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A Bispecific Antibody Enhances the Fibrinolytic Potency of Single-Chain Urokinase†

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Received August 30, 1989; Revised Manuscript Received January 24, 1990

ABSTRACT: A monoclonal antibody specific for an epitope at the amino terminus of the β chain of fibrin and a monoclonal antibody that binds both one- and two-chain high molecular weight urokinase were chemically cross-linked [using *N*-succinimidyl 3-(2-pyridyldithio)propionate and 2-iminothiolane]. The chemically modified material was heterogeneous, ranging in molecular size from tetramers to monomers and containing the two antibodies in various ratios. Nevertheless, fractions of a molecular size larger than a monomer were capable of binding fibrin and urokinase simultaneously in a radioimmunoassay. These fractions also enhanced fibrinolysis by high molecular weight single-chain urokinase (scuPA) by 50-fold and plasma clot lysis by 5-fold. Whereas scuPA significantly decreased the concentration of fibrinogen in plasma clot assay supernatants, scuPA in association with the bispecific antibody did not.

Tissue plasminogen activator (tPA)[1] and single-chain urokinase plasminogen activator (scuPA) interact with plasminogen to convert it to the active enzyme plasmin. Plasmin then lyses thrombi by degrading fibrin. Bleeding complications arise because at the doses required for the prompt lysis of thrombi the generation of excess plasmin may result in the degradation of fibrinogen, α_2-antiplasmin, factors V and VIII, and the platelet GPIIb/IIIa receptor complex that binds fibrinogen (Bennett et al., 1982; Verstraete & Collen, 1986). The degradation of these proteins (and others important in hemostasis) can induce a systemic lytic state. Increasing the fibrin specificity of plasminogen activators has been proposed as a mechanism by which plasmin generation at the site of a thrombus can be enhanced and systemic activation of the fibrinolytic system can be avoided (Collen et al., 1989; Dewerchin et al., 1989; Haber et al., 1989; Maksimenko & Torchilin, 1985; Nakayama et al., 1986; Robbins & Boreisha, 1987). The high affinity and specificity of antifibrin monoclonal antibodies makes them suitable agents for further increasing the fibrin specificity of plasminogen activators such as tPA and scuPA.

In this paper, we describe the preparation and biochemical characterization of a bispecific (antifibrin–antiurokinase) antibody. A high-affinity antifibrin monoclonal antibody, 59D8 (Hui et al., 1983), was chemically coupled to an antiurokinase monoclonal antibody, PEG2, that had been produced

† Supported in part by National Institutes of Health Grants HL-19259 and HL-28015.
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[1] Abbreviations: tPA, tissue plasminogen activator; scuPA, high molecular weight single-chain urokinase; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

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by fusing SP2/0 cells with spleen cells from mice immunized with human high molecular weight two-chain urokinase. We also characterize the ability of the bispecific antibody to enhance the fibrinolytic potency of scuPA in human plasma.

Materials and Methods

High molecular weight two-chain urokinase (100 000 IU/mg) was purchased from Serono Laboratories, tris(hydroxymethyl)aminomethane from Aldrich, fibrinogen from Kabi Vitrum, ^{125}I-labeled fibrinogen (IBRIN) from Amersham, IODO-GEN, 2-iminothiolane hydrochloride, and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) from Pierce Chemical, iodoacetamide from Sigma, DE52 from Whatman, and Sephacryl ACA-34, Sephacryl S-300, and Sepharose CL4B from Pharmacia Fine Chemicals. The high molecular weight scuPA was a generous gift of Dr. Dêsirê Collen, University of Leuven, Belgium.

Antibody Production. Monoclonal antibody 59D8, which is specific for an epitope at the amino terminus of the β chain of fibrin, was raised and purified as previously described (Hui et al., 1983). Urokinase-specific monoclonal antibody PEG2 was produced as follows. Female A/J mice were immunized with 25 μg/mouse of urokinase (human high molecular weight two-chain urokinase unless otherwise specified) in complete Freund's adjuvant and were rechallenged 4 weeks later with urokinase (25 μg/mouse) in incomplete Freund's adjuvant. Somatic cell fusion was performed according to the method of Köhler and Milstein (1975) as modified by Galfre et al. (1977). A solid-phase radioimmunoassay identified 12 clones positive for urokinase. PEG2 was selected for expansion and purification because of its high-affinity binding to both scuPA (high molecular weight unless otherwise specified) and urokinase.

