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SOME PROPERTIES OF TISSUE-TYPE PLASMINOGEN ACTIVATOR RECONSTITUTED ONTO PHOSPHOLIPID AND/OR GLYCOLIPID VESICLES

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Summary: Porcine heart tissue-type plasminogen activator (t-PA) was reconstituded onto large multilamellar liposomes with various lipid compositions and the kinetics of plasminogen activation by free or the reconstituted t-PA were studied. Negatively charged lipids, sulfatide and phosphatidylserine (PS), lowered the K_m values of t-PA for plasminogen activation (sulfatide, 20-fold; PS, 6-fold), whereas neutral lipid phosphatidylcholine raised the K_m. On the other hand, these lipid environments did not affect the amidase parameters and fibrin-binding potency of t-PA. The present results suggest that t-PA could function as a cell-associated form and its plasminogen activation may be regulated by the net charge of the head group of membrane lipids.

Tissue-type plasminogen activator (t-PA, EC 3.4.21.31) catalyzes the conversion of plasminogen into plasmin and is an important factor in fibrinolytic system. Because of its fibrin-directed action, t-PA has been used as a specific thrombolytic agent (1-3). An important link of plasminogen and t-PA with endothelial cells (4) and their extracellular matrix (5) has been also established, suggesting that the localized plasmin generation may play a pivotal role in modulating thrombotic events at the vessel wall.

Most of the information for the physiological function of t-PA comes from the action of secreted, one- or two-chain t-PA. In

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Abbreviations: t-PA, tissue-type plasminogen activator; Glu-plasminogen, native human plasminogen with NH_2-terminal glutamic acid; PC, phosphatidyl choline; PS, phosphatidylserine.

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various cells including the vasucular endothelial cells, t-PA probably exists as a cell-associated proenzyme. The single-chain precursor may be synthesized as an inactive form and partly converted into the active one-chain t-PA during its transfer from Golgi-derived secretory vesicles to plasma membrane or after it was exposed on the cell surface (reviewed in ref. 6). However, little is known about the function(s) of cell-associated t-PA. Therefore, we tried to reconstitute the active one-chain t-PA, which was solubilized and highly purified from porcine heart, with various phospholipids and/or glycolipids. In this communication, we describe the relationship between the physical state of t-PA and its enzyme activity in the defined lipid environments.

Materials and Methods

Materials

Porcine one-chain t-PA was solubilized and purified as described previously (7). Glu-plasminogen was prepared from human plasma by the method of Hoylaerts et al. (8). Egg york phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), bovine brain cerebroside and bovine brain sulfatide were purchased from Sigma. Sepharose 6B and n-octyl-β-D-glucoside were from Pharmacia and Wako Pure Chem. (Japan), respectively. Plasminogen-rich human fibrinogen, HD-Val-Leu-Lys-p-nitroanilide (S-2251) and HD-Ile-Pro-Arg-p-nitroanilide (S-2288) were products of Kabi.

Reconstitution of purified t-PA onto lipid vesicles

One-chain t-PA was reconstituted onto various lipid vesicles by a detergent-dialysis method (9). Ten mg of lipid was dissolved in 1 ml of chloroform/methanol (2:1, v/v) and the solvent was evaporated under a stream of nitrogen. The dry lipid film was dissolved in 1 ml of 40 mM Tris-H_2SO_4 buffer (pH 7.5)/0.4 mM EDTA/100 mM KCl/60 mM n-octyl-β-D-glucoside. To the solution t-PA (about 200 μg) in 500 μl of the same buffer was added and the mixture was dialyzed against 20 mM Tris-H_2SO_4 buffer (pH 7.5)/0.2 mM EDTA/50 mM KCl at 4 C for three days.

Separation of t-PA-entrapped lipid vesicles from free t-PA by Sepharose 6B chromatography

The above dialysate was applied to a Sepharose 6B column (2.8 x 32 cm) equilibrated with 20 mM Tris-H_2SO_4 buffer (pH 7.5)/0.2 mM EDTA/100 mM NaCl. The column was eluted with the same buffer at a flow rate of 20 ml/h and fractions of 4.1 ml each were collected. The fibrinolytic t-PA activity of each fraction was determined as described below. The fractions of t-PA-entrapped lipid vesicles were concentrated on a PM-10 membrane (Amicon) and stored at 4 C.

