Tissue Plasminogen Activator Catalyzed Lys-Plasminogen Activation on Heparin-Inserted Phospholipid Liposomes†

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ABSTRACT: We prepared heparin-inserted phospholipid liposomes as a functional model of heparan sulfate present on the vascular surface and examined tissue plasminogen activator (t-PA) catalyzed plasminogen activation on the liposome surface. Kinetic analyses showed a marked increase in the affinity of t-PA for Lys-plasminogen in the presence of heparin-inserted phosphatidylcholine (PC) liposomes. The catalytic efficiency (k_{cat}/K_m) of t-PA for the plasminogen activation on the surface of heparin-inserted PC liposomes was 5.4 times that on the surface of heparin-free PC liposomes. This stimulatory action of immobilized heparin was apparently affected by changing the phospholipid component of liposomes. Phosphatidylethanolamine or stearylamine, having a positively charged group, reduced the catalytic efficiency of t-PA by raising its K_m value (10-fold), whereas negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, did not affect the efficiency. t-PA and generated plasmin bound to the liposome surface heparin were protected from inhibition by plasminogen activator inhibitor type 1 and α_2-plasmin inhibitor, respectively. t-PA-induced clot lysis of euglobulin or whole plasma, which contained native (Glu-) plasminogen and the above inhibitors, was also accelerated by addition of heparin-inserted PC liposomes. These results suggest that the vascular surface heparin-like molecules may play an important role in modulating fibrinolytic events. The principles of conjugation of t-PA with a biologically active liposome will be applied to the construction of better thrombolytic agents.

Vascular endothelial cells synthesize heparan sulfate proteoglycans, which have the disaccharide structure of heparin (Nader et al., 1987) and exhibit anticoagulant activity indistinguishable from that of heparin (Marcum et al., 1987). It is widely accepted that the heparin-like molecules expressed on the cell surface play an important role in the regulation of coagulation by forming a complex with circulating antithrombin III. Recently, several laboratories (Paques et al., 1986; Andrade-Gordon & Strickland, 1986; Soeda et al., 1987) have shown that heparin can bind to endothelial cell derived tissue plasminogen activator (t-PA)[1] and plasminogen in the fluid phase, with consequent promotion of plasminogen activation. Therefore, these findings imply the possibility that the fibrinolytic components bind to the heparin-like molecules present on the endothelial cell surface and that the resultant complex modulates fibrinolysis.

In the present study, we prepared heparin-inserted phospholipid liposomes as a functional model of the endothelial heparin-like molecules and demonstrated that the immobilization of t-PA and Lys-plasminogen on the artificial membrane improves their kinetic parameters and protects the activities of t-PA and generated plasmin from inhibition by their primary inhibitors, plasminogen activator inhibitor type 1 (PAI-1) and α_2-plasmin inhibitor (α_2-PI). We also describe the stimulatory effect of heparin-inserted phosphatidylcholine liposomes on t-PA-induced clot lysis.

Experimental Procedures

Materials. The following reagents were commercially obtained: porcine intestinal mucosa heparin (181 USP units/mg), egg phosphatidylcholine (PC), bovine brain phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), bovine liver phosphatidylinositol (PI), and stearylamine from Sigma Chemical Co., St. Louis, MO; 1,9-dimethylmethylene blue from Polysciences Inc., Warrington, PA; Sepharose 6B and Sephadex G-75 from Pharmacia Fine Chemical Co., Uppsala, Sweden; H-D-valyl-L-Leucyl-L-lysine *p*-nitroanilide (S-2251) from Kabi Vitrum AB, Stockholm, Sweden; *n*-octyl β-D-glucoside and 3,3′-diaminobenzidine tetrahydrochloride from Wako Pure Chemical Co., Osaka, Japan; tosyl-glycyl-L-prolyl-L-arginine *p*-nitroanilide (Chromozyme TH) from Boehringer Mannheim Yamanouchi Co., Tokyo, Japan; cholesterol and cholesteryl chloroformate from Aldrich Chemical Co., Milwaukee, WI.

Purified Proteins. Glu-plasminogen was isolated from fresh human plasma by affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1970) in the presence of 1 mM benzamidine, followed by gel filtration on Ultrogel AcA 44 (Robbins & Summaria, 1976). Lys-plasminogen was prepared by the method of Lucas et al. (1983). Val_{442}-plasminogen was prepared by elastase digestion of Lys-plasminogen essentially as described by Sottrup-Jensen et al. (1978). α_2-PI was pu-

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[1] Abbreviations: t-PA, tissue plasminogen activator; α_2-PI, α_2-plasmin inhibitor; PAI-1, plasminogen activator inhibitor type 1; Glu-plasminogen, native human plasminogen with NH_2-terminal glutamic acid (residues 1–790); Lys-plasminogen, plasmin-cleaved human plasminogen with NH_2-terminal lysine (residues 77–790); Val_{442}-plasminogen, elastase-cleaved human plasminogen fragment with NH_2-terminal valine (residues 442–790); S-2251, H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide; Chromozyme TH, tosylglycyl-L-prolyl-L-arginine *p*-nitroanilide; Ig G, immunoglobulin G; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; WSC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DMSO, dimethyl sulfoxide; DMF, dimethylformamide.

