was increased.

Attenuation of fibrinolysis by tPA, but not uPA, under conditions of a well-mixed extrinsic phase reservoir of large volume. The diffusion/lysis front configuration does not allow a large mass of extrinsic tPA to adsorb to the fibrin since the tPA concentration at the lysis front was fed by diffusion from the stationary bulk fluid behind the front, and was subsequently at a lower concentration at the front. Therefore, unusually high concentrations of tPA, approaching the limits of tPA solubility, had to be placed adjacent to the fibrin under these unmixed conditions. In clinical situations, these unusually high concentrations are never used (C_{max} < 15 μM tPA at 1 mg/ml injected concentration). During thrombolytic therapy, substantial amounts of tPA (milligrams) at low concentration are present in the systemic circulation which is in contact with the clot. Continual binding of tPA from the systemic circulation by the clot can then create very high local concentrations of tPA particularly on the proximal face of the clot.

To explore the tPA inhibition of plasmin degradation with fibrin clots decorated with tPA without using high concentrations of tPA, we incubated a 2-mm thick layer of fibrin gel with 2.77 ml of tPA solution for 300 s to allow fibrin adsorption of the tPA from the fluid. After this incubation period, plasmin was added to the extrinsic phase. We found that preincubation of fibrin for 5 min with increasing concentrations of tPA from 0.1 to 1.0 μM tPA caused a dose-dependent inhibition of fibrinolysis when 0.1 μM plasmin was added to the well-mixed extrinsic phase (Fig. 3 *A*). When tPA was added to the extrinsic phase above Glu-plasminogen–laden fibrin (0.1 μM), an optimal tPA concentration was observed that led to rapid lysis, but increasing the tPA concentration above this optimal level results in slower lysis rates (Fig. 3 *B*). Addition of uPA (5 μM) in the extrinsic phase had no effect on fibrinolytic rates when 0.1 μM plasmin was subsequently added to the extrinsic phase (data not shown).

Attenuation of fibrinolysis by tPA, but not uPA, in uniform fibrin fiber suspensions. Since slower lytic rates were observed at high concentrations of tPA regardless of whether the plasminogen (or tPA) was intrinsically or extrinsically supplied, it appeared likely that these unexpected slow lysis rates were an inherent kinetic property of the biochemistry, and would also be observed in well mixed systems. Uniform suspensions of fibrin fibers formed by polymerization of dilute solutions of

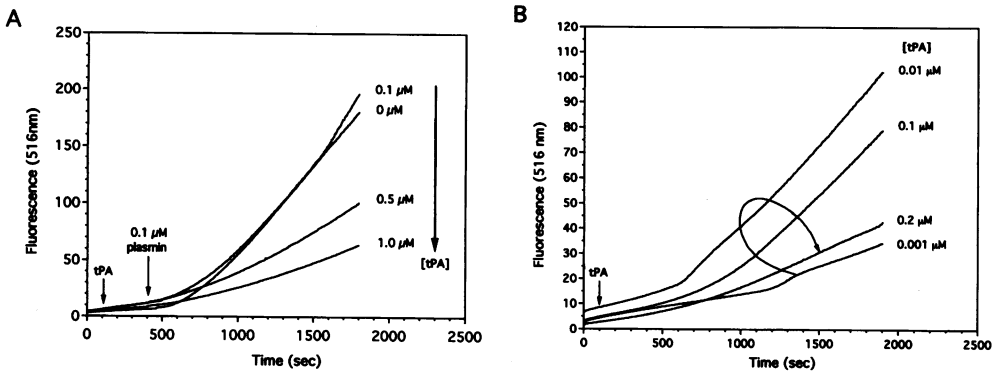

Figure 3. The presence of tPA in a well-mixed extrinsic phase caused a dose-dependent inhibition of plasmin-mediated lysis of fibrin. Various concentrations of tPA from 0.1 to 1 μM were placed in the extrinsic phase above FITC-fibrin gels before addition of 0.1 μM plasmin. Release of fluorescent degradation products into the extrinsic phase was monitored with time (*A*). Various concentrations of tPA from 0.001 to 0.2 μM were added to the extrinsic phase above FITC-fibrin gels that contained 0.1 μM plasminogen, and the release of fluorescent degradation products was monitored with time (*B*). For lysis experiments conducted with FITC-fibrin gels, 100% lysis corresponded to a fluorescence intensity in the extrinsic phase of ~ 550 U.

