# Preparation and Characterization of a Disulfide-Linked Bioconjugate of Annexin V with the B-Chain of Urokinase: An Improved Fibrinolytic Agent Targeted to Phospholipid-Containing Thrombi†

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ABSTRACT: A conjugate of annexin V and the B-chain of urokinase was prepared and its fibrinolytic properties were studied. First, a mutant of annexin V was constructed with an N-terminal extension of six amino acids (Met-Ala-Cys-Asp-His-Ser) and with $Cys_{316}$ mutated to Ser; this molecule was expressed in *Escherichia coli*. The urokinase B-chain was prepared by limited reduction of the interchain disulfide bond between the A- and B-chains of urokinase. These two molecules were then connected by a disulfide bond and purified to yield a 1:1 stoichiometric conjugate. The conjugate had the same catalytic activity as urokinase against a synthetic substrate, Glt-Gly-Arg-MCA, and a similar plasminogen activating activity. The conjugate showed the same binding affinity for phosphatidylserine-containing membranes as annexin V. The *in vitro* fibrinolytic activity of the conjugates on clots prepared from platelet-rich plasma was comparable to that of urokinase. However, the conjugate showed 3−4-fold stronger *in vivo* thrombolytic activity than urokinase in a rat pulmonary embolism model, while having essentially the same plasma clearance rate as urokinase or B-chain. These results show that annexin V is a useful agent for targeting plasminogen activators to phospholipid-containing thrombi.

Urokinase and t-PA[1] are currently used for thrombolytic therapy (deBono, 1994). These agents, however, have side effects largely due to the insufficient binding affinity for the fibrin clot and/or short half-lives in circulation. Systemic hemorrhage is the major problem, especially when large doses are administered. To solve these problems, improved fibrinolytic agents that have a higher binding affinity to thrombi or a prolonged half-life are needed. Newer agents, such as scuPA (the precursor of urokinase) and APSAC, are in clinical use. A number of mutants and hybrids of t-PA and scuPA have been prepared, and their fibrinolytic activities were tested (Lijnen & Collen, 1992). Chemical conjugates and chimeras, which have scuPA attached to antibodies against fibrin or platelet membrane proteins, have a higher affinity for thrombi and show an enhanced fibrinolytic activity (Runge *et al*., 1991; Lijnen & Collen, 1992).

Annexins, which are present in many cells, have $Ca^{2+}$-dependent phospholipid binding properties. Annexin V is the most abundant protein and has the highest binding activity in this family. Although a variety of functions have been proposed for annexins, *e.g*., anticoagulant, membrane fusion, or mediation of cytoskeleton−membrane interaction, their physiological functions are not clear (Walker *et al*., 1992; Huber *et al*., 1992). Nonetheless, annexin V strongly binds to negatively charged phospholipid vesicles ($K_d$ $10^{-9}$−$10^{-11}$ M) (Tait *et al*., 1989, 1992) and to activated platelets ($K_d$ 7 × $10^{-9}$ M) (Thiagarajan & Tait, 1990). It is a simple protein composed of 319 amino acids, 36 kDa, with a blocked N-terminus, containing neither disulfide bonds nor carbohydrate chains. Annexin V is composed of four internal repeating units, each with 70−80 amino acid residues, and an N-terminal tail (Funakoshi *et al*., 1987a). X-ray diffraction analysis shows that each repeat forms a compact domain with five α-helices. The protein binds to phospholipid membrane *via* its convex surface, where the $Ca^{2+}$ binding sites are located (Huber *et al*., 1990). The N-terminal tail (∼16 residues) is located outside of the membrane binding region. Annexin V could be an excellent agent for targeting to phospholipid since, besides its strong binding affinity for phospholipid, it is a human protein with a relatively small size and has a compact structure resistant to proteolytic degradation (unpublished observations). Consistent with these expectations, we have recently shown in animal models that intravenously administered annexin V accumulates selectively in arterial and intracardiac thrombi *in vivo* (Tait *et al*., 1994; Stratton *et al*., 1995).

Urokinase is a trypsin-type serine protease that is synthesized as a single-chain precursor (scuPA). Plasma kallikrein and plasmin can activate scuPA to urokinase (Ichinose *et al*., 1986; Nielsen *et al*., 1982), which is composed of two chains, an N-terminal regulatory unit (A-chain, 24 kDa) and a C-terminal catalytic unit (B-chain, 30 kDa). These two chains are held together by an interchain disulfide bond between $Cys_{148}$ in A-chain and $Cys_{279}$ in B-chain (Gunzler *et al*., 1982a,b). Urokinase has a potent activity to convert

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[1] Abbreviations: APSAC, anisoylated plasminogen streptokinase activator complex; BSA, bovine serum albumin; C-, carboxy-terminal; CNBr, cyanogen bromide; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; HMW, high molecular weight; HSA, human serum albumin; LMW, low molecular weight; N-, amino-terminal; PCR, polymerase chain reaction; scuPA, single-chain urinary-type plasminogen activator; PRP, platelet-rich plasma; PS, phosphatidylserine; PTH, phenylthiohydantoin; t-PA, tissue-type plasminogen activator.

