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Conjugation to an Antifibrin Monoclonal Antibody Enhances the Fibrinolytic Potency of Tissue Plasminogen Activator in Vitro†

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ABSTRACT: Tissue plasminogen activator (tPA) was covalently linked by disulfide bonds to a monoclonal antibody specific for the amino terminus of the β chain of fibrin (antibody 59D8). The activity of the tPA–59D8 conjugate was compared with that of tPA, urokinase (UK), and a UK–59D8 conjugate. For lysis of fibrin monomer, tPA was 10 times as potent as UK, whereas both UK–59D8 and tPA–59D8 conjugates were 100 times as potent as UK and 10 times as potent as tPA. Conjugation of tPA or UK to antibody 59D8 produced a 3.2–4.5-fold enhancement in clot lysis in human plasma over that of the respective unconjugated plasminogen activator. However, the UK–59D8 conjugate was only as potent as tPA alone. Antibody-conjugated tPA or UK consumed less fibrinogen, α_2-antiplasmin, and plasminogen than did the unconjugated activators, at equipotent fibrinolytic concentrations. Antibody targeting thus appears to increase the concentration of tPA in the vicinity of a fibrin deposit, which thereby leads to enhanced fibrinolysis.

The use of thrombolytic agents in the treatment of acute myocardial infarction is receiving increasing attention because of recent reports that both mortality and morbidity can be reduced by their early administration (GISSI, 1986; TIMI, 1985). However, the frequency of hemorrhagic complications resulting from this form of therapy has prompted investigators to seek more specific agents.

The principle of antibody targeting of cytotoxic agents to cell-surface antigens has been explored extensively (Letvin et al., 1986; Marsh & Neville, 1986; Ramakrishnan & Houston, 1985; Vitetta & Uhr, 1985). The proposed advantage is the selective ablation of a subset of cells identified by the antibody. We reasoned that the same concept could be applied to the directing of lytic agents; in particular, we sought to improve the specificity of tissue plasminogen activator (tPA).[1] Our laboratory has already demonstrated that a covalent (disulfide) complex of murine fibrin-specific antibody (antibody 64C5) and urokinase (UK) is 100 times more efficient than UK alone in an in vitro fibrinolytic system (Bode et al., 1985). The chemical coupling strategy employed at that time, however, was limited by poor yields of functional, coupled urokinase. Here we used a chemical coupling strategy that entails modification of the antibody with 2-iminothiolane and formation of a disulfide bond between it and the plasminogen activator (Runge et al., 1987). This strategy produces conjugates in yields sufficient for definitive testing. The fibrinolytic potency and specificity of conjugates of antifibrin monoclonal antibody 59D8 and either tPA or UK were enhanced in comparison with those of the unconjugated plasminogen activators.

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[1] Abbreviations: 59D8, antifibrin monoclonal antibody 59D8; S-2288, chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride; S-2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; PBSA, phosphate-buffered saline azide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; tPA, tissue plasminogen activator; UK, urokinase; Tris, tris(hydroxymethyl)aminomethane.

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Materials and Methods

Single-chain recombinant tPA[2] was obtained from Genentech (South San Francisco, CA), and two-chain, low molecular weight urokinase (Abbokinase) was obtained from Abbott Laboratories (North Chicago, IL). Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and 2-iminothiolane were purchased from Pierce Chemical (Rockford, IL), and Sepharose CL-4B was obtained from Pharmacia (Piscataway, NJ). The ^{125}I-labeled fibrinogen came from Amersham International (Arlington Heights, IL), and we bought plasma from the local blood bank. H-D-Isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2288), a chromogenic substrate for proteases, was obtained from Helena Labs (Beaumont, TX), as was H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251). All other chemicals came from Sigma (St. Louis, MO).

Preparation and Purification of Monoclonal Antifibrin Antibody 59D8. The identification, characterization (Hui et al., 1983), and purification (Runge et al., 1987) of monoclonal antibody 59D8 have been described. We chose antibody 59D8 over the previously used antibody 64C5 because 59D8 has a greater specificity for fibrin.