fibrinogen have no concentration gradients (and no macroscopic diffusional fluxes) and no extrinsic phase. Fibrin fiber suspensions have been previously used for kinetic analysis (12, 15) and binding studies (16). We have used a fluorescence approach to measure lysis under various reaction conditions. The fluorescence dequenching assay relies on the quenched state of FITCs bound to each fibrin monomer and additional quenching due to neighbor–neighbor interactions of the protofibrils in the fibrin fiber. As the fibrin fibers degrade, the FITCs on each fragment are dequenched due to loss of fibril–fibril interaction and loss of interactions within the fibrin monomer subunit. We have previously shown (12) that this increase in fluorescence intensity during lysis is correlated with the release of the α chain fragments during the generation of fragment X, with later stages of degradation contributing less to the overall dequench signal.

Addition of 5 nM plasmin to FITC-fibrin (10 nM) caused an immediate rise in fluorescence emission due to dequenching (Fig. 4). If the FITC-fibrin was preincubated with increasing amounts of tPA (0–160 nM) for 300 s before addition of the plasmin, a dose-dependent reduction in the dequenching rate was observed. At the highest concentration of 160 nM tPA, a time of 800 s was required to obtain a level of dequenching (lysis) that was achieved within 150 s by plasmin in the absence of tPA. Since plasmin was supplied directly into the assay, the tPA was not a catalyst for any reaction and a maximum was not expected. Only attenuation of lysis was found with increasing concentrations of tPA. The initial dequenching rate immediately after addition of plasmin was largely independent of prevailing tPA levels but this rate dropped substantially after ~ 30 s in the presence of tPA. Very similar findings as those seen in Fig. 4 were observed using 1,5-dansyl-Glu-Gly-Arg-chloromethyl ketone–inhibited tPA on fibrin polymerized via recalcification of a mixture of FITC-fibrinogen and platelet-poor plasma isolated from citrated human blood. Thus, tPA can interfere with plasmin activity on fibrin generated from a plasma milieu.

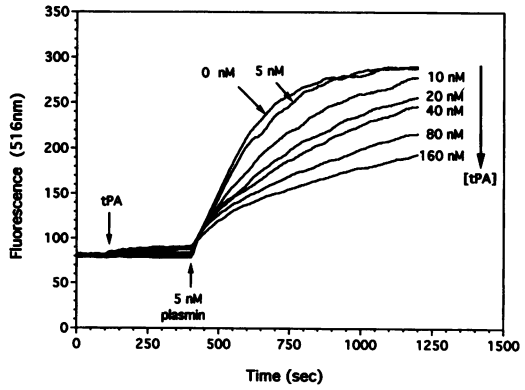

Figure 4. tPA caused a dose-dependent reduction of the lytic action of plasmin on fibrin suspension in the absence of an extrinsic phase. Change in fluorescence intensity (*at 516 nm*) was due to fluorescence dequenching during plasmin-induced lysis of FITC-fibrin (*10 nM*) that had been preincubated in increasing concentrations of tPA from 0 to 160 nM. The tPA was added at 100 s, incubated for 300 s, followed by addition of 5 nM plasmin.