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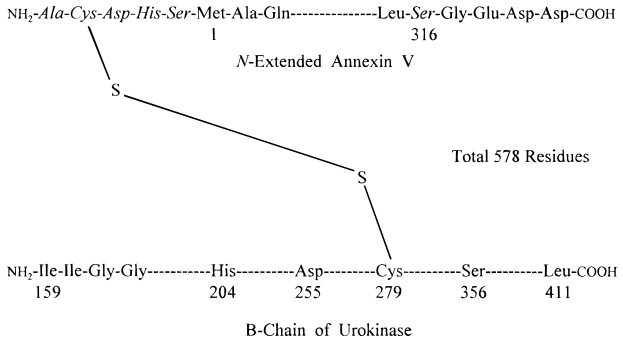


FIGURE 1: Schematic structure of annexin V/urokinase conjugate. Selected amino acid residues are numbered according to the sequences of the native proteins. The residues in the extension and mutated in annexin V are indicated in italics.

plasminogen to plasmin (Summaria *et al*., 1975), which is the enzyme that resolves fibrin clots. Urokinase does not bind to fibrin clots, although scuPA was reported to have a weak binding affinity for fibrin clots (Kasai *et al*., 1985).

In this paper, we present the preparation and characterization of a novel fibrinolytic agent, a chemical conjugate of B-chain of urokinase with annexin V that targets phospholipid-containing thrombi. The structure of this molecule is shown schematically in Figure 1.

## MATERIALS AND METHODS

*Materials*. Urokinase (two-chain high molecular weight urokinase) (150 000 international units/mg), Glu-plasminogen (25.1 casein units/mg), thrombin (1000 NIH units/mg), fibrinogen, and HSA were the products of The Green Cross Co., Osaka, Japan. Annexin V was purified from human placenta as described (Funakoshi *et al*., 1987b). DNase (10 units/mL) was the product of Boehringer Mannheim. DTT, Triton X-100, Tween 80, kanamycin, ampicillin, pepstatin, leupeptin, and DFP were purchased from Sigma. Benzamidine-Sepharose 6B was from Pharmacia. Chromogenic substrates, Glu-Gly-Arg-*p*NA (S-2444) and D-Val-Leu-Lys-*p*NA (S-2251), were obtained from KabiVitrum, Sweden. A fluorogenic substrate, Glt-Gly-Arg-MCA (3097-v), was from Peptide Institute, Inc., Osaka, Japan. $^{125}$I-Annexin V was prepared as described earlier (Tait & Gibson, 1994). For studies of *in vivo* clot lysis, $^{125}$I-fibrinogen was prepared as follows: Fibrinogen, 5 mg in 0.5 mL of 50 mM phosphate buffer, pH 7.4, 150 mM NaCl, was mixed with 50 $\mu$L of immobilized lactoperoxidase (Funakoshi, Tokyo, Japan), 5 $\mu$L of Na$^{125}$I (1 mCi), 5 $\mu$L of 20 mM KI, and 10 $\mu$L of 0.12% (w/v) $H_2O_2$ and left for 8 min at room temperature. The reaction product was then applied to a PD-10 column (Pharmacia) that was equilibrated with 0.5% HSA in 150 mM NaCl. Radioactive protein fractions were pooled and dialyzed overnight against 1 L of 150 mM NaCl. The radioactivity of the sample was then adjusted to 200 $\mu$Ci/mL by diluting with 150 mM NaCl. Radiolabeled urokinase, B-chain, and conjugate were prepared by essentially the same methods as the fibrinogen labeling. The specific activities of fibrinogen, urokinase, B-chain, and conjugate were 10, 7505, 9428, and 3780 cpm/ng, respectively. Protein assay reagent (BCA) and standard bovine serum albumin were purchased from Pierce. L-Broth was obtained from Gibco/BRL. Liposome-gel was prepared according to Meers *et al*. (1987) by mixing 500 mg of phosphatidylcholine, 500 mg of PS, and 250 mg of phosphatidylethanolamine (all from Sigma) with 30 mL of CNBr-activated Sepharose 6B (from Pharmacia).

*Isolation of B-Chain of Urokinase*. Urokinase (36.9 mg) was reduced at 37 °C for 90 min in 90 mL of 0.1 M phosphate buffer, pH 7.4, 1 mM DTT, and 20 mM benzamidine on a shaking rocker. The reaction mixture was immediately desalted by gel filtration on a Sephadex G-25 Superfine (5 × 12 cm) equilibrated with 20 mM sodium acetate, pH 4.0 , 0.4 M NaCl, and 0.05% $NaN_3$. The protein fractions were pooled, neutralized with 1 N NaOH, and applied to a benzamidine-Sepharose column (1.6 × 5 cm). After the column was washed with 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 0.05% $NaN_3$ to remove nonadsorbed A-chain, B-chain was eluted with 20 mM sodium acetate, pH 4.0, 0.4 M NaCl, and 0.05% $NaN_3$. It was stored frozen at −20 °C until use. The yield was 10.8 mg (52%).

*Preparation of N-Terminally Extended Annexin V (Annexin V-N6)*. First, a mutant annexin V cDNA (pANXVC-S) was designed in which the $Cys_{316}$ codon was replaced by a Ser codon, an *Nde*I site was introduced prior to the initiator Met codon, and a *Bam*HI site was introduced after the stop codon. This was constructed by PCR using an annexin V cDNA (pPAP-I-1.6; Funakoshi *et al*., 1987a; Cookson *et al*., 1994) as a template and two oligonucleotides, 5′-g·gaa·ttc·cat·atg·gca·cag·gtt·ctc·aga·ggc·act·gtg-3′ and 5′-cgc·gga·tcc·tta·gtc·atc·ttc·tcc·gga·gag·cag-3′. This cDNA was ligated into the *Nde*I and *Bam*HI cloning sites in the pET12a plasmid (Novagen, Madison, WI). An oligonucleotide (5′-t·atg·gca·tgt·gac·cat·tc-3′) and its inverse complement (5′-t·aga·atg·gtc·aca·tgc·ca-3′) were prepared to encode six amino acid residues (Met-Ala-Cys-Asp-His-Ser) with *Nde*I-compatible overhangs at both ends. The two complementary oligonucleotides were annealed and the product was ligated into pANXVC-S that was digested with *Nde*I to produce plasmid pANXVC-S-N6. DNA sequencing of this plasmid confirmed that the intended mutations had been correctly introduced.