Electrophoresis and Densitometric Scanning. SDS–PAGE was performed according to the method of Laemmli (1970), and the proteins were visualized with Coomassie Brilliant Blue R. For relative protein quantitation, the gels were scanned at 595 nm with a Zeinch "Soft Laser" scanning densitometer, Model SL-504-XL (Biomed Instruments). Because the correlation between Coomassie Brilliant Blue R binding and protein content is linear only between 1 and 20 μg in our hands, a determination of the relative ratios of tPA and antibody 59D8 was made only from gels that contained amounts of protein within this range. For these calculations, we assumed Coomassie Brilliant Blue R staining was equal for all proteins examined. Quantitation was made on reduced gels, which provided separate bands for tPA and the heavy and light chains of antibody 59D8.

Radiolabeling. Radioiodination of tPA and UK was carried out by the Chloramine-T method (Hunter & Greenwood, 1962), using ^{125}I obtained from Amersham International. Both ^{125}I-labeled UK and ^{125}I-labeled tPA gave single bands on SDS–PAGE.

Preparation and Purification of tPA–59D8 and UK–59D8 Conjugates. Disulfide-linked plasminogen activator–antibody conjugates were prepared by reacting an SPDP derivative of either tPA or UK with 2-iminothiolane-substituted antifibrin monoclonal antibody 59D8. The details of the preparation and purification are reported elsewhere (Runge et al., 1987).

Purification of Conjugates. The reaction mixtures were purified in two sequential affinity chromatography steps: after selection for plasminogen activator binding to benzamidine–Sepharose, the eluate was passed through a column of Sepharose conjugated to the synthetic peptide that had been used to raise antibody 59D8 (Gly-His-Arg-Pro-Leu-Asp-Lys-Cys, β peptide; the peptide represents the seven amino-terminal residues of the β chain of human fibrin). The tPA–antidigoxin antibody conjugate (control) was also purified by two affinity steps: first by benzamidine–Sepharose and second by a ouabain affinity column, from which bound antidigoxin antibody was eluted with 20 mM ouabain.

Quantitation of Plasminogen Activator in Various Preparations. To compare native plasminogen activators (UK or tPA) with antibody-linked plasminogen activators, the amidolytic activities and molar amounts were determined in the following manner. Standardized samples of UK in Plough units and milligrams per milliliter (as aliquots from a freshly resuspended vial of Abbokinase) and of single-chain recombinant tPA in International units and milligrams per milliliter (as aliquots from a freshly resuspended vial of tPA) were analyzed in a chromogenic substrate assay (S-2288 in 0.15 M Tris and 0.15 M NaCl, pH 8.4, with a substrate concentration of 1×10^{-3} mol/L and an enzyme concentration of 8×10^{-9} mol/L). For UK, 1 Plough unit was assumed to equal 1.8×10^{-4} nmol; for tPA, 1 International unit was assumed to equal 6.3×10^{-5} nmol. The correlation between the change in absorbance per minute reported for the S-2288 assay[3] and that for our samples was excellent, such that 100 units of UK (8×10^{-5} mmol) or 100 units of single-chain tPA (6.3×10^{-6} mmol) gave a change in absorbance at 405 nm of approximately 0.060/min. On the basis of these results, whenever a determination was made on the activity (in appropriate units as above) or molar amount (in millimoles) of active enzyme in an unknown sample of UK or tPA, the sample was diluted until an absorbance change of 0.060/min at 405 nm occurred in an S-2288 assay, as described above. The linear range of enzyme concentration to absorbance change was from 4×10^{-10} mol/L to 3.2×10^{-8} mol/L in our hands. If concentrations above this range were used in assays for fibrinolysis, appropriate dilutions were made from stock solutions containing higher concentrations (aliquots of which had been tested at a 1:10 dilution in the S-2288 assay).