Single chain tPA (sc-tPA) can be converted to two chain tPA (tc-tPA) through plasmin-mediated hydrolysis of the Asp_{275}-Ile_{276} bond. Using a plasmin chromogenic substrate assay, we tested if some component of the tPA solution (clinical grade activase, < 20% tc-tPA) or the tPA itself was interfering or competing with the active plasmin in solution. We found that addition of tPA (0.1 and 20 μM) had little effect on the activity of 10 nM plasmin on 10 μM D-Val-Leu-Lys *p*-nitroanilide (S-2251). Thus, the interaction of tPA with plasmin in solution without fibrin was weak compared to the relatively weak interaction of plasmin with S-2251 which is characterized by a K_m of 120 to 240 μM (17). Unlabeled fibrin at 50 nM (which we have found to be at least 20 times more potent as a competitive substrate than sc-tPA in attenuating plasmin activity on peptide substrates) at 10 times the concentration of tPA (of 5 nM) caused an identical level of inhibition in the dequenching assay. Thus, we conclude that sc-tPA is not a significant competitive substrate of plasmin in free solution. To further demonstrate that sc-tPA is a poor alternative substrate for plasmin in solution, we incubated plasmin with tPA (initially < 20% tc-tPA) for 90 min before addition of the incubated mixture to a 10 nM FITC-fibrin suspension. We found an identical time course and degree of attenuation of the dequenching signal when plasmin (5 nM) and tPA (80 nM), with and without 90-

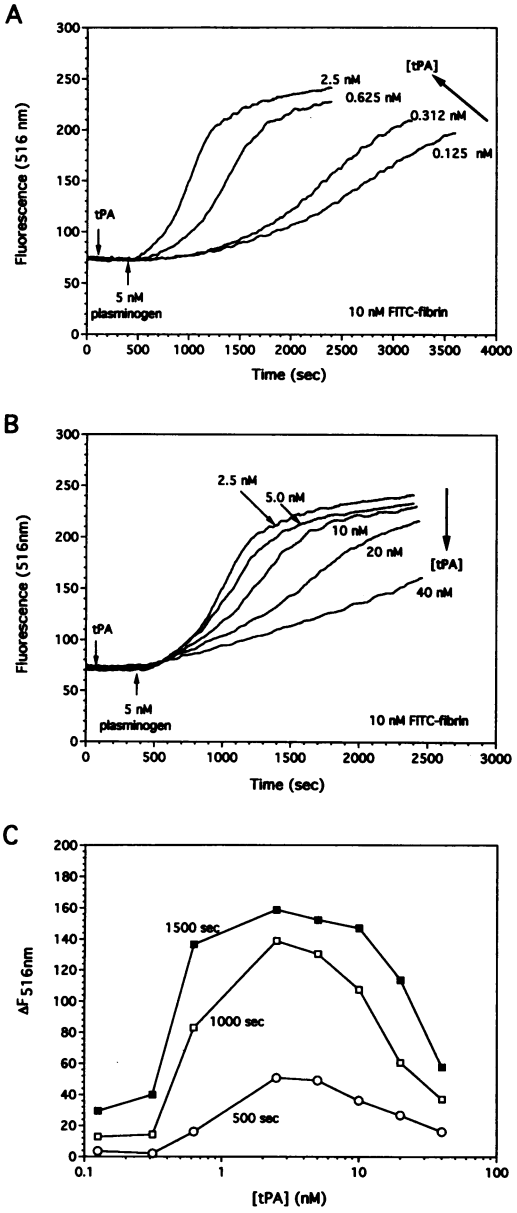

Figure 5. In the absence of an extrinsic phase, high levels of tPA attenuated fibrinolysis of fibrin suspensions containing plasminogen. Change in fluorescence intensity (*at 516 nm*) was due to fluorescence dequenching of FITC-fibrin (*10 nM*) that had been preincubated in increasing concentrations of tPA from 0.125 to 2.5 nM tPA (*A*) and 2.5–40 nM tPA (*B*). The tPA was added at 100 s, incubated for 300 s, followed by addition of 5 nM plasminogen. The $\Delta F_{516\,nm}$ (=F(t) − F_o) at t = 500, 1,000, and 1,500 s after addition of plasminogen demonstrated the maximum in lysis with increasing tPA levels (*C*).