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rified from human plasma by the method of Moroi and Aoki (1976). Recombinant human t-PA (TD-2061, 550000 IU/mg of protein) was kindly supplied by Daiichi Pharmaceutical Co., Tokyo, Japan. Since the t-PA preparation contained a stabilizing agent, it was further purified by affinity chromatography on heparin–Sepharose CL-6B (Soeda et al., 1986). Melanoma PAI-1, which remains essentially in the active form (Wagner & Binder, 1986), was purchased from American Diagnostica Inc., New York. Human thrombin (19 NIH units/mg of protein) and antithrombin III (40 units/mg of protein) were obtained from Boehringer Mannheim. Bovine serum albumin was from Wako Pure Chemical Co.

Antibodies. Sheep anti-human α_2-PI Ig G (20 mg/mL) and sheep anti-human plasminogen Ig G (20 mg/mL) were purchased from The Binding Site Ltd., Birmingham, UK. Peroxidase–rabbit anti-sheep Ig G conjugate (0.5 mg/mL) and mouse anti-human t-PA Ig G (1 mg/mL) were obtained from Zymed Labs. Inc., San Francisco, CA, and Monozyme ApS., Charlottenlund, Denmark, respectively. Peroxidase–goat anti-mouse Ig G conjugate (0.5 mg/mL) was from E-Y Labs. Inc., San Mateo, CA.

Synthesis of a Cholesterol Derivative of Heparin. This heparin derivative was synthesized by modifying the method of Sunamoto et al. (1987), which had been developed for the synthesis of cholesterol derivatives of pullulan. Heparin (250 mg, 16.7 μmol), whose molecular weight was estimated to be 15000 by gel filtration on Sephadex G-75, and ethylenediamine dihydrochloride (15 mg, 112.8 μmol) were dissolved in 5 mL of distilled water, and the pH of the solution was adjusted to 4.75 with 2 M NaOH. After addition of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (WSC) (20 mg, 104.3 μmol), the mixture was mechanically stirred at 25 °C for 2 h (the pH was maintained at 4.75) and dialyzed at 4 °C overnight against 1 mM HCl. The dialysate was lyophilized and dried over P_2O_5 at 25 °C in vacuo. The dried sample was suspended in 6 mL of water-free dimethyl sulfoxide (DMSO). To the suspension was added 16 mg (35.6 μmol) of cholesteryl chloroformate dissolved in 4 mL of water-free dimethylformamide (DMF). The reaction was carried out at 70 °C for 20 h and quenched by addition of 10 mL of distilled water. The resultant aqueous phase was washed three times with 20 mL of *n*-hexane to remove uncoupled cholesteryl chloroformate. A 2-mL portion of the aqueous phase was applied to a Sepharose 6B column (2.5 × 30 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/0.2 mM EDTA. The column was eluted with the buffer at a flow rate of 30 mL/h, and fractions of 3.2 mL each were collected. The fractions containing the heparin derivative (fractions 32–38) were pooled and concentrated to about 6 mL by ultrafiltration on a YM-10 membrane (Amicon Co.). The concentrate (1.2–1.5 mg/mL) was stored at −80 °C until use.

Determination of Cholesterol. The degree of introduction of the cholesterol moiety into heparin was determined as follows. After addition of concentrated HCl (3 mL), the above concentrate (3 mL) was hydrolyzed at 110 °C for 16 h. The hydrolysate was adjusted to pH 7.0 with 12 N NaOH and extracted with 10 mL of *n*-hexane. The extract was evaporated to dryness and dissolved in 300 μL of *n*-hexane (the recovery of standard cholesterol by these procedures was 95%). Together with 10 μL of a standard cholesterol solution (1 mg/mL in *n*-hexane), 50 μL of the sample solution was spotted on a silica gel TLC plate (5 × 20 cm, Merck). The solvent system used was *n*-hexane/diethyl ether (1:1 v/v). The chromatograms were visualized by heating at 100 °C for 5 min with the Zatkis reagent [50 mg of $FeCl_3 \cdot 6H_2O$ in 100 mL of a mixture of H_2O/concentrated H_2SO_4/acetic acid (18:1:1 v/v)]. The amount of the colored cholesterol on each spot was measured with a dual-wavelength flying spot scanner (CS-9000, Shimadzu, Kyoto, Japan) in the reflectance mode at 500 nm with integrated areas.

Preparation of Heparin-Inserted Phospholipid Liposomes. *n*-Octyl β-D-glucoside (120 mg) was added to 8 mg of phospholipid(s) dissolved in 1 mL of chloroform/methanol (19:1 v/v). The solvent was evaporated under a stream of nitrogen. The resultant lipid/detergent film was dissolved in 6 mL of a heparin derivative solution [1.2 mg/mL in 20 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/0.2 mM EDTA]. The solution was dialyzed three times against 3 L of the same buffer for 24 h at 4 °C to remove the detergent. The dialysate was applied to a Sepharose 6B column (2.5 × 30 cm) equilibrated with the Tris-buffered saline. The column was eluted with the buffer at a flow rate of 30 mL/h, and fractions of 3.2 mL each were collected. The fractions containing heparin-inserted phospholipid liposomes were combined, concentrated on a PM-10 membrane (Amicon Co.), and stored at 4 °C.

