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Prolonged Systemic Delivery of Streptokinase Using Liposome

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To prolong the biological half-life of streptokinase, a thrombolytic agent, streptokinase-bearing liposome with and distearolyphosphatidyl ethanolamine-N-poly (ethylene glycol) 2000 (DSPE-PEG 2000) was prepared and evaluated. Streptokinase-bearing liposomes composed of distearolyphosphatidylcholine (DSPC), cholesterol and cholesterol-3-sulfate with DSPE-PEG 2000 was prepared by the freeze-thawing method and administered via femoral vein to rats (15000 IU/kg). The activity of streptokinase in plasma was determined by the method based on the amidolytic activity of streptokinase-plasminogen complex. Pharmacokinetic parameters of streptokinase incorporated in liposomes were compared with those of streptokinase alone. The $T_{1/2}$ and AUC_{∞} of streptokinase incorporated in DSPC-PEG liposome increased 16.3- and 6.1-fold, respectively, compared with those of streptokinase alone. Streptokinase-bearing long-circulating liposome could increase the circulation time of streptokinase in blood and expect longer thrombolytic activity compared with streptokinase alone.

Key words : Streptokinase, Long-circulating liposome, Pharmacokinetics

INTRODUCTION

In recent, many peptides and proteins have been available with the advance in biotechnology, but in general, the application of these bioactive materials is limited due to short biological half-life in many cases (Zhou and Po, 1991). Liposomes have been used to improve the systemic delivery of such susceptible drugs by protecting them from degradation *in vivo*. However, conventional liposomes are rapidly recognized and cleared by reticuloendothelial system (RES) from blood stream and thus its application as a drug delivery system has not been extended (Patel, 1992). This defect could be partially relieved by controlling the size, lipid composition and charge of liposomal bilayers (Senior *et al.*, 1985; Allen *et al.*, 1989). But the achieved longevity was effective only at the high lipid dose and the extent was not enough for drug delivery. Novel liposomes composed of polyethylene glycol (PEG)-phospholipid derivatives have been developed to reduce the RES uptake and prolong the duration of liposomes in the circulatory system (Woodle *et al.*, 1992).

Streptokinase (m.w. 47 kDa) is an antigenic single-chain bacterial protein having fibrinolytic properties in human. It has been used as a thrombolytic agent for the treatment of deep vein thrombosis, pulmonary embolism, acute myocardial infarction, etc. (Martindale, 1993) However, i.v. infusion for 12-72 h is usually required because of short biological half-life due to rapid elimination from the circulatory system by antibodies and the RES (Fletcher *et al.*, 1959).

In this study, to develop a delivery system for streptokinase which can prolong the duration of streptokinase in the circulation, streptokinase-bearing long-circulating liposomes were prepared by the freeze-thawing method, and then the pharmacokinetic parameters of streptokinase incorporated in liposome were compared with those of streptokinase alone.

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METERIALS AND METHODS

Materials

Streptokinase (SK, 100000 IU/ml) was kindly supplied by Cheiljedang (Icheon, Korea). Human plasminogen (20 U) was purchased from Boehringer Mannheim (GmbH, Germany). Cholesterol (CH), cholesterol-3-sulfate (CS), D-Val-Leu-Lys-p-nitroanilide·dihydrochloride (S-2251) and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Distearolyphosphatidylcholine (DSPC) and distearolyphosphatidylethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG 2000) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

Preparation of streptokinase-bearing liposomes

Streptokinase bearing liposomes (DSPC-PEG liposomes) were prepared by the freeze-thawing method (Mayer *et al.*, 1985). Briefly, lipid mixture (90 μmoles total lipid) composed of DSPC, CH, CS and DSPE-PEG 2000 in a molar ratio of 1.85:0.85:0.15:0.15 was dissolved in organic solvent mixture ($CHCl_3$:MeOH=2:1, v/v). The organic phase was removed on a rotary evaporator under reduced pressure (360 mmHg). The lipid film was hydrated with 2 ml of Tris-HCl buffer (0.05 M Tris, 0.01 M NaCl, pH 7.4) and subsequently frozen in liquid nitrogen and thawed at room temperature. 100 μl of streptokinase (100000 IU/ml) was added and vortexed for 30 sec. Freeze-thawing cycle was repeated 5 times and the resulting liposomes were extruded several times through stacked polycarbonate filters (0.4 μm pore size, Nucleopore, Costar, Cambridge, MA, USA). The unincorporated streptokinase was then removed by ultracentrifugation (180000×g, 1 h) at 4°C. The particle size distribution of liposomes was determined by the photon correlation spectroscopy (LPA-3000, Otsuka Electronics, Japan). The amount of protein and lipid were determined by the Lowry method (Lowry *et al.*, 1951) and Stewart method (Stewart, 1959) with slight modifications, respectively. The physical stability of liposomes was estimated by measuring the size change of liposomes during storage at 4°C.