Fibrin Monomer–Sepharose Assay. The preparation of ^{125}I-labeled fibrin–Sepharose has been previously described (Bode et al., 1987). To assess fibrinolytic activity, unconjugated tPA or UK, tPA–59D8, UK–59D8, or the tPA–antidigoxin conjugate (units as indicated in 100 μL) was incubated with 100 μL of fibrin–Sepharose for from 2 to 4 h. The Sepharose was washed first with 3 mL of a solution containing 0.1 M Tris, 0.1 M NaCl, 0.5% Triton X-100, 0.1% Tween-80, and 0.5% bovine serum albumin and then 3 times with 3-mL aliquots of 0.1 M Tris, 0.1 M NaCl, and 0.02% NaN_3 (pH 7.4). Thereafter, the resin was incubated at room temperature with 1 mL of purified plasminogen (0.15 mg/mL in 50 mM phosphate buffer, pH 7.4). After 15 h, the mixture was centrifuged, and radioactivity in the supernatant was determined in a γ scintillation counter. The percentage of fibrin lysis was determined from the ratio of radioactivity present in the supernatant to the total initial radioactivity.

Fibrinogen Assays. Two methods were used to determine the fibrinogen content of the samples of citrated human plasma. Clottable fibrinogen was measured by the method of Clauss (1957), and the total amount of fibrinogen was determined by sodium sulfite precipitation (Rampling & Gaffney, 1976).

α_2-Antiplasmin and Plasminogen Assays. S-2251 was used to determine α_2-antiplasmin (Edy et al., 1976) and plasminogen (Friberger & Kiros, 1979) levels as a percentage of normal levels in citrated human plasma.

Plasma Clot Assay. The method of Lijnen et al. (1984b) was used with the following modifications. Fresh-frozen human plasma obtained from four donors was pooled, aliquoted, and refrozen. Immediately before each experiment, the activities of tPA, UK, and their conjugates were calibrated by using the S-2288 assay (i.e., the peptidase activities of the native activators and their conjugates were determined and

[2] tPA is measured in international units; urokinase is measured in Plough units.

[3] Kabi Vitrum, S-2288 data sheet.

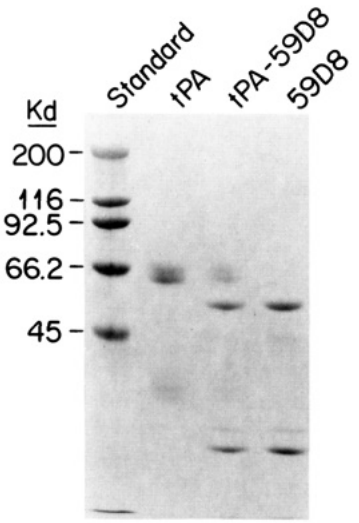

FIGURE 1: SDS–PAGE of tPA–antifibrin antibody conjugate. Molecular weight standards, tPA, purified tPA–antifibrin antibody conjugate (tPA–59D8), and antibody 59D8 were electrophoresed on a 7.5% polyacrylamide gel under reducing conditions.

related to a standardized concentration of UK, and appropriate dilutions were made so that the peptidase activity, in units per milliliter, was identical for each sample). Plasma clots were made by adding each of the following to fresh–frozen plasma: thrombin, 8 NIH units/mL; 0.5 M $CaCl_2$, 100 μL/mL; and ^{125}I-labeled human fibrinogen, 100000 cpm/mL. The solution was immediately drawn into Silastic tubing (inner diameter 4 mm) and incubated at 37 °C for 30 min. Silastic tubing containing clotted fresh–frozen plasma was cut into 1-in. sections, yielding clots of 0.2 mL. These were then washed in 0.15 M NaCl before use. Each clot was placed in a plastic tube, counted, and suspended in 1 mL of fresh–frozen plasma (from the same pool). Experiments were initiated by the addition of a plasminogen activator (or conjugate of plasminogen activator and antibody). At 30-min intervals, an aliquot of the fresh–frozen plasma was removed from each tube for counting. Samples were saved at the end of the experiment for determination of fibrinogen, α_2-antiplasmin, and plasminogen levels.