Enzyme Assays. All enzyme assays were carried out in 96-well plates, and their activities were measured at 25 °C with a microplate reader (EIA Reader Model MPR-A4, Toyo Soda, Tokyo, Japan). The heparin-catalyzed antithrombin III–thrombin reaction was measured as follows. Thrombin (0.5 NIH units) and antithrombin III (0.05 units) were incubated for 5 min with various amounts of heparin or its derivative in a total volume of 150 μL of 50 mM Tris-HCl buffer (pH 7.5)/0.1 M NaCl/0.1% (w/v) poly(ethylene glycol) 6000. To the reaction mixture was added 50 μL of 0.6 mM Chromozyme TH, and the change in absorbance at 405 nm was monitored for 15 min.

Kinetic analyses of Lys-plasminogen activation in the presence and the absence of heparin-inserted phospholipid liposomes were as follows. Various amounts of Lys-plasminogen (0.09–1.42 μM) were incubated with t-PA (36 IU), S-2251 (final concentration 0.75 mM), and, if indicated, the liposomes (70 μg) in a total volume of 200 μL of 50 mM sodium phosphate buffer (pH 7.5)/0.15 M NaCl. At specified time intervals, the absorbance at 405 nm was measured. An initial rate of plasminogen activation was obtained by plotting A^{1cm}_{405nm} versus t^2. The actual reaction rates were also determined by comparing the relative rates of increase of relative absorbance units with those of known plasmin standards.

For the α_2-PI assay, plasmin (16 pmol), which was prepared by the activation of Lys-plasminogen with immobilized urokinase, was incubated for 5 min in the presence of heparin-inserted or heparin-free PC liposomes (70 μg). To the reaction mixture were added various amounts of α_2-PI and S-2251 (final concentration 0.75 mM) in a total volume of 200 μL of 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl, and then the absorbance at 405 nm was measured up to 20 min.

Inhibition of t-PA by PAI-1 was determined as follows. The assay mixture contained the following components in a total volume of 200 μL: 0.75 mM S-2251, 0.5 μM Lys-plasminogen, 0–29.4 nM PAI-1, 14.7 nM t-PA, 20 mM sodium phosphate buffer (pH 7.2), and 70 μg of heparin-inserted or heparin-free PC liposomes. The assay was started by addition of t-PA, and then the absorbance at 405 nm was measured up to 20 min.

Determination of Euglobulin Clot Lysis. Human plasma (10 mL) was diluted 20-fold with 10 mM sodium acetate buffer (pH 5.2) at 4 °C. After 10 min, the euglobulin precipitated was collected by centrifugation at 1500*g* for 15 min at 4 °C and dissolved in 10 mL of 50 mM Tris-HCl buffer

(pH 7.5)/0.1 M NaCl. In a plastic tube, 300 μL of this solution was clotted at 4 °C by addition of 50 μL of thrombin solution (100 NIH units/mL). t-PA (600 IU) was mixed with various amounts of heparin-inserted or heparin-free PC liposomes in 300 μL of 50 mM Tris-HCl buffer (pH 7.5)/0.1 M NaCl, and the mixtures were overlaid on the clots. The length of time required for the completion of clot lysis was determined at 30 °C.

Immunoblotting. A euglobulin sample (16.2 μg of protein) was loaded on a 12% SDS–polyacrylamide gel according to the method of Laemmli (1970). The separated proteins were then transferred to a nitrocellulose membrane (pore size 0.22 μm, Millipore Co.) by the immunoblot technique of Towbin et al. (1979) using a semidry electroblot system (Sartoblot, Sartorius Co.). The nitrocellulose membrane was incubated at 25 °C for 18 h with 3% (w/v) bovine serum albumin in 20 mM sodium phosphate buffer (pH 7.4)/0.15 M NaCl (PBS) and washed 3 times with 20 mM PBS containing 0.05% (v/v) Tween 20 (TPBS) (100 mL each, 15 min). The membrane was further incubated at 25 °C for 1 h with sheep anti-human α_2-PI (500-fold dilution with 20 mM TPBS). After the membrane was washed with TPBS as described above, a detecting antibody, peroxidase–rabbit anti-sheep IG G conjugate (1000-fold dilution with 20 mM TPBS), was added to the membrane and it was incubated at 25 °C for 1 h. After the membrane was washed with 20 mM TPBS, the peroxidase reaction was carried out at 25 °C for 10 min with Karnovsky's solution (25 mg of 3,3′-diaminobenzidine tetrahydrochloride and 0.02 mL of 30% hydrogen peroxide in 100 mL of 50 mM Tris-HCl buffer, pH 7.6). The membrane was washed extensively with water, air-dried, and stored.