Assay of t-PA activity

The fibrinolytic activity of free or reconstituted t-PA was determined on plasminogen-rich human fibrin plates as described previously (7), except that the sample was treated with an equal volume of 1% (v/v) Triton X-100 solution to fuse the liposomes and applied to the well. The amidolytic activity of t-PA was assayed with S-2288 as the substrate. In a typical assay, the enzyme (15 IU) was incubated at 25 C for 60 min in 250 µl of 0.1 M Tris-HCl buffer (pH 7.5) containing 2.5 mM substrate. The reaction was stopped by addition of 500 µl of 2% (w/v) citric acid/1% (v/v) Triton X-100 and then the absorbance at 405 nm was measured. For kinetic analyses, S-2288 concentration was varried from 0.6 to 4.6 mM. The kinetic constants were calculated from the conventional Lineweaver-Burk transformation. Kinetics of plasminogen activation by t-PA were determined by the method of Hoylaerts et al. (8) with a slight modification. A reaction mixture contained 5 IU of free or reconstituted t-PA, 0-100 µM Glu-plasminogen, 0.6 mM S-2251 and, if indicated, fibrin monomer (1 mg/ml) in a total volume of 250 µl of 50 mM Tris-HCl buffer (pH 7.5). An initial activation rate was obtained from a plot of generated plasmin concentration versus activation time.

Others

Protein concentration was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard. The concentrations of each phospholipid and sulfatide were measured by the methods of Duck-Chong (11) and Kean (12), respectively.

Results and Discussion

Figs. 1A and 1B show typical elution profiles of sulfatide vesicles and t-PA on Sepharose 6B columns. Sulfatide vesicles and t-PA were eluted at the positions of apparent Mr= 300,000 and 70,000, respectively. When the dialysate of t-PA-inserted sulfatide was applied to this column, about 60% of the fibrinolytic activity of t-PA applied was found in the vesicle fractions (Fig. 1C). The activity of this final vesicle preparation was 20% of starting t-PA activity. Namely, these results indicate that most of t-PA used was lost during dialysis and gel filtration, because t-PA shows a strong tendency to bind to various surfaces in the absence of detergent. When t-PA was mixed with pre-prepared sulfatide vesicles and the mixture was subjected to the column, a negligible activity of t-PA was found in the vesicle fractions (Fig. 1D). Thus, t-PA activity in the lipid fractions shown in Fig. 1C is due to the incorporation of t-PA into sulfatide vesicles. Under

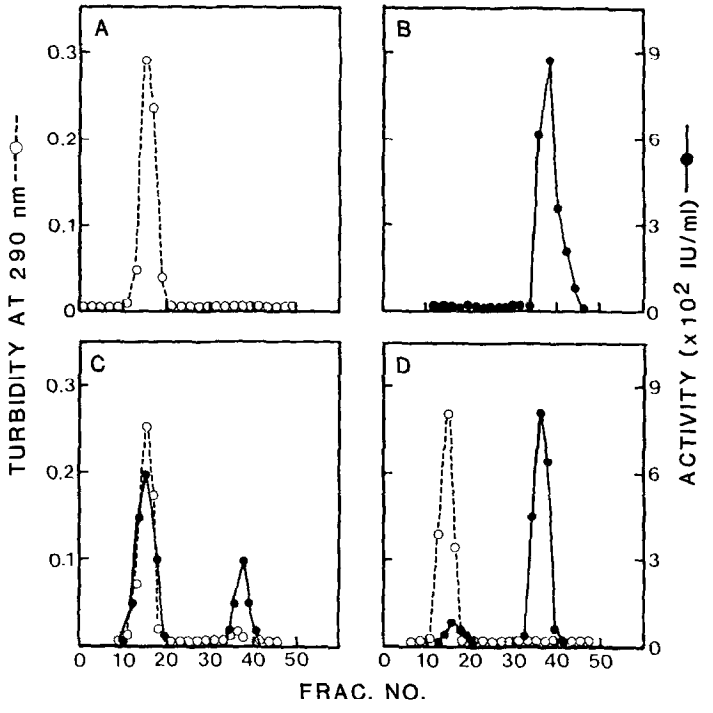

FIGURE 1. Elution profiles of Sepharose 6B chromatography. (A), sulfatide vesicles (10 mg) alone; (B), purified t-PA (200 μg) alone; (C), t-PA (200 μg)-inserted sulfatide vesicles (10 mg); (D), pre-prepared sulfatide vesicles (10 mg) plus free t-PA (200 μg).

these conditions, t-PA was incorporated into liposomes with various lipid components. Table I shows that fibrinolytic activity of t-PA per mg of each lipid vesicle was not different one another. The amidase parameters (K_m and V_{max}) of these reconstituted t-PAs toward S-2288 were also similar to those of free t-PA. This result indicates that the catalytic activity of t-PA toward such a small peptide substrate was not affected by its insertion into these lipid vesicles.