This plasmid (pANXVC-S-N6) was then transformed into *Escherichia coli* strain K-38 containing the pGP-1 plasmid for production of recombinant protein by expression from the T7 promoter of the vector. The cells were grown at 30 °C in 3 L of 2% L-broth in 50 mM potassium phosphate, pH 7.4, 25 $\mu$M ampicillin, and 25 $\mu$M kanamycin. After 4 h ($OD_{600}$ = 0.4), cells were given a heat shock by placing culture flasks in a 42 °C water bath for 20 min and the cultivation was continued at 37 °C for 2 h. Cells were then harvested and stored frozen overnight at −20 °C.

Thawed cells were suspended in 100 mL of 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.1 mM DFP containing 1 mg of leupeptin and 1 mg of pepstatin and sonicated for 5 min. The cell lysate was treated with 5 $\mu$L of DNase (10 units/mL) for 10 min at room temperature. Then, pellets were collected by centrifugation, and annexin V-N6 was extracted from the pellets by stirring for 1 h at 4 °C in 70 mL of 50 mM Tris-HCl, pH 8.0, 8 M urea, 0.1 M $NH_4Cl$, 10 mM EDTA, 0.1 mM DFP containing 1 mg of leupeptin and 1 mg of pepstatin. The supernatant obtained by centrifugation was diluted 2-fold with 50 mM Tris-HCl, pH 8.0, and 0.1 mM DTT and left for 48 h at 4 °C. The sample was then dialyzed successively against 150 mL, 500 mL, and 2 L of 0.1 M sodium-phosphate, pH 6.0. The dialyzed sample was applied to a DEAE-Sepharose column (1.4 × 18 cm) that was equilibrated with 0.1 M phosphate, pH 6.0. After the

column was washed, proteins were eluted by a linear gradient formed by 100 mL each of 0 M and 0.5 M NaCl in the phosphate buffer. The major protein peak that contained anticoagulant activity was pooled and ammonium sulfate was added to 80% saturation. The precipitates collected by centrifugation were dissolved in 5 mL of 50 mM sodium-acetate, pH 5.6, 150 mM NaCl, and 10 mM DTT and applied to a Sephadex G-75 column (2.5 × 90 cm) equilibrated with the same buffer except for DTT. Annexin V-N6 eluted as a single peak and it was homogeneous on SDS−PAGE with a molecular mass of 36 kDa. The yield was 35 mg of protein from 3 L of culture medium.

*Preparation and Isolation of Annexin V-N6/B-Chain Conjugate*. Annexin V-N6 was treated for 1 h with 0.1 mM DTT and desalted on a Sephadex G-25 superfine column (5 × 12 cm) with 50 mM Tris-HCl, pH 7.4, and 50 mM NaCl. The stock solution of B-chain was thawed and trace insolubles were removed by centrifugation. B-chain (0.69 mg) and annexin V-N6 (4.5 mg) were mixed in 4 mL of 50 mM Tris-HCl buffer, pH 7.4, 20 mM benzamidine hydrochloride, 5 mM $CaCl_2$, and 50 mM NaCl and incubated overnight at 37 °C with gentle stirring. After the reaction, the sample was applied to a liposome-gel column (1.6 × 2.7 cm) equilibrated with 50 mM Tris-HCl, pH 7.4, 2.5 mM $CaCl_2$, and 0.5 M NaCl. After extensive washing, adsorbed proteins were eluted with the same buffer except that $CaCl_2$ was replaced by 3 mM EDTA. The eluate was then applied to a benzamidine-Sepharose column (1 × 2.8 cm) that was equilibrated with 50 mM Tris-HCl, pH 7.4, and 50 mM NaCl. After the column was washed with the same buffer, the conjugate was eluted with the same buffer containing 20 mM benzamidine hydrochloride. The yield of the purified conjugate was 0.47 mg with an overall yield of 32% based on the amidolytic activity.

*Assay of Amidolytic Activity*. The amidolytic activities of the conjugate, B-chain and urokinase were measured using Glt-Gly-Arg-MCA; 50 μL of sample (activity ranging from 30 to 1000 international units/mL) in 50 mM Tris-HCl, pH 7.4, 0.4% HSA, 50 mM NaCl, and 0.05% $NaN_3$ was mixed with 50 μL of substrate (0.5 mM in the same buffer) in a microtiter plate and incubated for 5 min at room temperature. The reaction was terminated by adding 50 μL of 50% acetic acid. The intensity of fluorescence was then measured (excitation at 355 nm and emission at 460 nm); results were expressed in international units of urokinase activity. In some experiments, the amidolytic activity of urokinase or B-chain was assayed using S-2444. Test samples (3−5 μL) were incubated for 3−5 min at room temperature with 80 μL of 0.1 M Tris-HCl, pH 7.8, and 20 μL of 10 mM S-2444. Reactions were terminated by adding 1 mL of 0.2 M citric acid, and the adsorbance at 405 nm was measured.

*Assay of Plasminogen Activating Activity*. The stock solution of conjugate was first quantitated by assay with S-2444, and it was then incubated at room temperature at a concentration of 1.85 nM with plasminogen (0−30 μM) in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween-80. The rate of plasmin generation and Michaelis−Menten kinetic parameters were then determined as described (Tait et al., 1995).