RESULTS

Characterization of Conjugates. The nature of the disulfide-linked conjugates obtained by the SPDP–iminothiolane coupling protocol used here is defined in part by their purification (Runge et al., 1987). Because of the two-step affinity purification procedure, conjugates must contain both the ability to bind to benzamidine and the ability to bind to the β-peptide column (i.e., antibody activity). When electrophoresed under reducing conditions, the disulfide bond is broken, and one can visualize the individual components (Figure 1). A 0.99:1.0 molar ratio of tPA to 59D8 resulted from this coupling procedure, as determined by densitometric quantitation of the scans. A 2.8:1 molar ratio of UK to 59D8 was obtained (Bode et al., 1987). Radioautograms of nonreducing gels comparing ^{125}I-labeled tPA (or UK) with ^{125}I-labeled tPA–59D8 (or ^{125}I-labeled UK–59D8)—only the plasminogen activator was labeled—demonstrate that the major product is of a higher molecular weight than that of either the activator or the antibody alone. The most prominent band corresponded to a 1:1 complex of tPA–59D8; however, larger species were also present.

Fibrin Monomer–Sepharose Assay. UK, tPA, and their conjugates were compared in the fibrin–Sepharose assay as follows. The concentration of each activator was first determined as described under Materials and Methods. Samples were diluted so that the activator concentration ranged from 0.1 unit/100 μL (6.3×10^{-9} mmol of tPA or 1.8×10^{-8} mmol of UK) to 1000 units/100 μL (6.3×10^{-5} mmol of tPA or 1.8×10^{-4} mmol of UK) as indicated. Figure 2 shows that, in the fibrin–Sepharose assay, tPA is approximately 10-fold more active in fibrinolysis than UK. For example, about 100 units of UK (1.8×10^{-5} mmol) are required to obtain approximately 15% lysis, whereas only 10 units (6.3×10^{-5} mmol) of tPA are required to obtain a similar degree of fibrinolysis. There is no significant difference between the fibrinolytic activity of the UK–59D8 and tPA–59D8 conjugates. Both are 100-fold more potent than UK and 10-fold more potent than tPA, as evidenced by Figure 2.

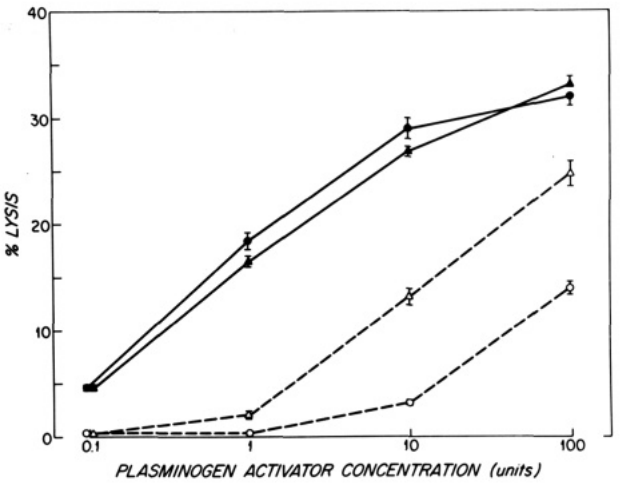

FIGURE 2: Analysis of conjugates in a fibrin–Sepharose assay. tPA–59D8 (closed circles), tPA alone (open triangles), UK–59D8 (closed triangles), and UK alone (open circles) were tested. Means and standard deviations from three samples are shown.

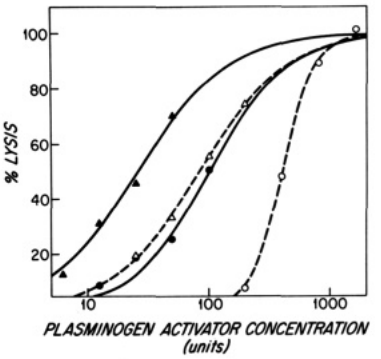

FIGURE 3: Clot lysis in plasma. tPA–59D8 (closed triangles), tPA alone (open triangles), UK–59D8 (closed circles), and UK alone (open circles) were tested as described under Materials and Methods. Before each experiment, the concentration of peptidase activity in each sample was calibrated by the S-2288 assay. Shown here are the results at 90 min. Each point represents the mean of four different samples. When analyzed by the Fit–Function program (BBN Research Systems, 1983), all curves differed from one another significantly ($p < 0.0001$), with the exception that those for tPA alone and UK–59D8 did not significantly differ.