min preincubation, were added to fibrin suspensions. To test for the role of nonspecific protein interactions, we preincubated 10 nM fibrin with 80 and 160 nM of BSA before addition of 5 nM plasmin to recreate the protein content of the high concentration of tPA used in the experiment shown in Fig. 5. We found that this large molar excess of BSA had no effect on plasmin-mediated degradation. Also, we dialyzed the recombinant tPA (1 mg/ml) in the presence of 0.2 M arginine for 24 h. The dialyzed tPA solution caused identical inhibition as was seen with undialyzed tPA (Fig. 4), thus ruling out a role for preservatives or stabilizers in the pharmaceutical preparation. Addition of 100 mM L-arginine, a level exceeding those present in the experiment shown in Fig. 4, had no effect on plasmin-mediated fibrinolysis.

To test whether high concentrations of tPA can interfere with plasminogen activation, we repeated the dequenching experiment by adding plasminogen (instead of plasmin) to the fibrin suspension that had been preincubated with various concentrations of tPA. Since tPA catalyzes plasminogen activation but hinders plasmin-mediated fibrinolysis as seen in Fig. 4, a maximum in lysis rates was expected as the tPA concentration was increased. Indeed, an optimum in the lytic rate was observed (Fig. 5 *A*) when 5 nM of Glu-plasminogen was added to 10 nM of fibrin that had been preincubated with 2.5 nM tPA. In Fig. 5 *A*, increasing the tPA concentration from 0.125 to 2.5 nM led to more rapid lysis. However, when the ratio of tPA to plasminogen was increased up to 8:1, fibrinolysis was significantly impaired (Fig. 5 *B*). Under all conditions, a lagtime of at least 200 s was required before substantial lysis took place. Several kinetic steps account for this lag including: (*a*) plasminogen adsorption to fibrin, (*b*) plasminogen activation, and (*c*) partial degradation of fibrin required before an accelerated phase of lysis (18, 19). In this well-mixed system, a maximum in lysis rates was apparent (Fig. 5 *C*), and was not due to leakage of plasminogen out of the reaction mixture.

However, this dequenching experiment with plasminogen does not resolve whether excess tPA interfered with plasminogen activation. To resolve this issue, we measured the amount of plasminogen that was activated over time. Fibrin (10 nM) was preincubated for 5 min with various concentrations of tPA (0.25–40 nM tPA). Plasminogen (5 nM) was then mixed into the solution and lysis was allowed to proceed. Samples were removed from the reaction mixtures at which time epsilon amino caproic acid (ϵACA) was added to the sample at a final concentration of 100 mM. This concentration of ϵACA dissociated plasminogen and plasmin from the fibrin, causing an end to fibrinolysis, but did not inhibit plasmin. We then immediately assayed each sample for plasmin activity using the chromogenic substrate S-2251 assay. We found that plasminogen activation proceeded more rapidly with increasing concentrations of tPA at all stages of the reaction, and was fastest at the highest levels of tPA used (Fig. 6). Thus, excess tPA had no pronounced inhibitory effect on plasminogen activation. The maximum in lysis observed in Fig. 5 *C* was due to the reduced action of plasmin on fibrin in the presence of tPA.

In similar dequenching experiments using uPA, we preincubated a 10-nM fibrin fiber suspension with up to 80 nM of uPA before addition of 5 nM plasmin. A large molar excess of uPA over plasmin had no effect on plasmin-mediated lysis of fibrin (data not shown). In this experiment, the sc-uPA in the solution could serve as a competitive substrate for plasmin, yet the avidity of plasmin for fibrin was dominant in determining the kinetics of dequenching measured in the assay. This experiment with uPA suggested that tPA inhibition of plasmin degradation of fibrin required the ability of tPA to bind fibrin, as well as ruling against the role of nonspecific protein interactions, or competitive substrate effects. Also, preincubation of 10-nM fibrin suspensions with increasing concentrations of uPA from 1 to 160 nM for 300 s before addition of 5 nM Glu-plasminogen did not cause an inhibition of fibrinolysis as indicated by the rate of dequenching. The rate of fibrinolysis continually increased as