Antibody Expansion and Purification. Ascites containing PEG2 were produced in pristane-primed A/J mice. Antibodies were purified by DEAE-cellulose ion-exchange chromatography (DE52). Ascites containing 59D8 were produced in pristane-primed BALB/C retired breeder mice. Antibody 59D8 was further purified by affinity chromatography on a column of Sepharose linked to the peptide against which it had been raised [Gly-His-Arg-Pro-Leu-Asp-Lys-Cys: peptide Bβ(15–21) plus Cys (Hui et al., 1983)].

Conjugation of Antifibrin Antibody to Antiurokinase Antibody. SPDP-modified 59D8 was conjugated to 2-iminothiolane-modified PEG2 by disulfide bond formation, according to the method of Liu et al. (1985) with the following modifications. Purified PEG2 (15 mg) was dialyzed against 0.14 M sodium chloride, 1 mM potassium chloride, and 3.7 mM sodium phosphate, pH 7.4 (conjugation buffer), and then reacted with a 200-fold molar excess of 2-iminothiolane hydrochloride dissolved in 25 mM sodium borate, pH 9.1, for 30 min. At the end of the incubation, the modified PEG2 was dialyzed into phosphate-buffered saline azide (PBSA), pH 6.6.

Affinity-purified 59D8 (15 mg) was dialyzed against conjugation buffer and incubated with a 10-fold molar excess of SPDP dissolved in ethyl alcohol for 30 min. At the end of the incubation, the modified 59D8 was dialyzed into conjugation buffer overnight.

2-Iminothiolane-modified PEG2 was mixed with SPDP-modified 59D8 in an equimolar ratio and incubated overnight at 4 °C. The reaction was terminated by the addition of a 100-fold molar excess of iodoacetamide. The reaction mixture was then fractionated by gel filtration on a Sephacryl S-300 column (2.5 × 60 cm) and equilibrated with PBSA and 1 M urea, pH 6.6. Fractions were collected, and aliquots of those absorbing at 280 nm were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and radioimmunoassay.

Characterization of Bispecific Antibody. (*A*) *Dual Antigen Binding Radioimmunoassay.* Microtiter plates were coated with 25 μL of either fibrin monomer (10 μg/mL) or scuPA (10 μg/mL), blocked with 10% horse serum, and washed 10 times with deionized water to remove nonspecifically bound ligand. Culture supernatants containing 59D8, PEG2, or samples of fractions presumed to contain bispecific antibody were plated in duplicate wells and allowed to react for 1 h. After the plates had been washed 10 times with water, 25 μL of either ^{125}I-labeled goat anti-mouse Fab′ (approximately 50 000 cpm/25 μL) or ^{125}I-labeled scuPA (approximately 50 000 cpm/25 μL) was placed in the wells. Control wells contained no radiolabeled probe. After a 1-h incubation, excess probe was washed from the plates with water, and the wells were measured for specifically bound radioactivity in a γ counter.

(*B*) *Fibrin–Sepharose Assay.* Fibrin monomer–Sepharose was prepared by coupling ^{125}I-labeled fibrinogen (IBRIN) to cyanogen bromide activated Sepharose CL4B. The immobilized fibrinogen was converted to fibrin by the addition of human thrombin in the presence of 100 mM $CaCl_2$ (Bode et al., 1985). The assay was conducted as follows. ^{125}I-Labeled fibrin–Sepharose (100 μL) containing 60 000 cpm/100 μL was pipetted into 5-mL Sarstedt tubes. A solution (100 μL) of scuPA (yielding final concentrations of 1.852×10^2, 1.852×10^1, 1.852×10^0, 1.852×10^{-1}, 1.852×10^{-2}, or 1.852×10^{-3} pM) and either 100 μL of bispecific antibody (0.01 mg/mL) in PBSA or 100 μL of PBSA alone was added to the test tubes and allowed to incubate for 3 h. After an initial washing step with 0.1 M Tris-HCl, 0.1 M sodium chloride, 0.1% Tween-80, 0.5% Triton X-100, and 0.5% bovine serum albumin, pH 7.4 (3 mL/tube), the tubes were washed 3 times with Tris-buffered saline azide (TBSA), pH 7.4. At the end of each washing step, the tubes were spun in a centrifuge for 5 min at 2000 rpm, and supernatant was carefully removed. After the last wash, all but 100 μL of the supernatant was removed. Lysine–Sepharose-purified plasminogen (1 mL/tube of 0.15 mg/mL plasminogen) was then added to each sample and incubated at room temperature overnight. The tubes were counted in the γ counter, and 600 μL of supernatant (50% of total volume) was pipetted out of each, transferred to a second tube, and counted in the γ counter. Percent lysis for each sample was calculated as (supernatant counts × 200)/total counts.