Binding of Lys-Plasminogen and t-PA to Immobilized Heparin Derivative. A cholesterol derivative of heparin was immobilized on glass beads ($^1/_4$ inch, Wako Pure Chemical Co.). Ten beads (0.4 g each) were soaked in 5 mL of a heparin derivative solution (1.1 mg/mL in 20 mM PBS) at 25 °C overnight. The heparin derivative coated beads were washed 3 times with 20 mM TPBS and stored at 4 °C in 20 mM PBS until use. The amount of heparin derivative on the bead surface was determined after solubilization with 250 μL of 1% (v/v) acetic acid solution, and it was greater than 25 μg (0.71 nmol)/bead (the coated heparin derivative was not solubilized with 20 mM TPBS). In a typical binding assay, various amounts of Lys-plasminogen (or t-PA) were dissolved in 20 mM PBS, and 200 μL of each solution was incubated with or without a heparin derivative coated bead at 30 °C for 45 min. The reaction mixture was then diluted 80-fold (or 40-fold) with 20 mM PBS, and 200 μL of the diluted sample was put into the well of a polystyrene microtiter plate (Sumitomo Bakelite Co., Tokyo, Japan). After incubation at 4 °C overnight, wells were washed 3 times with 200 μL of 20 mM TPBS. Wells were then incubated at 25 °C for 4 h with 200 μL of 20 mM PBS containing 1% (w/v) bovine serum albumin to saturate nonspecific protein-binding sites and washed 3 times with 20 mM TPBS. To each well was added 200 μL of a sheep anti-human plasminogen Ig G (or mouse anti-human t-PA Ig G) solution (10 μg/mL in 20 mM TPBS). After incubation at 25 °C for 4 h, wells were washed 3 times with 20 mM TPBS. To detect the bound sheep (or mouse) antibody, 200 μL of a peroxidase–rabbit anti-sheep Ig G conjugate (or peroxidase–goat anti-mouse Ig G conjugate) solution (1.5 μg/mL in 20 mM TPBS) was added to each well, and the plate was incubated at 25 °C for 2 h. After the wells were washed with 20 mM TPBS, the peroxidase reaction was started by addition of 150 μL of 2 mM *o*-phenylenediamine in 0.2 M sodium phosphate buffer (pH 5.0)/0.1 M citrate/0.003% (v/v) H_2O_2. After a 5-min incubation at 25 °C, the reaction was stopped by addition of 50 μL of 4 M H_2SO_4 and the absorbance at 492 nm was measured by using the plate reader.

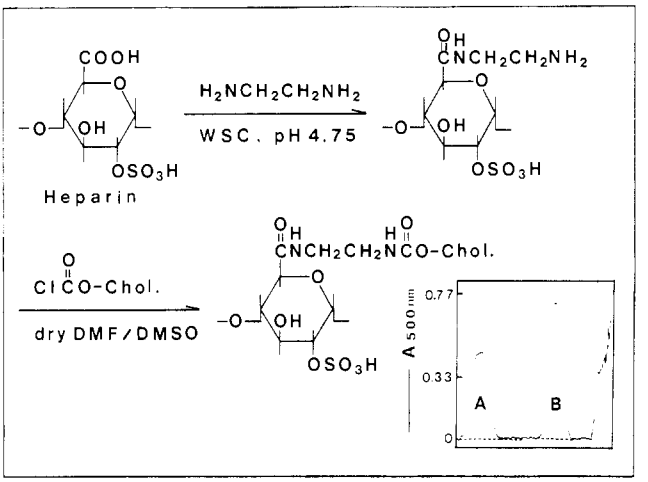

FIGURE 1: Synthesis of a cholesterol derivative of heparin. The inset shows a flying spot scanner chromatogram of the colored spots of the synthetic preparation (peak A) and standard cholesterol (10 μg, peak B) on a TLC plate.

Determination of Protein, Phospholipid, and Heparin. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as the reference. The amount of phospholipid was measured by the method of Duck-Chong (1979), but the concentration of phospholipid in column fractions was estimated by determining its turbidity at 300 nm. The concentration of heparin or its derivative was determined by the method of Farndale et al. (1986), using 1,9-dimethylmethylene blue as a color-producing reagent.

RESULTS

Characterization of a Synthesized Cholesterol Derivative of Heparin and Its Insertion onto PC Liposomes. To modify the surface of PC liposomes with heparin, we synthesized a cholesterol derivative of heparin and tried to penetrate its hydrophobic leg into the lipid bilayer. Figure 1 shows the synthesis of a cholesterol derivative of heparin. The carboxyl groups of heparin were first ethylaminated, and then the amino group of the introduced spacer arm was coupled with cholesteryl chloroformate. The degree of introduction of the cholesterol moiety was determined by the TLC technique as described under Experimental Procedures. the inset of Figure 1 shows a flying spot scanner chromatogram of the synthetic sample (peak A) and standard cholesterol (peak B) spots on a TLC plate. The sample spot contained 8.3 μg of cholesterol. Therefore, the amount of cholesterol moiety introduced to heparin was calculated to be 0.9 mol/mol of native heparin (M_r = 15 000).