The kinetics of plasminogen activation by various reconstituted t-PAs were studied in the absence or presence of fibrin. In the absence of fibrin, the lipid environment greatly affected the plasminogen activation kinetics of t-PA. Neutral phospholipid PC considerably prevented the affinity of t-PA for plasminogen, because t-PA-PC (100%) liposomes were not saturated even at 100 μM

TABLE I. Kinetic parameters for the hydrolysis of S-2288 by free and reconstituted t-PAs

Composition of liposome	Encapsulated t-PA	K_m	V_{max}
	IU of t-PA/mg of lipid[3]	mM	nmol/min and IU
None (free t-PA)	--	1.1	0.26
PC (100%)[1]	494	1.8	0.18
PS (100%)[1]	555	1.3	0.32
Sulfatide (100%)[1]	627	0.9	0.29
PC/PS (1:1)[2]	498	1.4	0.33
PC/sulfatide (1:1)[2]	536	1.2	0.22
PC/cerebroside (1:1)[2]	506	1.7	0.20

1) Liposomes were prepared with 10 mg of lipid.
2) Liposomes were prepared with each 5 mg of lipid.
3) Assay was performed on human fibrin plates as described in the Materials and Methods.

of Glu-plasminogen. In contrast, the K_m values of t-PA-PS (100%) and t-PA-sulfatide (100%) lowered to 1/6 and 1/20 of that of free t-PA, respectively. The insertion of these negatively charged lipids into PC liposomes (PC : PS or sulfatide = 1 : 1) restored the preventive effect of PC on the affinity of t-PA for plasminogen. However, the insertion of cerebroside into PC liposomes did not modify the effect of PC. These results suggest that the negatively charged groups (especially sulfate groups) of liposomes may be important to promote the affinity of reconstituted t-PA for plasminogen. In addition, the Vmax value for plasminogen activation by t-PA-sulfatide (100%) liposomes was 3-fold higher than that by free t-PA. On the other hand, the K_m values of these reconstituted t-PAs and free t-PA in the presence of fibrin were similar. These results suggest that a large part of the bound t-PA molecule may be exposed on the outer surface of each lipid vesicle and

TABLE II. Kinetics of Glu-plasminogen activation by free and reconstituted t-PAs

Sample	K_m value	
	- Fibrin	+ Fibrin
	μM Glu-plasminogen	
Free t-PA	85	0.9
t-PA bound to PC (100%) liposomes	>100	1.8
t-PA bound to PS (100%) liposomes	15	1.4
t-PA bound to sulfatide (100%) liposomes	4.3	1.0
t-PA bound to PC/PS (1:1) liposomes	67	1.3
t-PA bound to PC/sulfatide (1:1) liposomes	45	1.2
t-PA bound to PC/cerebroside (1:1) liposomes	>100	1.6

thereby t-PA could form a cyclic ternary complex with plasminogen on fibrin matrix (Table II).

Sulfatide vesicles were the best surface for plasminogen activation by t-PA in fibrin-noncontainig microenvironment. The stimulatory effect of sulfatides on the plasminogen activation was clearly diminished by the presence of 5 mM or more 6-aminohexanoic acid (data not shown). Miles and Plow (13) have recently suggested that, since plasminogen bound to unknown site on platelete surface through its lysine-binding site, the interaction may serve to localize and promote plasminogen activation. Roberts et al. (14) have reported that sulfatides are present on the surface of platelets. Therefore, it is conceivable that plasminogen could bind to the sulfate groups of sulfatides surrounding t-PA molecules through the cationic group(s) (e.g. lysine-binding site(s)) and this interaction may improve the plasminogen activation kinetics. Mammalian brain is characterized by an abundance of comlex lipids, particularly sulfatides and cerebrosides. Zisapel et al. (15) have shown that two-species of plasminogen activator are enriched in the synaptosomal plasma membrane of bovine brain and suggested that the plasminogen activators may have a role in the processing of pre-

cursors for peptide hormones and/or neurotransmitters. The mechanism of the modulating effect of lipids on t-PA activity has yet to be sufficiently clarified. It largely depends on future multilateral studies.

The present results are the first for the reconstitution of t-PA into liposomes. Liposomes are now one of the most promising candidates for microcapsules which can deliver therapeutic agents into the desired organs. Human t-PA is now available as a thrombolytic agent (1-3). However, a serious problem in t-PA therapy is that the enzyme is recognized by the liver primarily through its heavy chain and rapidly removed from the blood circulation (16). Our findings will provide useful clues for the development of improved thrombolytic agents with a long half-life and a potency to pass through the blood-brain barrier.

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