(*C*) *Plasma Clot Assay.* The in vitro plasma clot assay was performed essentially as described by Lijnen et al. (1984). To thawed fresh-frozen human plasma (FFP) was added ^{125}I-labeled human fibrinogen (IBRIN; 100 000 cpm/mL FFP), calcium chloride (to 50 mM), and 8 NIH units of thrombin/mL of FFP. This mixture was drawn into Silastic tubing (inner diameter 4 mm) and incubated at 37 °C for 30 min. One-inch clot segments were cut from the tubing, and the clots were extruded into 5-mL Sarstedt tubes. The clots were washed in saline and counted in a γ counter before use. To each tube was added 2 mL of thawed FFP and a solution containing 100 μL of scuPA (yielding final concentrations of 3704, 1852, 926, 463, 185.2, or 92.6 pM) with or without 100 μL of bispecific antibody. Every hour 750 μL of supernatant was removed from each tube, counted, and replaced. Percent lysis for each tube was calculated as (supernatant counts × 300)/total counts. Samples were saved at the end of the experiment for determination of fibrinogen levels.

(*D*) *Fibrinogen Assay.* The fibrinogen content of samples of citrated human plasma was determined by two methods:

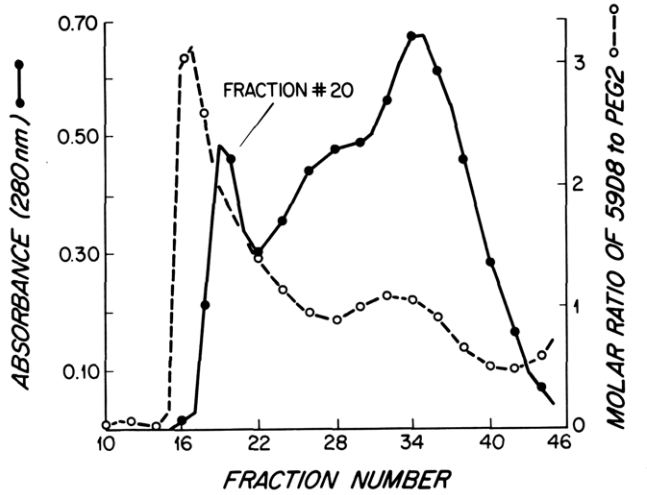

FIGURE 1: ACA-34 gel filtration of the conjugate reaction mixture. The solid curve represents the absorbance at 280 nm that is proportional to the protein content in each fraction. In each column fraction, the ratio of ^{125}I to ^{131}I was calculated. This number represents the mean ratio of antibody 59D8 to antibody PEG2 in each fraction, shown by the dashed curve.

Clottable fibrinogen was measured by the method of Clauss et al. (1957), and precipitable fibrinogen content was determined by the sodium sulfite method (Rampling & Gaffney, 1976).

Quantitation of 59D8:PEG2 Ratio in Bispecific Antibody Preparations. PEG2 (100 μg) was radiolabeled with ^{131}I and 59D8 (100 μg) with ^{125}I by the IODO-GEN method (Fraker & Speck, 1978). The specific radioactivity of each iodination was calculated by the trichloroacetic acid protein precipitation method. Chemical conjugation of ^{131}I-labeled PEG2 to ^{125}I-labeled 59D8 was performed as described for the unlabeled species. The crude reaction mixture was then fractionated on a calibrated ACA-34 gel filtration column for purification. The protein content of each fraction was measured by absorbance at 280 nm, and the amount of ^{131}I and ^{125}I radioactivity in the peak protein fractions was simultaneously measured by dual-label γ counting. The samples were also subjected to SDS–PAGE on 7.5% and 5% gels, followed by autoradiography.