Heparin forms a ternary complex with antithrombin III and thrombin and thereby promotes the inhibition of thrombin by its inhibitor. Therefore, to assess the degree of denaturation of the modified heparin, we compared the catalytic efficiency of the cholesterol derivative of heparin on the thrombin inhibition with that of native heparin. As shown in Figure 2, the maximal thrombin inhibition (87%) by addition of the heparin derivative was almost the same as that (92%) by addition of native heparin. However, the concentration of heparin derivative (4 μg/mL) that caused maximal inhibition was 4 times that of native heparin. Also, 80% of the thrombin activity was lost by addition of 1 μg of the heparin derivative or 0.25 μg of native heparin per milliliter. Therefore, these

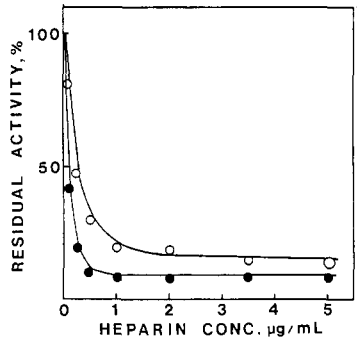

FIGURE 2: Effect of native heparin (●) or its cholesterol derivative (○) concentration on thrombin inhibition by antithrombin III. Thrombin (0.5 NIH units) was incubated with antithrombin III (0.05 units) in the presence of various concentrations of native or modified heparin, and then its residual activity was measured with Chromozyme TH. Experimental details were as described under Experimental Procedures. Each point represents the mean of duplicate experiments.

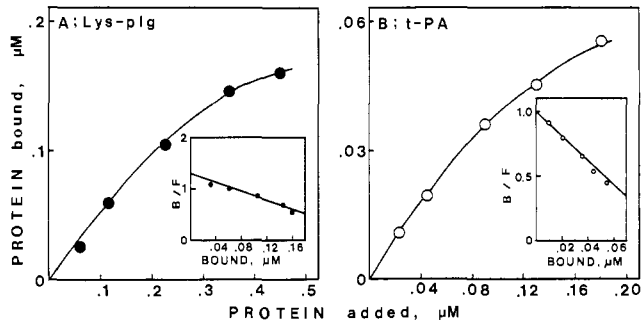

FIGURE 3: Concentration-dependent binding of Lys-plasminogen (panel A) or t-PA (panel B) to the heparin derivative immobilized on glass beads and Scatchard analysis (inset of each panel). Various concentrations of Lys-plasminogen (0.06–0.45 μM) or t-PA (0.023–0.18 μM) were incubated at 30 °C for 45 min with a heparin derivative coated glass bead, and the bead surface-bound protein was determined as described under Experimental Procedures. Each point represents the mean of duplicate experiments.

data indicate that the catalytic efficiency of the heparin derivative for the antithrombin III–thrombin reaction decreased to one-fourth that of the native heparin.

Heparin also forms a ternary complex with the fibrinolytic components plasminogen and plasminogen activator. Figure 3 shows the concentration-dependent binding of Lys-plasminogen or t-PA to the heparin derivative immobilized on glass beads. Apparent K_D values for Lys-plasminogen and t-PA binding to the immobilized ligand were 0.23 and 0.11 μM, respectively. We also determined the binding affinities of these proteins for native heparin immobilized on glass beads (data not shown). Lys-plasminogen and t-PA bound to the immobilized heparin with K_D values of 0.55 and 0.32 μM, respectively. These results suggest that, in contrast to the low catalytic efficiency of the heparin derivative in the antithrombin III–thrombin reaction, the derivative can bind to Lys-plasminogen and t-PA more effectively than can native heparin. On the other hand, Glu-plasminogen under these assay conditions did not bind to either the immobilized heparin or its derivative (data not shown).

Figure 4 shows a typical elution profile of the dialysate (see Experimental Procedures) containing heparin derivative inserted PC liposomes on a Sepharose 6B column. The liposomes were eluted at the position of apparent M_r = 300 000 and separated from free (noninserted) heparin derivative. The amount of inserted heparin derivative was 40 μg/mg of PC. The inset of Figure 4 shows the result of a molecular weight determination of the heparin derivative on a Sephadex G-75

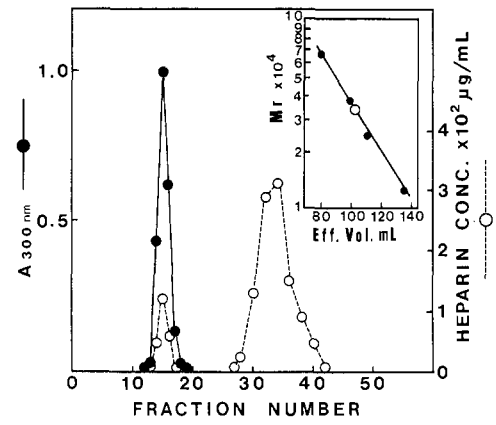

FIGURE 4: Elution profile on Sepharose 6B of heparin-inserted PC liposomes. The preparation and chromatography conditions of heparin-inserted PC liposomes are described under Experimental Procedures. The inset shows the molecular weight determination of the heparin derivative on a Sephadex G-75 column. The column (2.5 × 45 cm) was equilibrated with 20 mM Tris-HCl buffer (pH 7.5)/0.3 M NaCl. Bovine serum albumin (M_r = 68 000), Val_{442}-plasminogen (M_r = 38 000), chymotrypsinogen (M_r = 25 000), and cytochrome *c* (M_r = 12 500) were used as standards.

column. The derivative was eluted in the region of apparent M_r = 18 000–45 000 with a peak at M_r = 35 000.