*Membrane Binding and Anticoagulant Activities*. The binding affinity of the conjugate was determined by competition assay against $^{125}$I-annexin V (5 nM) using preserved erythrocytes (5 × $10^6$ cells/mL) with surface-exposed PS as a phospholipid source as described (Tait & Gibson, 1994); samples were incubated at 37 °C for 15 min in 10 mM Na-HEPES, pH 7.4, 136 mM NaCl, 2.7 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 5 mM glucose, and 5 mg/mL BSA. Anticoagulant activity of annexin V-N6 was determined by clotting assay as described earlier (Funakoshi *et al*., 1987b) using placental annexin V as a standard.

*Measurement of in Vitro Fibrinolytic Activity*. *In vitro* lysis of clots prepared from human PRP and human $^{125}$I-fibrinogen was assayed exactly as described (Tait et al., 1995), except for use of a different concentration of activator (18.5 nM in this study). Results are expressed as percentage of soluble radioactivity, corrected for volume changes over the course of the assay.

*Measurement of in Vivo Fibrinolytic Activity*. Blood was drawn from Wistar rats into 0.1 volume of 0.1 M trisodium citrate and centrifuged at 1000*g* for 3 min to yield PRP in the supernatant. Radiolabeled clots were formed by incubating 2 mL of PRP for 30 min at 37 °C with $^{125}$I-fibrinogen (1.4 μCi/mL), $CaCl_2$ (25 mM), and thrombin (10 units/mL). After the clot was washed with 150 mM NaCl, it was cut into small pieces, frozen in liquid $N_2$, crushed in a mortar, and suspended in 5 mL of 150 mM NaCl. The clot was injected into the tail vein, and test samples (urokinase or conjugate, 1.5 mL/rat) were injected 5 min later, also into the tail vein. After 1 h, blood was drawn from posterior vena cava for the determination of fibrinogen and α2-antiplasmin, and the animals were sacrificed. Lung was removed and total radioactivity was measured (counts per minute after 1 h). In each experiment, the initial radioactivity (145 161 ± 3375 cpm) was measured in the lungs of five animals sacrificed 5 min after administration of fibrinogen. Lysis activity was calculated as follows:

$$\% \text{ lysis} = [1 - (\text{cpm after 1 h/initial cpm})] \times 100$$

The plasma concentrations of fibrinogen and α2-antiplasmin were determined using Data-Fi fibrinogen determination reagent set (Baxter) and COATEST antiplasmin kit (Helena Laboratories), respectively, according to the manufacturers' instructions.

*Measurement of in Vivo Plasma Clearance*. The radiolabeled conjugate (520 ng), urokinase (350 ng), or B-chain (430 ng) was injected into the tail vein of a rat. Blood samples were drawn into anticoagulant at the indicated times after injection and the radioactivity present in a 0.1-mL aliquot was determined. Radioactivity present in blood at time zero was estimated by dividing total radioactivity injected by (rat weight in grams × 0.06 mL/g). Results were then expressed as percentage of radioactivity remaining at each time point.

*Other Procedures*. SDS−PAGE was carried out with a standard Laemmli system using 10% or 8−16% acrylamide gel and proteins were visualized either with Coomassie brilliant blue or by silver staining. Protein concentration was determined by amino acid analysis or with the BCA protein assay using BSA as a standard.

## RESULTS

*Partial Reduction of Urokinase and Isolation of B-Chain of Urokinase*. Van der Graaf *et al*. showed that benzamidine, a competitive inhibitor of trypsin-type serine protease, protects the catalytic units of factor XIa (van der Graaf *et*

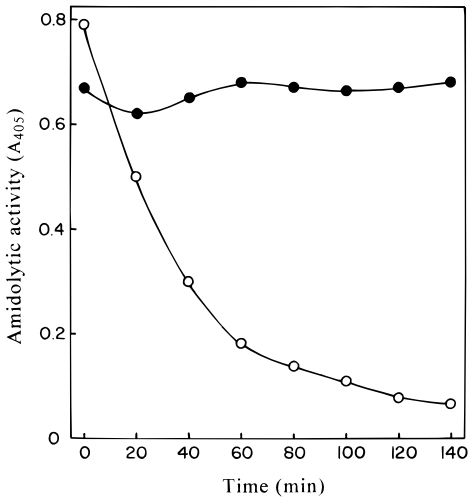


FIGURE 2: Partial reduction of urokinase. Urokinase (3.2 mg) was incubated at room temperature with 1 mM DTT in 0.4 mL of 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA in the presence or absence of 20 mM benzamidine. At desired times, the reduction was terminated by the addition of monoiodoacetamide and the amidolytic activity against S-2444 was assayed as described in Materials and Methods. Reactions with (●) and with (○) benzamidine are shown.

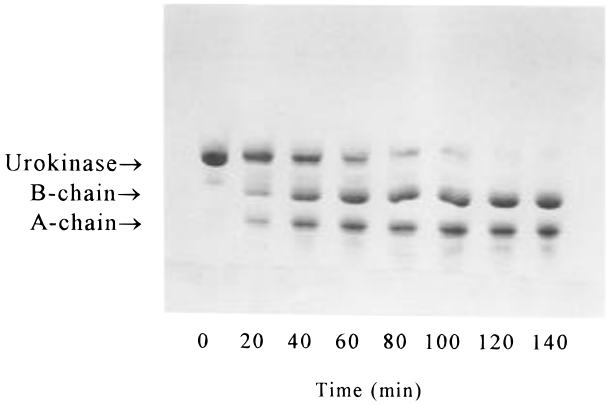


FIGURE 3: SDS−PAGE pattern for the reduction of urokinase. The samples (20 $\mu$L) obtained in the presence of benzamidine as in Figure 2 were applied to a 10% Laemmli gel without reduction. The gel was stained with Coomassie brilliant blue.