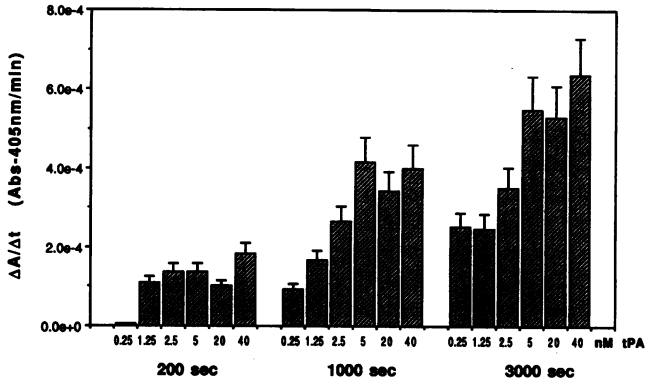

Figure 6. High levels of tPA do not interfere with plasminogen activation in the presence of fibrin. Fibrin (*10 nM*) was preincubated for 5 min with various concentrations of tPA (*0.25–40 nM tPA*). Plasminogen (*5 nM*) was then mixed into the solution and lysis was allowed to proceed. Samples were removed from the reaction mixtures at 200, 1,000, and 3,000 s at which time epsilon amino caproic acid was added to the sample at a final concentration of 100 mM to dissociate plasmin from the fibrin. The measurement of plasmin activity ($\Delta A/\Delta t$) using the chromogenic substrate S-2251 assay demonstrated that plasminogen activation proceeded normally (without inhibition) with increasing concentrations of tPA at all stages of the reaction, and was fastest at the highest levels of tPA (*40 nM*) used.

the uPA concentration was increased, with essentially no lysis detected when fibrin was incubated at the lowest concentration of 1 nM uPA (data not shown).

We carried out SDS-PAGE analysis of fibrin(ogen)olytic reactions. Preincubation of 1.5 μM fibrinogen with 6 μM tPA for 5 min before addition of 10 nM plasmin caused a substantial delay in the appearance of fibrin degradation products as compared to equivalent fibrinogenolysis with no tPA present (Fig. 7, *A* and *B*). During fibrinogenolysis without tPA, fragment X and Y appeared within 2 min (Fig. 7 *A*). However, in the presence of tPA, less fragment X was generated by 2 min and fragment Y did not appear until 10 min into the reaction (Fig. 7 *B*). By 5 min into either reaction (with and without tPA), the highest molecular fibrinogen was degraded with subsequent generation of late fragment X (~ 220 kD). The initial generation rate of late fragment X proceeded slightly slower in the presence of tPA, however the half-life of late fragment X was substantially prolonged due to the tPA in the reaction, and this fragment accumulated to levels greater than those seen when no tPA was present. By 60 min, no fragment X and very little fragment Y were left in the reaction without tPA, while significant quantities of fragments X and Y were present at this time when tPA was present. In the fibrinogenolysis reaction, the tPA was undergoing conversion to tc-tPA by the plasmin. This conversion of sc-tPA (~ 65 kD) to tc-tPA (~ 35 kD, each chain) by proteases is well established (20). Lyophilized activase is < 20% tc-tPA, and less than about half the tPA in the fibrinogenolytic reaction at 1 h was converted to tc-tPA. It appeared that the maximal rate of tc-tPA generation occurred between 2 and 5 min into the reaction (Fig. 7 *B*) concomitant with the appearance of the 220-kD fragment X which is suggestive of close assembly of fibrin-bound plasmin and tPA bound to carboxyl terminal lysines.

We conducted SDS-PAGE analysis of a 1.5-μM solution of fibrin preincubated with and without 6 μM tPA before addition of 10 nM plasmin. During fibrinolysis with and without tPA, fibrin monomer was converted to early and late fragments X within 2–5 min (Fig. 7, *C* and *D*). In the presence of tPA, late fragment X accumulated to higher levels and persisted for longer times (up to 20 min into the reaction) compared to plasmin-mediated fibrinolysis without tPA. Fragment D appeared within 1 min during fibrinolysis without tPA but required over 10 min to first appear in the presence of tPA. Similar to fibrinogenolysis, less than about half of the tPA was converted to tc-tPA during fibrinolysis over 60 min.