Kinetics of Plasminogen Activation on Heparin-Inserted Phospholipid Liposomes. Activation of Lys-plasminogen by t-PA in the presence of heparin derivative inserted or heparin-free PC liposomes was examined with a chromogenic substrate, S-2251. The plasminogen activation obeyed Michaelis–Menten kinetics. The K_m and k_{cat} values for the plasminogen activation in the presence of heparin-free PC liposomes were 6.6 μM and 0.017 s^{-1}, whereas those in the presence of heparin-inserted PC liposomes were 0.5 μM and 0.007 s^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) of t-PA for Lys-plasminogen activation in the presence of heparin-inserted PC liposomes was 5.4 times greater than that in the presence of heparin-free PC liposomes. This seems to be primarily due to the remarkable lowering of K_m (1/13.2) in the assay system containing heparin-inserted liposomes, since the V_{max} and k_{cat} values in this system were not much different from those in the heparin-free system. On the other hand, k_{cat}/K_m for Lys-plasminogen activation in the absence of liposomes was 0.0035 μM^{-1} s^{-1} (these data are summarized in Table I). These results clearly indicate that heparin molecules on the surface of liposomes bind t-PA and Lys-plasminogen and promote t-PA-catalyzed plasminogen activation via the lowering of K_m, while the liposome surface itself has little or no effect on the plasminogen activation kinetics.

We next inserted the heparin derivative onto liposomes composed of PC/PS (1:1), PC/PI (1:1), PC/PE (1:1), or PC/stearylamine (4:1) and examined the effect of surface charge on the immobilized heparin catalyzed stimulation of Lys-plasminogen activation. As shown in Table I, the presence of a negatively charged phospholipid, PS or PI, did not affect any kinetic parameters given for the plasminogen activation on heparin derivative inserted PC liposomes. However, the introduction of PE or stearylamine, with a positively charged head group, resulted in a decrease in the catalytic efficiency (k_{cat}/K_m) of t-PA, mainly by raising the K_m value (10-fold).

Protection of Liposome Surface Bound Plasmin or t-PA from Inhibition by Each Primary Inhibitor. Both Lys-plasminogen and t-PA bound to the glass bead immobilized heparin derivative with low K_D values of 0.23 and 0.11 μM, respectively (Figure 3). Furthermore, the complex formation of t-PA and Lys-plasminogen with the heparin derivative on

Table I: Kinetic Parameters of Lys-Plasminogen Activation by t-PA in the Presence of Heparin-Inserted Phospholipid Liposome Preparations[a]

addition	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
none	10.0	0.035	0.0035
heparin-free PC liposomes	6.6	0.017	0.0026
heparin-inserted PC liposomes	0.5	0.007	0.014
heparin-inserted PC/PS (1:1) liposomes	0.6	0.009	0.015
heparin-inserted PC/PI (1:1) liposomes	0.6	0.008	0.013
heparin-inserted PC/PE (1:1) liposomes	5.0	0.017	0.0034
heparin-inserted PC/stearylamine (4:1) liposomes	5.0	0.017	0.0034

[a] Kinetic parameters were obtained from Lineweaver–Burk plot analyses. K_m and V_{max} were calculated from the plot x and y intercepts, respectively. The catalytic rate constant (k_{cat}) was derived from the expression $V_{max}/[E_0]$, where $[E_0]$ = 4.8 nM t-PA. Catalytic efficiency was represented as k_{cat}/K_m. Each reaction mixture (200 μL) contained 70 μg of heparin-free or heparin-inserted liposomes (heparin derivative content 2.5–2.8 μg). Other experimental details were as described under Experimental Procedures.

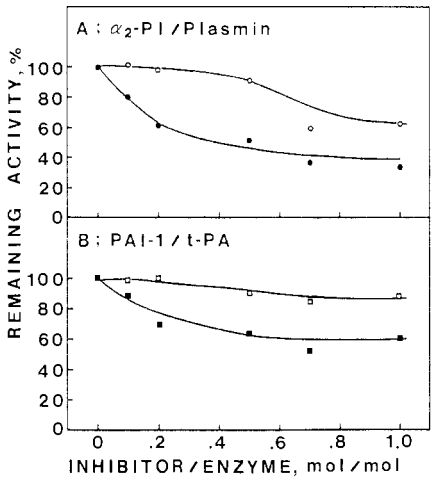

FIGURE 5: Protective effect of heparin-inserted PC liposomes on plasmin or t-PA inhibition by each primary inhibitor. Panel A, inhibition of plasmin by α_2-PI in the presence of heparin-inserted (○) or heparin-free (●) PC liposomes; panel B, inhibition of t-PA by PAI-1 in the presence of heparin-inserted (□) or heparin-free (■) PC liposomes. Experimental details were as described under Experimental Procedures. Each point represents the mean of duplicate experiments.