*al*., 1983) and plasma kallikrein (van der Graaf *et al*., 1982) during the reduction of the interchain disulfide bonds. Following their methods, urokinase was incubated with 1 mM DTT in 50 mM Tris-HCl buffer, pH 8.0, in the presence of 20 mM benzamidine hydrochloride. At desired times, the reduction was terminated by adding excess amounts of iodoacetamide. The amidolytic activity of the reaction products was assayed using Glu-Gly-Arg-*p*NA. In the presence of benzamidine, the amidolytic activity remained unchanged during the reduction, whereas in the absence of benzamidine the enzyme activity declined and disappeared at the end of the reaction (Figure 2). The band of urokinase progressively diminished and disappeared in 140 min on SDS−PAGE without reduction, and concomitantly the bands of A- and B-chain increased (Figure 3). During the reduction, not only the enzyme activity but also the structure of B-chain was protected by benzamidine; the band of B-chain was intact during the reduction, while the A-chain partially degraded into at least two fragments.

After reduction of 3.2 mg of urokinase, A-chain and B-chain were separated on a benzamidine-Sepharose column. The reduced sample was applied to the column with a pH 8.0 buffer. After nonadsorbed A-chain was removed by washing, adsorbed B-chain was eluted with 0.1% acetic acid. The isolated B-chain gave a single band on SDS−PAGE (*see* lane 4 in Figure 5). The yield of B-chain was 1.7 mg with 94% recovery. Sulfhydryl content of B-chain was determined by the Ellman method (Ellman, 1959) and a value of 1.04 mol of SH/mol of protein was found. This result indicates that under the above conditions the reduction was restricted to the interchain disulfide bond, which connects $Cys_{148}$ in A-chain and $Cys_{279}$ in B-chain. For a large-scale preparation of B-chain, slightly modified conditions were used as described in Materials and Methods.

*Preparation and Characterization of Annexin V-N6.* Annexin V has one half-cystine residue at position 316 near the C-terminus, which is buried inside the molecule (Funakoshi *et al*., 1987a) and inaccessible to disulfide bond formation. Therefore, a mutant protein (annexin V-N6) was expressed in *E. coli*; this molecule has a Cys residue in an expression of six amino acids attached to the N-terminus along with a mutation of $Cys_{316}$ to Ser (Figure 1). After the cells were disrupted by sonication, annexin V-N6 was recovered in the insoluble membrane fraction in the presence of $Ca^{2+}$. It was then extracted from the membrane fraction with 8 M urea/EDTA. The 8 M urea/EDTA extract was first diluted to 4 M urea and the concentration of urea was gradually decreased to near 0 M by several steps of dialysis. It was then purified by DEAE-Sepharose column chromatography followed by gel filtration. The purified annexin V-N6 showed a single band on SDS−PAGE (*see* lane 5 in Figure 5) with a migration slightly slower than that of wild-type annexin V. Sequence analysis yielded Ala-Cys-Asp-His-Ser-Met, indicating that the initiator Met residue was removed by posttranslational events. In an exploratory test, it formed a dimer in a neutral or slightly alkaline buffer. Thus, it was confirmed that the Cys residue introduced in the extension is exposed outside the molecule and accessible to conjugation through a disulfide bond. By clotting assay, annexin V-N6 has a specific activity equal to that of placental annexin V. More than 10 mg of annexin V-N6 could be obtained from 1 L of culture.

*Preparation and Isolation of Annexin V-N6/B-chain Conjugate.* We first studied homodimer formation of annexin V-N6 and B-chain separately and found that annexin V-N6 forms a dimer faster than B-chain. When the two components were incubated individually in a pH 7.4 buffer, approximately 60% of annexin V-N6 and 20% of B-chain converted to the homodimer in 6 h (data not shown). Therefore, to compensate for the loss of annexin V-N6 caused by homodimer formation, an excess of annexin V-N6 was used (ratio of annexin V-N6 to B-chain was 15:1) for the preparation of the conjugate. When the two components were mixed and exposed to air oxidation, multiple bands of ~65 kDa were seen on SDS−PAGE after overnight reaction. These bands represent the conjugate and the homodimers of annexin V-N6 and B-chain. Unfortunately, these three bands migrated close to each other and could not be distinguished clearly on the gel. After oxidation, the formation of products with molecular masses higher than ~65 kDa was not observed (*see* Figure 5). This indicated that the reduction was limited to the interchain disulfide bond and the intrachain disulfide bonds of B-chain were not reduced.

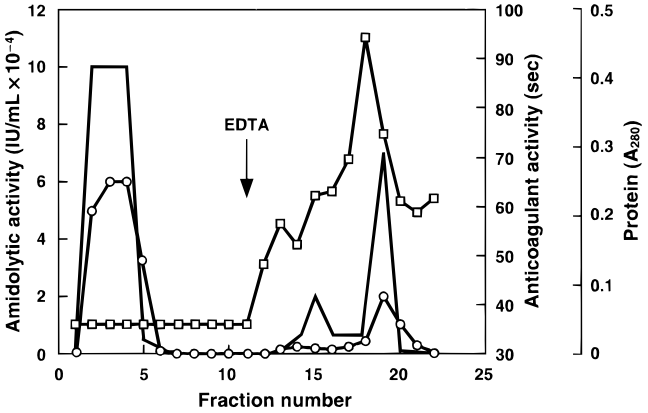


FIGURE 4: Separation of the conjugate on liposome-gel column. The conjugate prepared as described in Materials and Methods was applied to a liposome-gel column (1.6 × 2.7 cm) that was equilibrated with 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 2.5 mM $CaCl_2$. The conjugate was eluted with the same buffer containing 3 mM EDTA instead of $CaCl_2$. The fractions from 17 to 20 were pooled and applied to a benzamidine-Sepharose column for further purification. Anticoagulant activity (□) and amidolytic activity (○) were measured as described in Materials and Methods. Protein concentration is shown by a solid line (—).