Results

Monoclonal antibody PEG2 was selected from a panel of 12 antibodies to urokinase on the basis of 3 criteria: PEG2 binds to both urokinase and scuPA, it does not inhibit the enzymatic activity of urokinase in an amidolytic or fibrin-plate assay, and its serotype is identical with that of 59D8 (IgG_1-κ).

After the conjugation of 59D8 to PEG2, the crude reaction mixture was size-fractionated by S-300 chromatography. The fraction eluting in the void volume contained aggregates of approximately 300 000–600 000 daltons and greater (data not shown). The major peak within the column volume corresponded to approximately 150 000 daltons. Radioimmunoassay demonstrated that 59D8 and PEG2 both retained their ability to bind antigen after chemical modification and that the material in the void volume was capable of simultaneously binding both urokinase and fibrin monomer, while the included material was not (data not shown).

The success of the chemical coupling strategy was assessed in experiments with bispecific antibody formed by conjugating ^{131}I-labeled PEG2 to ^{125}I-labeled 59D8 (Figures 1–3). The autoradiograph (Figure 2) demonstrates the marked heterogeneity of conjugate species. Early fractions from the ACA-34 gel filtration column contained a single band with

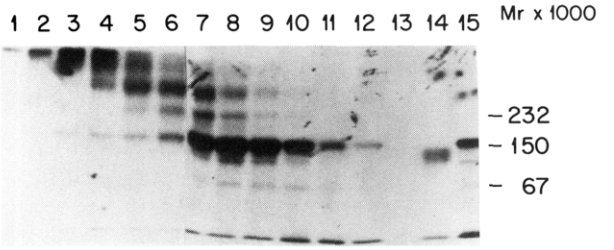

FIGURE 2: Autoradiography of samples of crude bispecific antibody separated by gel filtration on an ACA-34 column. Aliquots of protein-containing fractions (from the gel filtration profile shown in Figure 1) were subjected to SDS–PAGE on a 5% polyacrylamide gel. Lanes 1–12 contain aliquots of fractions 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, and 40, respectively. Lane 13 is blank. The 150 000-dalton unconjugated constituent immunoglobulins, ^{131}I-labeled PEG2 and ^{125}I-labeled 59D8, are shown in lanes 14 and 15, respectively.

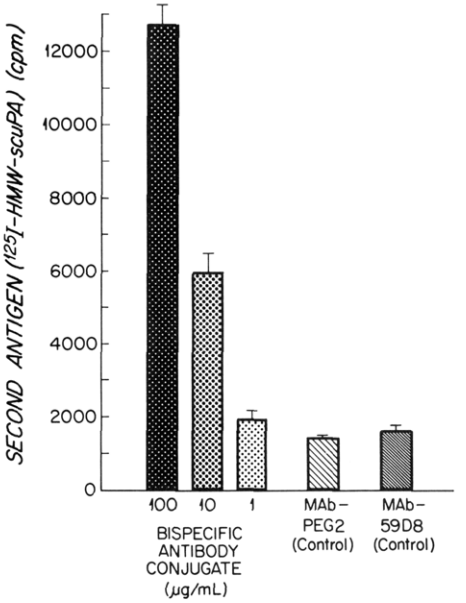

FIGURE 3: Binding of bispecific antibody to fibrin monomer and scuPA. Microtiter plates were coated with fibrin monomer (10 μg/mL) and incubated with bispecific antibody (100, 10, and 1 μg/mL), undiluted culture supernatant containing antiurokinase antibody PEG2, or undiluted culture supernatant containing antifibrin antibody 59D8. After the plates had been washed to remove excess and nonspecifically bound ligand, ^{125}I-labeled scuPA was added to each well and allowed to incubate for 1 h at 37 °C. The bars represent the mean of three determinations ± SEM (standard error of the mean).

a molecular weight of approximately 600 000 probably representing tetramers of the constituent 150 000-dalton immunoglobulins 59D8 and PEG2. Late fractions contained protein bands probably representing tetramers, trimers, dimers, and unconjugated monomers. The included fractions showed primarily a single protein band at 150 000 daltons that represents a mixture of unconjugated 59D8 and PEG2. These conclusions are supported by a graphic representation of the ratio of ^{125}I to ^{131}I in each column fraction (Figure 1, dashed curve). Fractions 16–18 from the ACA-34 column contained bispecific antibody with a 59D8 to PEG2 ratio of 3 to 1. Therefore, the bispecific antibody contained in these fractions should consist of three 59D8 molecules bound to a single PEG2 antibody. Fractions 19–30 contained bispecific antibody in 59D8:PEG2 ratios of 2:1 and 1:1.