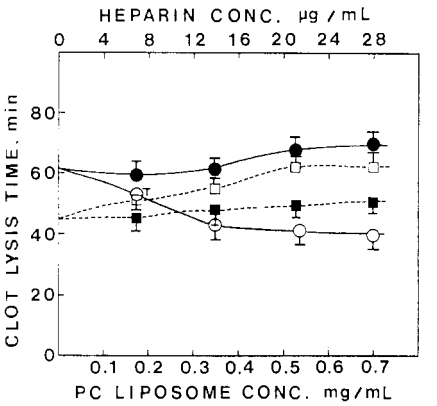

FIGURE 6: Effect of heparin-inserted or heparin-free PC liposome concentration on euglobulin or purified fibrin clot lysis by t-PA. Experimental details were as described under Experimental Procedures. Euglobulin clot lysis: (○), heparin-inserted liposome; (●), heparin-free liposomes. Purified fibrin clot lysis: (□), heparin-inserted liposomes; (■), heparin-free liposomes. Each point represents the mean ± SD of four experiments.

the PC liposome surface resulted in a 5.4-fold stimulation of plasminogen activation (Table I). Therefore, we conclude that the liposome surface bound t-PA retains full functional capcity and that its catalytic activity is at least as strong as that of t-PA in the fluid phase. Plasmin and t-PA are known to be susceptible to rapid inhibition by α_2-PI and PAI-1, respectively. To further characterize the bound plasmin(ogen) and t-PA, we tested the inhibitory effect of α_2-PI or PAI-1 on each enzyme bound to heparin-inserted PC liposomes.

As shown in Figure 5A, at molar ratios of up to 1:0.5 (plasmin:α_2-PI), the heparin liposome bound plasmin was almost insensitive to α_2-PI. However, plasmin in the presence of heparin-free PC liposomes was inhibited by 50% at an estimated molar ratio of 1:0.5 (plasmin/α_2-PI). The activity of heparin liposome bound t-PA was also protected from PAI-1 inhibition at molar ratios of up to 1:1 (Figure 5B). Thus, these results indicate that, as a result of the binding of t-PA or plasmin to heparin-like molecules on the liposome surface, the active site of each enzyme becomes inaccessible to the inhibitor, suggesting that the fibrinolytic potency would be enhanced.

Fibrinolysis by t-PA in the Presence of Heparin-Inserted PC Liposomes. Figure 6 shows clot lysis by t-PA in the presence of heparin-inserted or heparin-free PC liposomes. When t-PA was mixed with various amounts of heparin-inserted PC liposomes and overlaid on euglobulin clots, the clot lysis time of t-PA was clearly shortened with an increase in the amount of the liposomes. The maximal effect was obtained with 0.53–0.7 mg of the liposomes/mL, which contained 21.3–28.0 μg of heparin derivative/mL. Under these conditions, the activity of the liposome-bound t-PA was 4.5-fold higher than that of free t-PA, as judged from the calibration curve of t-PA dose-dependent lysis time (data not shown). In contrast, the fibrinolytic potency of t-PA was gradually decreased with increasing amounts of heparin-free PC liposomes added.

We prepared purified fibrin clots with 0.5% (w/v) plasminogen-rich human fibrinogen and a catalytic amount of thrombin and carried out the same experiment as described above. In contrast to the above result, the clot lysis time of t-PA was significantly prolonged with an increase in the amount of heparin-inserted PC liposomes. The maximal prolongation of lysis time was obtained with 0.5–0.7 mg of the liposomes/mL (Figure 6). This discrepancy may be explained as follows. Plasminogen and t-PA form a ternary complex with fibrin through their kringle domains, and the generation of plasmin is greatly enhanced (Hoylaerts et al., 1982; Ichinose et al., 1986). However, heparin diminishes the stimulation of plasminogen activation by fibrin (Andrade-Gordon & Strickland, 1986). The assay system used purified fibrin clots; therefore, fibrin may compete with the PC liposome surface bound heparin derivative for some of the same binding sites on plasminogen and/or t-PA. Fibrinolysis is also regulated by PAI-1 and α_2-PI. Immunoblot analysis of the euglobulin fraction indicated that the fraction contained at least α_2-PI, consisting of a major form (M_r = 75 000) and a minor form (M_r = 160 000), which may be a complex with plasmin (data not shown). Therefore, it is concluded that the enhancement of euglobulin clot lysis was due to a predominant protection of the liposome surface bound t-PA and plasmin from their inhibitors rather than inhibition of the localization of these fibrinolytic components to fibrin that augments the generation

of plasmin.

Discussion

We synthesized a cholesterol derivative of heparin and inserted it onto liposomes of different phospholipid composition. Both Lys-plasminogen and t-PA bound to the heparin derivative immobilized on glass beads with significantly low K_D values of 0.23 and 0.11 μM, respectively (Figure 3). Furthermore, the immobilization of Lys-plasminogen and t-PA on heparin derivative inserted PC liposomes resulted in a 5.4-fold increase in the catalytic efficiency of t-PA (Table I). The precise mechanism by which Lys-plasminogen and t-PA bound to the liposome surface heparin and enhanced plasminogen activation remains to be fully elucidated. However, we have recently shown that the Val_{442}-plasminogen fragment (residues 442–790) and the B (light) chain of t-PA contain the binding sites for heparin (Soeda et al., 1987). Moreover, we have found that the heparin-binding site of plasminogen is located in its B chain and that one or two lysine residues in the B chain are essential to the heparin binding (Soeda et al., 1989). Therefore, it is considered that these fibrinolytic components can form a complex with the heparin derivative on PC liposome surfaces through their catalytic regions. The heparin connection between the catalytic sites of plasminogen and t-PA may improve the plasminogen activation kinetics by making a situation in which t-PA is easily approachable to plasminogen. Furthermore, the stimulatory effect of liposome surface heparin was clearly reduced by the introduction of positively charged PE or stearylamine to the liposome, whereas the negatively charged phospholipids PS and PI did not alter the effect of immobilized heparin. Since lysine (one or more residues) is crucial for the binding of plasminogen to heparin, the positively charged head group of PE or stearylamine may interfere with the interaction of plasminogen with the negatively charged sulfate groups of heparin.