Discussion

We have used three distinct types of in vitro fibrinolysis assays: (*a*) diffusional penetration of species into fibrin gels from an unmixed extrinsic phase, (*b*) penetration of species into fibrin gels from a well-mixed extrinsic phase, and (*c*) lysis of uniform dilute suspensions of fibrin fibers. Although the transport phenomena differs in the systems, the biochemical results were identical in all systems when identical reacting components were established in them. tPA significantly reduced in a dose-dependent manner the extent of fibrinolysis when plasmin was added directly to fibrin. A maximum in lytic efficiency during plasminogen activation by increasing concentrations of tPA was observed due to competing effects: as tPA levels increased at constant plasminogen concentration, plasmin was generated faster, but the plasmin was less effective because of the tPA. No maximum was observed with increasing concentrations of uPA in the assays. The observation of reduced lytic efficiency at high concentrations of tPA was independent of α_2-antiplasmin and did not require leakage of intrinsic plasminogen from the fibrin.

The reduced fibrinolysis rate observed at high tPA concentration was not due to decreased plasminogen activation, but was likely due to events relating to molecular assembly on fibrin. This slower fibrinolysis may be due to several distinct mechanisms: (*a*) fibrin-bound tPA hindered or modulated the ability of fibrin-bound plasmin to cleave certain sites, (*b*) soluble tPA out competed plasmin for newly revealed binding sites, and/or (*c*) fibrin-bound sc-tPA acted as a competitive substrate for fibrin-bound plasmin. From SDS-PAGE analysis of plasmin-mediated fibrinolysis (Fig. 7), it appeared that the half-life of fragment X was prolonged in the presence of high tPA levels. This was consistent with the dequenching experiment (Fig. 4) where the initial rate was largely independent of tPA level, but declined shortly thereafter when the prevailing tPA level was at elevated levels. We conducted a preliminary study of plasmin binding to the surface of fibrin gels (3 mg/ml) with and without plasmin nicking. We found that 160 nM of 1,5-dansyl-Glu-Gly-Arg-chloromethyl ketone–inhibited tPA caused an 80% reduction of the binding of D-Val-Phe-Lys-chloromethyl ketone-inhibited [^{125}I]-Glu-plasmin (5 nM) to the plasmin-generated binding sites in nicked fibrin.

Sobel et al. (1) first described "plasminogen steal" as a situation of attenuated lysis at high plasminogen activator levels where depletion of systemic plasminogen reduced plasminogen in the clot. Torr et al. (2) found that high concentrations of tPA up to 8,000 ng/ml (0.1 μM tPA) could reduce by 90% after 30 min, the amount of clot-bound plasminogen extractable from washed, homogenized human whole blood clots. Addition of plasminogen to the system enhanced lysis rates at the very high levels of tPA where unexpectedly slower lytic rates had been