Several fractions from the ACA-34 column were tested for antigen binding to fibrin monomer and scuPA. Figure 3 shows the results from an early fraction, 20, that contained bispecific antibody on the basis of its molecular weight by SDS–PAGE. The antigen binding assay included the unconjugated immu-

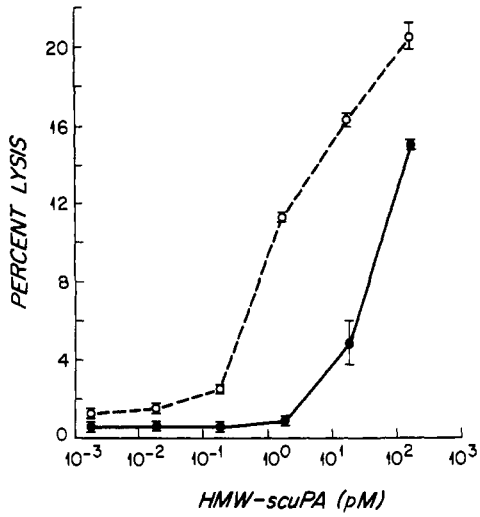

FIGURE 4: Effect of the bispecific antibody on fibrinolysis by scuPA in the fibrin–Sepharose assay. The enhancement of fibrinolysis by scuPA in the presence of the bispecific antibody (open circles) is shown with fibrinolysis by scuPA alone (filled circles). Each point represents the mean of three determinations ± SEM.

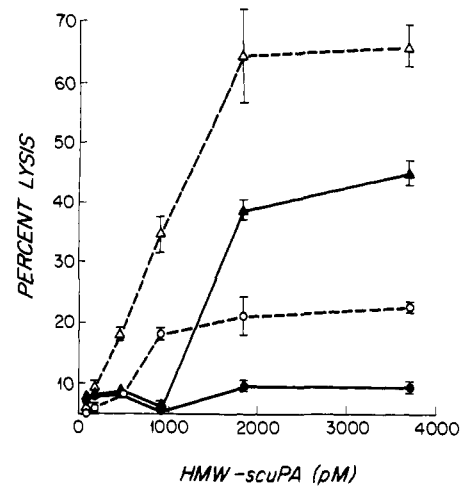

FIGURE 5: Effect of bispecific antibody on human plasma clot lysis by scuPA. The enhancement of human plasma clot lysis by scuPA in the presence of the bispecific antibody (dashed lines, open symbols) is plotted against the effect of scuPA alone (solid lines, filled symbols) at 1 (circles) and 2 (triangles) h. Each point represents the mean of three determinations ● SEM.

noglobulins 59D8 and PEG2. Fraction 20 and 59D8 (but not PEG2) bound to fibrin monomer in a direct assay in which ^{125}I-labeled goat anti-mouse Fab′ was used as probe. Similarly, fraction 20 and PEG2 (but not 59D8) bound to scuPA (^{125}I-labeled goat anti-mouse Fab′ probe). However, only fraction 20 simultaneously bound to fibrin monomer and ^{125}-I-labeled scuPA.

Throughout the range of scuPA concentrations tested in the fibrin–Sepharose assay, lysis by scuPA in the presence of the bispecific antibody was greater than lysis by scuPA alone. For example, in samples containing the bispecific antibody, the fibrinolysis observed at a scuPA concentration of 1.85 pM was 12.8-fold higher than in samples lacking the bispecific antibody [i.e., the dose of scuPA, in the presence of the bispecific antibody, could be decreased to less than 2% of the dose of scuPA alone to achieve the same degree of fibrinolysis (Figure 4)]. The bispecific antibody also increased the lytic efficacy of scuPA in the human plasma clot assay at scuPA concentrations above 463 pM. At a scuPA concentration of 463 pM, plasma clot lysis by scuPA in the presence of bispecific antibody (measured as the release of iodinated fibrin degradation products into the plasma supernatant) increased by 5.6-fold relative to that for scuPA alone at 2-h incubation (Figure 5).