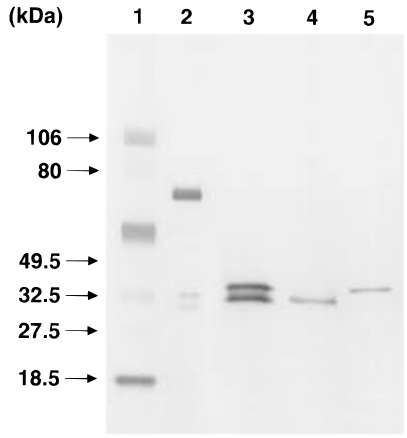


FIGURE 5: SDS−PAGE pattern of the conjugate. Samples were applied to an 8−16% Laemmli gel. Lane 1, molecular weight standard; lane 2, nonreduced conjugate; lane 3, reduced conjugate; lane 4, B-chain; lane 5, annexin V-N6. The bands were visualized by silver staining.

In order to purify the conjugate, the oxidized product was applied to the liposome-gel column in the presence of $CaCl_2$. After the column was washed, adsorbed proteins were eluted with a buffer containing EDTA (Figure 4). SDS−PAGE showed that the eluate contained the conjugate and the monomer and dimer of annexin V-N6. The eluate was then applied to the benzamidine-Sepharose column and eluted with a buffer containing benzamidine hydrochloride. The monomer and dimer of annexin V-N6 were found in the nonadsorbed fraction, and only the conjugate bound to the column and eluted as a single peak. The purified conjugate migrated as a single band on SDS−PAGE with a molecular mass of 69 kDa (Figure 5), which agrees with the sum of the molecular masses of annexin V-N6 (37 kDa) and B-chain (30 kDa). Upon reduction, it migrated as two bands corresponding to annexin V-N6 and B-chain. This gel pattern demonstrated that the product was the annexin V-N6/B-chain conjugate (1:1 molar ratio) held together by a disulfide bond. Sequence analysis of the conjugate (63 pmol) gave two sequences, Ala(30.9)-X-Asp(9.1)-His(4.0)-Ser(17.5)-Met(5.9)-Ala(25.2)-Gln(11.4) of annexin V-N6 and

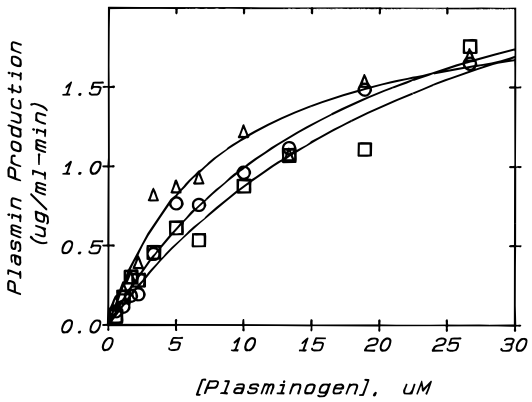


FIGURE 6: Plasminogen activation by the conjugate, urokinase, and B-chain. Plasminogen at the indicated concentration was added to urokinse (△), conjugate (○), or B-chain (□), and the rate of plasmin generation was measured as described under Materials and Methods. The lines are the fitted functions used to determine the kinetic parameters.

Ile(21.8)-Ile(22.6)-Gly(26.9)-Gly(28.7)-Glu(16.0)-Phe(24.0)-Thr(16.4)-Thr(17.3) of B-chain, approximately at a 1:1 ratio (numbers in parentheses are the picomoles of PTH-amino acids detected). The blank in the second cycle of the annexin V-N6 sequence indicates that the Cys residue in this position is connected with B-chain by a disulfide bond.

*Enzyme Activities of the Conjugate, B-Chain, and Urokinase*. As mentioned above, the amidolytic activity of urokinase remained intact throughout the reduction period, indicating that B-chain retains full amidolytic activity after being separated from the A-chain. The specific activities of the conjugate, B-chain, and urokinase were determined against Glu-Gly-Arg-MCA, and the following values were obtained: conjugate, 11.8; B-chain, 11.0; and urokinase, 8.4 (in international units per picomole). These results indicate that B-chain also retains its full amidolytic activity even after being conjugated with annexin V-N6.

We then tested the plasminogen-converting activities of the conjugate and B-chain and compared them with urokinase (Figure 6). The rate of plasminogen activation was similar for the three proteins tested. Curve-fitting of these data yielded these kinetic parameters: for urokinase, $K_m = 8.0 \pm 1.8$ $\mu$M and $V_{max} = 2.1 \pm 0.2$ $\mu$g/(mL·min); for B-chain, $K_m = 26.4 \pm 9.7$ $\mu$M and $V_{max} = 3.2 \pm 0.7$ $\mu$g/(mL·min); and for conjugate, $K_m = 18.6 \pm 3.5$ $\mu$M and $V_{max} = 2.8 \pm 0.3$ $\mu$g/(mL·min). The estimated standard deviations of the parameters are lower bounds, since the error analysis of the curve-fitting program does not reflect systematic and inter-experimental sources of error. Thus, these values indicate that the conjugate has plaminogen-activating activity similar to that of urokinase.