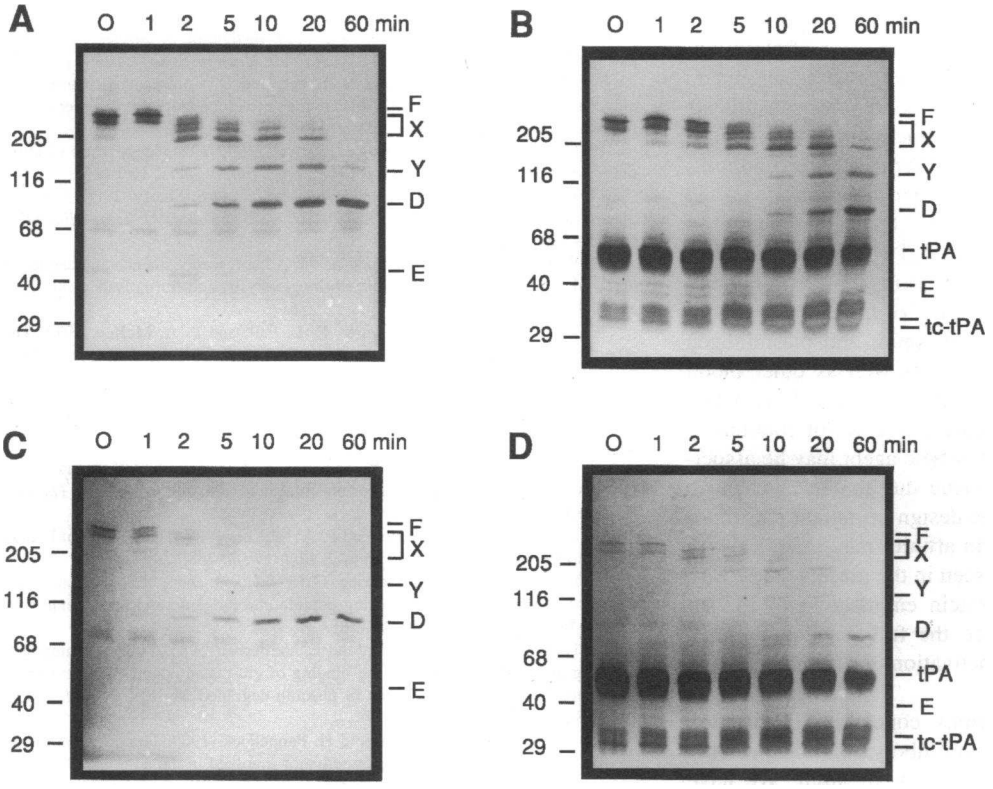

Figure 7. SDS-PAGE analysis of fibrin degradation products generated by the action of plasmin (*10 nM*) on 1.5 μM fibrinogen (*A* and *B*) or 1.5 μM fibrin (*C* and *D*) in the presence of no tPA (*A* and *C*) or 6 μM tPA (*B* and *D*) at various time points indicated at the top of each lane. Molecular weight in kilodaltons (*left*) and fragment identification (*right*) are indicated for each gel based on size and order of appearance (13, 14).

observed. Similarly, Onundarson et al. (3) found a maximum lysis rate of 2-h old, retracted and washed whole blood clots incubated in a well-mixed solution of 1,000 ng/ml tPA, while the lysis rate was slower at 5,000 and 10,000 ng/ml tPA. No maximum was observed when the incubating fluid contained 0.5 U/ml plasminogen ($\sim$ 1 μM). Nishino et al. (4) found bell-shaped dose-response curves for uPA- and tPA-mediated lysis of compacted fibrin thrombi (from cross-linked plasma) incubated in lytic mixtures. In this system, lysis was optimal at 50 U/ml tPA and 50 U/ml sc-uPA, and was greatly decreased at 5,000 U/ml of tPA or sc-uPA. Similar maxima were seen with plasma gel clots, but were less pronounced since lysis was only slightly slower at 5,000 U/ml of tPA or sc-uPA. By increasing the intrinsic Lys-plasminogen to 50 μg/ml, bell-shaped dose-response curves were not found even at 5,000 U/ml tPA. The authors indicated that the slow rate of lysis observed in their experiments at high tPA levels arises from depletion of extrinsic plasminogen, with possible compounding effects depending on α_2-antiplasmin to plasminogen ratios.

Using purified systems, we did not find a maximum in lysis rates for administration of uPA to fibrin gels or to uniform suspensions of fibrin fibers. It is possible that the interaction of uPA with fibrin generated during the coagulation of whole blood is different than the interaction with fibrin polymerized from purified fibrinogen. For example, high levels of Zn^{2+} ($>$ 20 μM) believed to be present in clots have been shown to promote urokinase association with fibrin in vitro (21). Using the fluorescence dequench assay conducted in the presence of 500 μM Zn^{2+}, we found that uPA at a 32-fold molar excess over plasmin had no inhibitory effect when plasmin (5 nM) was added to 10 nM fibrin suspension. We conclude from this preliminary experiment that urokinase bound to fibrin in the presence of Zn^{2+} had no inhibitory effect on plasmin. The mechanism(s) whereby slower lysis rates are found at high concentrations of uPA remain to be elucidated.