Plasmin and t-PA bound to the heparin derivative on PC liposome surfaces were protected from inhibitionby α_2-PI and PAI-1, respectively (Figure 5). The active site of each enzyme may become relatively inaccessible to the inhibitor by interacting with the immobilized heparin. This phenomenon has been observed not only in an endothelial cell system (Hajjar et al., 1987) but also in other cell or matrix systems such as fibroblasts (Plow et al., 1986), endothelial cell produced extracellular matrix (Knudsen et al., 1986), and immobilized thrombospondin or histidine-rich glycoprotein (Silverstein et al., 1985). Miles et al. (1988) have shown that a major recognition site of plasminogen for platelet receptor is located in its kringle 1–3 fragment, which binds to fibrin. This region is also involved in accelerating the interaction of plasmin with α_2-PI. They have suggested that an engagement of this region by the receptor may render plasminogen unavailable to the inhibitor. From the present and previous (Soeda et al., 1987) findings, we suggest that, in addition to the fibrin-binding sites, plasminogen and plasminogen activators have other regulatory sites, heparin-binding sites, in their catalytic regions. The functions of these heparin-binding sites that modulate the activation of plasminogen and the action of inhibitor are very similar to those of fibrin (lysine) binding sites located in their N-terminal regions (kringle domains). However, the real physiological function of the heparin-binding site is at present unknown.

Endothelial cells, forming the luminal vascular surface, are strategically located to play an important role in the regulation of coagulation and fibrinolysis. They synthesize and express heparan sulfate proteoglycan that apparently forms a complex with circulating antithrombin III and exhibits anticoagulant activity (Marcum et al., 1987). However, other physiological functions of this proteoglycan have not yet been well understood. The present finding shows the possibility that the heparin-like molecules expressed on the endothelial cell surface may bind fibrinolytic components and promote the breakdown of locally deposited fibrin clots via the mechanism described above. In fact, the binding of t-PA to heparin derivative inserted PC liposomes resulted in a 4.5-fold increase in the euglobulin clot lysis potency of the enzyme (Figure 6). The heparin derivative inserted PC liposomes were also effective on t-PA-induced whole plasma clot lysis (3.2-fold stimulation, data not shown). These phenomena may be mainly due to the protective effect of the liposome surface heparin on the inhibition of t-PA and plasmin by their fast-acting inhibitors (PAI-1 and α_2-PI), because, in a purified fibrin clot system, the immobilized heparin derivative retarded t-PA-induced fibrinolysis (Figure 6).

Plasma plasminogen exists in the form of Glu-plasminogen. The catalytic efficiency of t-PA for Glu-plasminogen activation in the presence of heparin-inserted PC liposomes was 3.2 times that in the presence of heparin-free PC liposomes (data not shown). Andrade-Gordon and Strickland (1986) have shown that the heparin-binding affinity of Glu-plasminogen (K_D = 120 μM) is largely different from that of Lys-plasminogen (K_D = 2.1 μM). We have also found that Glu-plasminogen had no affinity for heparin–Sepharose (Soeda et al., 1989) or the heparin derivative coated glass beads. Therefore, these findings suggest that the heparin-binding site (or sites) of native (Glu-) plasminogen is present as a latent region and becomes exposed or operational by proteolytic modification with plasmin. To bind heparin-like molecules on the endothelial cell surface, Glu-plasminogen may need to be converted to Lys-plasminogen, which is also a principal fibrin-bound species (Thorsen et al., 1984). Indeed, we confirmed the conversion of Glu-plasminogen to Lys-plasminogen during ongoing euglobulin clot lysis (Figure 6) as a M_r = 86000 band using Western blot analysis similar to that described for α_2-PI under Experimental Procedures (data not shown). In the euglobulin or whole plasma clot system, therefore, the principal liposome-bound species of plasminogen may by the Lys form.

Because of its fibrin-directed action, t-PA has recently been used as a specific thrombolytic agent. A disadvantage of t-PA in thrombolytic therapy is that the protein is rapidly removed from the blood circulation by hepatic clearance (Korninger et al., 1981). Therefore, various mutants or hybrid types of t-PA have been synthesized to resolve this problem [reviewed by Haber et al. (1989)]. In contrast to their clinical utilities, plasminogen activators that include t-PA and urokinase are thought to have a central role in initiating tumor cell invasion and metastasis [reviewed by Moscatelli and Rifkin (1988)]. Therefore, for the development of safe and effective t-PA, we will have to take into account possible side effects of this enzyme. Liposomes are now one of the most promising candidates for microcapsules that can deliver therapeutic agents into the desired organs. Heparin, which was used as the surface constituent of liposomes, inhibits lung metastasis of melanoma cells (Nakajima et al., 1988). Stassen et al. (1987) have reported that heparin fragments can potentiate thrombolysis by t-PA as measured by use of a rabbit jugular vein model. Therefore, our present findings will provide useful clues for the development of an improved thrombolytic agent with a high degree of safety and effective fibrinolytic potency.

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