*Phospholipid Binding Activity of the Conjugate, B-Chain, and Urokinase*. We designed annexin V-N6 to have the extension at the N-terminus because the N-terminal tail of annexin is not involved in the phospholipid binding site (Huber *et al*., 1990). As expected, annexin V-N6 had the same phospholipid binding activity and anticoagulant activity as wild-type annexin V (not shown). Binding activity of the conjugate to cell membranes containing exposed PS was then quantitatively measured by competition assay with $^{125}$I-annexin V. A 50% inhibition of binding was obtained at a concentration of 8 nM of the conjugate, essentially the same as a value of 6 nM for wild-type annexin V (Figure 7),

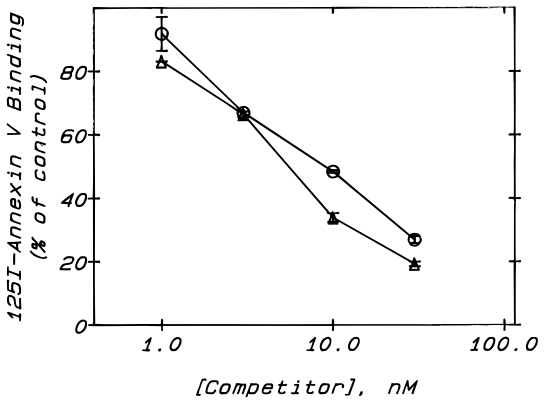


FIGURE 7: Competitive binding of the conjugate and annexin V to PS-containing membranes. Conjugate (○) or annexin V (△) was added at the indicated concentrations and binding activity was measured as described under Materials and Methods. Error bars are given where larger than size of symbols.

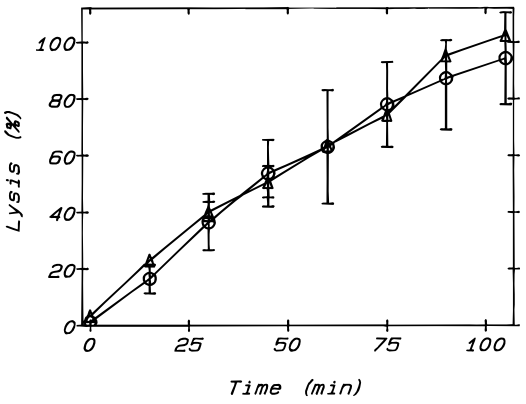


FIGURE 8: *In vitro* fibrinolytic activity of the conjugate and urokinase. Lysis of a clot prepared from human PRP was determined as described under Materials and Methods. Lysis with urokinase (△) or conjugate (○), both added at 18.5 nM, is shown. Lysis was minimal (<5%) in the absence of activator.

indicating that the conjugate had the same binding affinity as the wild-type annexin V. These results indicate that binding affinity of the annexin V moiety for phospholipid is not affected by conjugation with B-chain.

*Fibrinolytic Activity and Plasma Clearance of the Conjugate*. The conjugate promoted *in vitro* lysis of a clot formed from human platelet-rich plasma. Complete lysis was achieved after about 90 min under these conditions; the rate and extent of clot lysis were similar to results obtained with natural urokinase, indicating that the conjugate retained the ability to activate plasminogen and promote clot lysis (Figure 8). *In vivo* fibrinolytic activity of the conjugate was studied in a rat pulmonary embolism model. Although the conjugate and urokinase showed similar *in vitro* fibrinolytic activity, these two molecules gave significantly different *in vivo* lytic activities. The conjugate lysed 47.1% ± 3.5% and 66.1% ± 0.5% of clot prepared from PRP at doses of $2.5 \times 10^4$ and $5 \times 10^4$ international units/kg, respectively, whereas urokinase lysed 45.2% ± 7.2% and 77.1% ± 1.3% at $1 \times 10^5$ and $2 \times 10^5$ international units/kg (Figure 9). These numbers indicate that the conjugate has 3−4-fold higher fibrinolytic activity than urokinase. With administration of the same two concentrations of the conjugate, the fibrinogen and α2-antiplasmin levels remained unchanged after 1 h; fibrinogen was found at 1.4 ± 0.04 and 1.33 ± 0.02 mg/mL (control 1.39 ± 0.08 mg/mL) and α2-antiplasmin at 103.2% ± 2.7% and 98.4% ± 2.8%. With urokinase

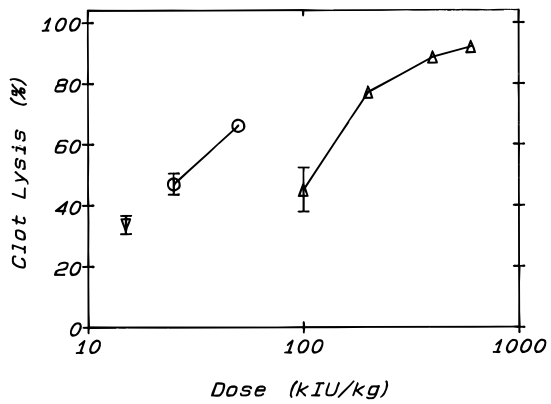


FIGURE 9: *In vivo* fibrinolytic activity of the conjugate and urokinase. Lysis of a clot prepared from rat PRP was determined in a rat pulmonary embolism model as described under Materials and Methods. The results are shown as the average of five experiments, with error bars given where larger than size of symbol. Lysis with urokinase (△), conjugate (○), or control with injection vehicle alone (▽) is shown.