The finger (F) and the kringle 2 (K2) domains of tPA are involved in the high affinity binding to internal lysine residues of the D domain of undigested fibrin ($K_d \sim 0.5$ μM; $\sim$ two sites per monomer) (19, 22). The interaction of tPA with fibrin involves interactions with D domain epitopes α(148–160) and γ(311–379) some of which are hidden in fibrinogen (23–25). However, K1 of tPA may also contribute to some extent toward fibrin binding (26). The binding of tPA to undegraded fibrin is not blocked by Glu-plasminogen (27). Plasminogen binding to undigested fibrin is of moderate affinity (Lys-plasminogen, $K_d \sim 0.2$–10 μM; Glu-plasminogen, $K_d \sim$ 20–40 μM) (22), and is not blocked by tPA (27). Inner chain lysines in undigested fibrin are believed to be specific for the F/K2 domains of tPA and K1–K5 domains of plasminogen (27).

The degradation pathway during fibrinolysis is well established (13, 14, 28). Plasmin cleaves several sites in the COOH terminus of the α chains of fibrin (including: $\alpha Lys_{206,219,230}$) and the $\beta(Arg_{42}–Ala_{43})$ bonds to yield fragment X (240–320 kD) polymer. Fragment X polymer generation in fibrin is considered to be the critical stage for enhanced tPA binding to partially degraded fibrin (29), and this augmented tPA binding requires carboxy-terminal lysine residues (19). Plasminogen also displays augmented binding to partially degraded fibrin (18). It has been shown that Glu-plasminogen can compete with tPA for the carboxy-terminal lysine residues in digested fibrin (27). We suggest that a similar competition may exist between plasmin and tPA for these revealed sites in partially degraded fibrin.

There is growing awareness that plasma can permeate through interstitial regions of blood clots and fibrin, driven by

arterial pressures (5–8). In clinical situations, depletion of circulating plasminogen will result in the permeation of plasminogen-poor plasma into the front of the clot with a subsequent efflux out the back of the clot of fluid containing constituents of the coagulation milieu. Also, clot retraction may exude plasminogen-rich fluid from the clot (30). Under these conditions of low prevailing inner clot plasminogen concentrations and low plasmin generation, the interference of plasmin action on fibrin by excess tPA may be pronounced. To optimize an administration regime of thrombolytic agents to achieve minimum reperfusion times requires consideration of the prevailing concentrations of plasminogen, α_2-antiplasmin, and plasminogen activator inhibitor type 1 (3, 9, 31), as well as other blood constituents (lipoprotein [a], platelets, etc.) (32). Even when systemic side effects are not considered, the use of continually increasing concentrations of thrombolytic agent may be associated with decreased therapeutic value due to the mechanism described in the present study. The design of mutant plasminogen activators with increased fibrin affinity may interfere with the action of plasmin on fibrin as seen in the present study with recombinant human tPA. The protein engineering of mutant activators should seek to optimize the full reaction network which includes both fibrin phase activation of plasminogen and plasmin degradation of fibrin.

We have presented observations concerning a paradox called "plasminogen steal" where thrombolysis is unexpectedly slower when high concentrations of tPA are used. We have found that α_2-antiplasmin is not required for the phenomenon. The term "plasminogen steal" may not best describe the actual mechanism(s) that result in the observed reduction in lysis at high concentrations of tPA since leakage of intrinsic plasminogen out of the clot is not required. Distinct from important transport processes which alter prevailing concentrations of fibrinolytic mediators, the direct biochemical effects of tPA on the action of plasmin on fibrin should also be considered. We have shown that tPA can directly interfere with the ability of plasmin to degrade fibrin.

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