Clot Lysis in Plasma. UK, tPA, and their conjugates were then compared in an assay for human clot lysis. Immediately before each plasma clot assay, the peptidase activity of each sample was determined by using the S-2288 assay as described. Appropriate dilutions were made so that comparable samples of unconjugated activator or activator–antibody conjugate contained identical peptidolytic activities. Figure 3 shows the means and fitted curves for clot lysis in this plasma system. At equal UK concentrations, there is a mean gain in potency

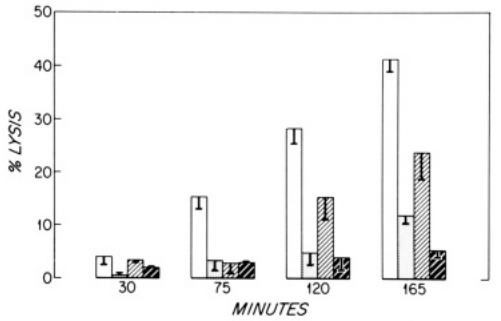

FIGURE 4: Time course of clot lysis in plasma. Clot lysis throughout the course of an experiment is shown at one activator concentration: 50 units of tPA–59D8 (open bar), tPA (stippled bar), or UK–59D8 (finely hatched bar), and at 200 units of UK (broadly hatched bar). Means and standard deviations are shown.

(over all concentrations) of 4.5-fold for the UK–59D8 conjugate ($p < 0.0001$). In the plasma clot assay, in contrast to the fibrin–Sepharose assay, UK–59D8 is only as effective as tPA alone (p not significant). The tPA–59D8 conjugate is 3.2-fold more effective than tPA alone (and than UK–59D8) throughout the concentration range ($p < 0.0001$). When one compares the relative advantage of tPA–59D8 over tPA (or that of UK–59D8 over UK) as a function of the percent lysis, a greater enhancement in potency is seen at the lower levels of lysis. Figure 4 demonstrates that the enhanced fibrinolytic potency seen at 75 min in Figure 2 is present throughout the course of the experiment. In similar assays, the tPA–anti-digoxin antibody conjugate displays clot lysis potency identical with that of tPA alone, and it is 3–5-fold less efficient than the tPA–59D8 conjugate.

Fibrinogen, α_2-antiplasmin, and plasminogen levels determined from the supernatants of human plasma clot assay samples (exposed to either tPA–59D8 or tPA alone) are shown in Figure 5. In each instance, the amount of fibrinogenolysis seen at approximately equal levels of clot lysis is substantially less for tPA–59D8 than for tPA alone. Similarly, less α_2-antiplasmin was consumed by the tPA–59D8 conjugate. Differences in plasminogen levels were less dramatic. The results comparing UK–59D8 with UK alone displayed a similar trend (data not shown).

Discussion

We report the synthesis and characterization of a disulfide-linked conjugate between tPA and an antifibrin monoclonal antibody (59D8). The SPDP–iminothiolane coupling strategy used here (Runge et al., 1987) is significantly more efficient than that previously reported for the coupling of UK to an antifibrin monoclonal antibody (Bode et al., 1985). Yields of from 15% to 25% of functional coupled activator can be obtained with the SPDP–iminothiolane method, as opposed to the 1–2% yields obtained by the previously described method (Bode et al., 1985). This enhanced yield makes the experiments described here technically feasible. Others have recently used the SPDP–iminothiolane coupling strategy to conjugate specific antilymphocyte antibodies to cytotoxic agents (Letvin et al., 1986; Marsh & Neville, 1986). In our hands, as in theirs, the strategy is reproducible and provides conjugates stable in vivo and in vitro (for 1 week) (Marsh & Neville, 1986).

Comparisons between native plasminogen activators and activators conjugated to antibody 59D8 were made at equal molar amounts (and at equal Plough units for UK or International units for tPA). Enzyme and enzyme–antibody conjugates were calibrated by an amidolytic assay with the colorimetric substrate S-2288. We considered this assay most appropriate because the interest of these experiments is in the comparison of plasminogen activation in the presence of fibrin (as measured by fibrinolysis) by different enzymes with the same amidolytic activity. We reasoned that physical methods of measuring the relative concentrations of enzyme protein could be misleading. Some of the conjugates are clearly heterogeneous because of variable degrees of cross-linking as well as varying target-reactive groups. This could result in partial inactivation of enzymatic activity in some species, and thus in misinterpretation.