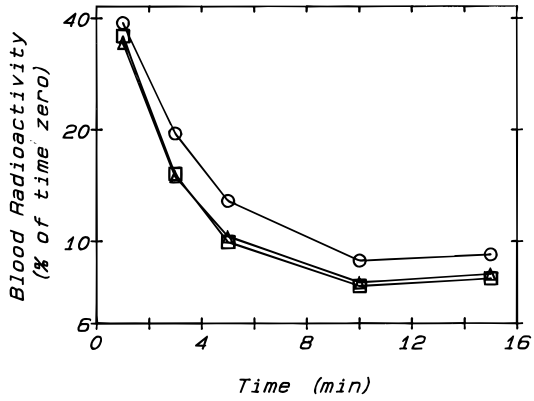


FIGURE 10: Plasma clearance of the conjugate, urokinase, and B-chain. The radiolabeled conjugate, urokinase, or B-chain was injected into the tail vein of a rat. Blood samples were drawn at the indicated times and radioactivity was measured. Results shown are mean values for three rats for each protein tested. Conjugate (○), urokinase (△), and B-chain (□) are shown.

the fibrinogen level was unchanged up to $6 \times 10^5$ international units/kg. However, the α2-antiplasmin level was decreased to 87.8% ± 5.0% and 85.9% ± 2.4% at $1 \times 10^5$ and $2 \times 10^5$ international units/kg, respectively. These two doses of urokinase showed the similar fibrinolytic activity with the conjugate at the above two concentrations.

The plasma clearance rates of the proteins were determined in rats (Figure 10). When data for 1−5 min were analyzed according to a model of monoexponential clearance, the $t_{1/2}$ values were 2.0 ± 0.4 min for urokinase, 1.9 ± 0.3 min for B-chain, and 2.3 ± 0.3 min for the conjugate. Plasma clearance calculated from these data were also identical within experimental error. Thus, these proteins have essentially the same initial rates of plasma clearance.

## DISCUSSION

Bioengineered fibrinolytic agents can be grouped in two categories: those with affinity for activated platelets, and those with affinity for the fibrin component of the thrombus. Agents of the first group could be suitable to resolve arterial thrombi, in which aggregated platelets are the major component (Cahill & Newland, 1993). A chemical conjugate of urokinase with an antibody against a platelet membrane

glycoprotein, IIb/IIIa, which targets activated platelets, showed enhanced fibrinolytic activity (Bode *et al*., 1991; Dewerchin *et al*., 1991). Chemical conjugates of urokinase with an antibody to thrombospondin also showed higher fibrinolytic activities in animal models (Dewerchin *et al*., 1991). The annexin V/B-chain conjugate is a new member of this group and its proposed fibrinolytic mechanism is as follows: the conjugate binds to anionic phospholipids (activated platelets) that are associated with the fibrin clot *via* the binding capability of the annexin V moiety, while the complementary part, B-chain, activates plasminogen to plasmin, which in turn dissolves the fibrin clot.

In the present study, the annexin V/B-chain conjugate showed *in vivo* fibrinolytic activity 3−4-fold higher than that of urokinase in a rat pulmonary embolism model. This effect was due to an increased specific thrombolytic activity, since the plasma clearance rates were essentially identical for the two proteins. These results are different from the *in vitro* tests, which showed that the conjugate and urokinase had similar fibrinolytic activity. It is not unusual that *in vitro* tests do not quantitatively reflect *in vivo* activity in the evaluation of fibrinolytic agents. Bode *et al*. (1990) reported that a chemical conjugate of scuPA with anti-GPIIb/IIIa antibody had a lower *in vitro* activity than scuPA but severalfold higher *in vivo* activity. Systemic fibrinolysis, which occurs with unconjugated urokinase (Gurewich *et al*., 1984), was not observed in the animal test with the conjugate, judging from the fact that fibrinogen and $\alpha_2$-antiplasmin levels remained unchanged 1 h after administration.

The second group of fibrinolytic agents use the fibrin-binding affinity of the kringle domains of t-PA or of antibodies specific to fibrin. Among a number of t-PA mutants tested, chimeric molecules of t-PA/scuPA, rtPA-DFE/scuPA-e (Collen *et al*., 1991) and $K_1K_2P_u$ (Lu *et al*., 1992) and a t-PA mutant in which the free $Cys_{84}$ is replaced by Ser (Suzuki *et al*., 1993) have a substantially longer half-life in circulation. Fibrin-targeted fibrinolytic agents, based on fibrin-specific antibodies, were developed by Haber *et al*., (1989). Runge *et al*. (1987) showed the enhanced thrombolytic activity of a chemical conjugate of t-PA and anti-fibrin monoclonal antibody in a rabbit model. A chemical conjugate of scuPA with an antibody against D-dimer of fibrin (Dewerchin *et al*., 1989) and a chimera expressed in insect cells of scuPA ($Ala_{132}$−$Leu_{144}$) with the Fv fragment of an antifibrin monoclonal antibody (Holvoet et al., 1991) also showed enhanced *in vitro* fibrinolytic activity. A hybrid molecule consisting of $Leu_{144}$−$Leu_{411}$ of prourokinase with the antigen-binding domain of a monoclonal anti-fibrin antibody showed higher fibrinolytic activity and higher fibrin selectivity than scuPA (Runge *et al*., 1991).

Recently, annexin V/scuPA fusion proteins were expressed in a prokaryotic system and we found that these molecules have the same plasminogen-activating activity and *in vitro* fibrinolytic activity as scuPA (Tait *et al*., 1995). Expression of the fusion proteins in a eukaryotic secretion system is also in progress to provide large amounts of materials for further animal studies and possible human trials.

The length of spacers between two molecules should be considered for future constructs. In the present conjugate, B-chain is connected to annexin V through an extension of six amino acid residues at the N-terminal tail of annexin V. It has the same binding activity to phospholipids and plasminogen-activating activity comparable to B-chain. Therefore, these two activities do not interfere with each other when they are tested separately *in vitro*. However, the binding and catalytic activities need to act simultaneously to resolve fibrin clots efficiently. A longer spacer may be required to get maximum *in vivo* activity. The DNA sequence of the expression vector used in this study allows the insertion of various lengths of spacers with ease.

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