The fibrinogen content of samples from the plasma clot assay was also determined. In samples containing scuPA and the bispecific antibody, there was no degradation of fibrinogen after 3 h of incubation, whereas in samples containing only scuPA there was a significant decrease in plasma fibrinogen. This absence of fibrinogen degradation demonstrates the fibrin specificity of the bispecific antibody. For example, when 1850 or 3700 pM scuPA was added to the assay solution, clottable fibrinogen decreased from an initial level of approximately 266 mg/dL (measured in a sample without scuPA) to less than 87.3 and 84.4 mg/dL, respectively, and precipitable fibrinogen decreased from an initial level of approximately 202 mg/dL (measured in a sample without scuPA) to 101 and 64.0 mg/dL, respectively. Figure 6 shows the fibrinogenolysis observed over the entire range of scuPA concentrations tested in this assay.

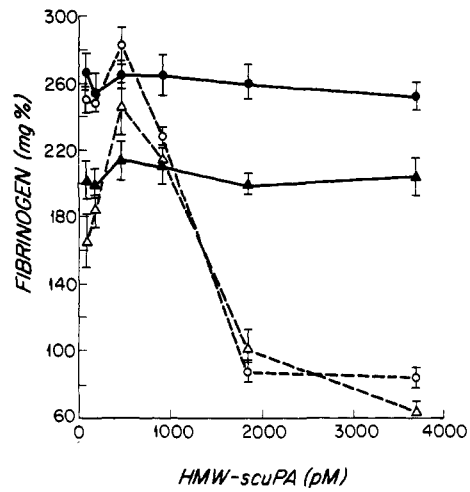

FIGURE 6: Effect of bispecific antibody on residual fibrinogen concentration in the human plasma clot assay. Fibrinogen levels were measured by the Clauss (circles) and precipitable fibrinogen (triangles) methods. Solid lines and filled symbols show residual fibrinogen levels in clots lysed in the presence of both scuPA and bispecific antibody. Dashed lines and open symbols show residual fibrinogen levels in clots lysed with scuPA alone. The points represent the means of two determinations ± SEM.

Discussion

A bispecific antibody was formed by chemically coupling antifibrin monoclonal antibody 59D8 to antiurokinase monoclonal antibody PEG2. When tested for the ability to lyse fibrin monomer and human plasma clots, the bispecific antibody increased both the fibrinolytic efficacy and fibrin specificity of scuPA. By chemically coupling radiolabeled forms of the two antibodies, it was possible to demonstrate that, as purified, the bispecific antibody consisted of a heterogeneous mixture of reaction products containing the two constituent antibodies in various ratios. Although it was not necessary to further purify the bispecific antibody mixture to demonstrate that it bound both antigens simultaneously and enhanced the fibrinolytic potency of scuPA, it may be possible to optimize the ratio of 59D8:PEG2 for fibrinolysis.

Runge et al. (1988) and Bode et al. (1985, 1987) have demonstrated that covalent (disulfide) conjugates of 59D8 and either urokinase or tPA are, respectively, 100 times more efficient than low molecular weight two-chain urokinase (Abbokinase, Abbott Laboratories) and 10 times more efficient than tPA in vitro. In the plasma clot system, the plasminogen activator–antifibrin antibody conjugates are 4–6-fold more

potent than the parent plasminogen activators. These authors further demonstrated that the conjugates enhance clot lysis in an in vivo rabbit model (Runge et al., 1987).

The approach described here further explores the concept of chemical conjugation. We demonstrate that fibrin binding can be imparted to a fibrin-selective plasminogen activator (scuPA) that does not directly bind to fibrin. Although it is not suited for clinical studies in its present form, the properties of the chemical conjugate described in this report suggest that the principle of using bispecific antibodies to target plasminogen activators merits further investigation.

Registry No. scuPA, 105913-11-9; urokinase, 9039-53-6.

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