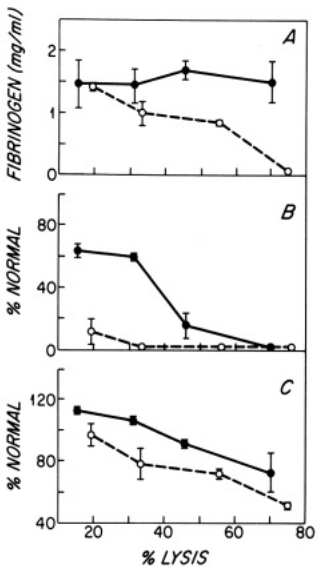

FIGURE 5: General lytic effects of tPA (open circles) and of tPA–59D8 conjugate (closed circles). The fibrinogen, α_2-antiplasmin, and plasminogen contents in aliquots of the plasma clot assay shown in Figure 3 were determined. (A) Fibrinogen (in milligrams per milliliter) is shown as a function of the percent clot lysis in each sample. α_2-Antiplasmin (B) and plasminogen (C) levels are shown as their percentage of normal levels compared to control aliquots of human plasma. In all cases, the points represent the mean of three separate determinations. Standard deviations are shown ($n = 3$).

When tested in the fibrin–Sepharose assay, which is carried out in the absence of plasma, both tPA–59D8 and UK–59D8 were 100 times as potent as UK and 10 times as potent as tPA. In the plasma clot assay, the tPA–59D8 conjugate is, on the average, 3.2-fold more potent than either tPA alone or UK–59D8 and is approximately 10-fold more potent than UK alone. Previous reports document that, in plasma, UK is less effective in clot lysis than tPA (Lijnen et al., 1984a; Matsuo et al., 1981). Interestingly, although the UK–59D8 conjugate was significantly more potent in clot lysis than UK alone, it was only as effective as tPA. This suggests that despite the increased fibrin specificity obtained by conjugation to 59D8, inhibition of the fibrinolytic activity of UK persists. Several plasma inhibitors of plasminogen activators have been described (Colucci et al., 1985; Erickson et al., 1984); any of these could be responsible for the effects observed. The relatively greater inhibition of the urokinase conjugage in comparison with the tPA conjugate cannot now be explained.

It is of critical importance to determine whether the increase in fibrinolytic potency is accompanied by an increase in specificity. In human plasma, there is a marked decrease in the consumption of fibrinogen and α_2-antiplasmin, and a less marked decrease in the consumption of plasminogen, when either tPA–59D8 or UK–59D8 is used rather than the respective unconjugated activator (at equal fibrinolytic potency). For example, 50 units (3.1×10^{-6} mmol) of tPA–59D8 caused plasma fibrinogen levels to decrease about 25%, whereas 200

units (1.2×10^{-5} mmol) of tPA alone (which achieves the same degree of clot lysis) caused plasma fibrinogen levels to decrease approximately 90% (Figure 5).

Our data suggest that the increased potency and specificity of tPA–59D8 reside in the conjugate's increased affinity for fibrin. The dissociation constant of tPA for fibrin is 0.16×10^{-6} M (Holyaerts et al., 1982), while that of antibody 59D8 is 0.77×10^{-10} [based on the binding to a firbin-like fragment called (T)N-DSK (Bohdan J. Kudryk, personal communication)], nearly 1000-fold greater. Somewhat surprisingly, fibrinolytic potency appears to neither increase nor decrease when tPA [or UK (Bode et al., 1987)] is conjugated to an antibody of irrelevant specificity (antidigoxin antibody). This may reflect the removal of inactive species during affinity chromatography on benzamidine–Sepharose.

High-affinity binding to fibrin appears to increase the local concentration of the antibody-bound plasminogen activator, resulting in a higher local concentration of plasmin. Thus, fibrinolysis at the clot is favored over fibrinogenolysis in the supernatant plasma. The plasminogen that does become activated is probably bound to the fibrin clot and is thus less available for fibrinogenolysis or inactivation of α_2-antiplasmin in the supernatant plasma. Conjugates formed on the basis of these principles may have advantages when used in vivo.

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