Assay of streptokinase in rat plasma

The assay for streptokinase activity was performed according to the previously reported method (Grierson *et al.*, 1987) with slight modifications. The determination of plasma streptokinase activity in plasma is based on the rate of amidolysis of the chromogenic substrate, S-2251, by the streptokinase-plasminogen activator complex. The assay procedure was as follows: 470 μl of Tris-HCl buffer, (pH 7.4, 0.15 mol/L), 80 μl of SBTI (5 mg/10 ml), 100 μl of plasminogen (10 U/ml) and 50 μl of pretreated plasma were mixed in a silanized glass tube in water bath at 37°C. 100 μl of streptokinase standard was added and timer started. The plasma concentration range of streptokinase standard was in between 20 and 800 IU/ml. At 4 min after the addition of standard streptokinase, the mixture was transferred to a 1 ml cuvette in a thermostated cuvette holder in a spectrophotometer and at 5 min, 200 μl of S-2251 (3.3 mmol/L) was added, and then mixture was shaken and the rate of amidolysis was recorded over 5 min. Maximum optical density change (ΔOD) at 405 nm was determined. The blank was prepared in the same way except that the streptokinase standard was substituted with buffer. Since streptokinase is not effective on the rat plasminogen, human plasminogen was added for the assay to form an active streptokinase-plasminogen complex (Cooley *et al.*, 1983). To determine the proper amount of human plasminogen for the streptokinase assay in rat plasma, 1.75, 3.5, 7 and 14 U/ml (Tris-HCl buffer, pH 7.4) of human plasminogen was mixed with streptokinase standards (50, 200, 400, 800, 1600 IU/ml) separately. The resulting maximal ΔOD was converted to plasma streptokinase activity in units/ml according to the standard curve equation. To determine the activity of liposomal streptokinase, streptokinase-incorporated liposomes were incubated with Triton X-100 (final 0.1%) prior to assay and then total activity of streptokinase liberated from liposomes was measured with same way as streptokinase alone.

Animal experiment

The healthy male Sprague-Dawley rats (240~280 g) were purchased from Experimental Animal Breeding Center of Seoul National University (Seoul, Korea). The femoral artery and vein were catheterized with polyethylene tubing (PE-50, Naume Co., Tokyo, Japan) under light ether anesthesia. Each rat was allowed to recover from anesthesia before study. The rats were in supine position during the study. Streptokinase alone (in Tris-HCl buffer, pH 7.4) and liposomal streptokinase equivalent to 15000 IU/kg were administered via the femoral vein of six rats, respectively. To investigate the effect of liposome itself on the behavior of streptokinase *in vivo*, the physical mixture of streptokinase and blank DSPC-PEG liposome was also administrated. Blood samples were collected via femoral artery at designated time intervals after the dose and anticoagulated with 130 mM sodium citrate to yield blood-citrate ratio (9:1). The plasma was separated by centrifugation and stored in the freezer prior to assay. The activity of streptokinase in collected samples was determined.

Pharmacokinetic data analysis

The pharmacokinetic parameters such as area under the plasma concentration-time curve from time zero to infinity (AUC_∞, $IU \cdot h \cdot ml^{-1}$), mean residence time (MRT, h), time-average total body clearance (CL, $L \cdot h^{-1} \cdot kg$) and apparent volume of distribution at steady state (Vss, $L \cdot kg^{-1}$) were calculated using standard methods (Gibaldi and Perrier, 1982). The data were compared for statistical significance by t-test. All results were expressed as mean±standard deviation (S.D.).

RESULTS AND DISCUSSION

Preparation and characterization of liposomes

The activity of streptokinase was not significantly changed after the freeze-thawing cycles. Repetition of

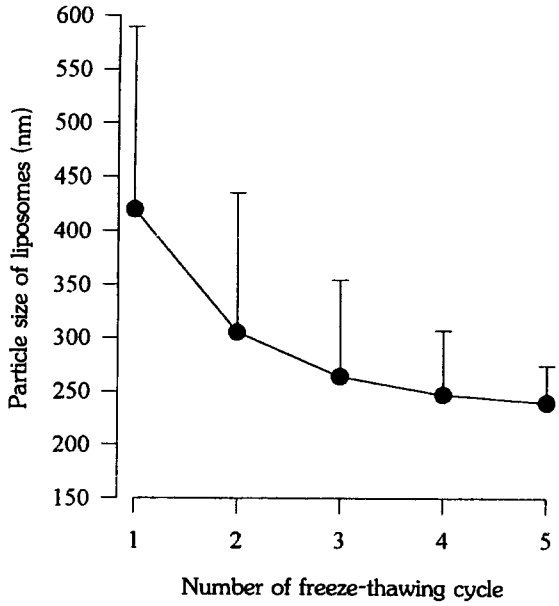

Fig. 1. Effect of the repeated number of freeze-thawing cycle on the size of streptokinase-incorporated DSPC-PEG liposome.

freeze-thawing cycle of MLVs promotes the physical disruption of phospholipid bilayers and serves to break apart the closely spaced lamellae of vesicle thereby raising the encapsulation efficiency. Therefore, in this study, the effect of the repeated number of freeze-thawing cycle on the size of liposome was investigated. Although the liposomal size was thought to be increased by the fusion between liposomal vesicles during freeze-thawing cycles, the size of streptokinase-incorporated liposomes decreased by increasing the number of freeze-thawing cycle as shown in Fig. 1. It is assumed that this process, which can result in the reduction of liposomal size, breaks up the aggregates. Moreover, streptokinase, the hydrophilic protein, was thought to be exposed on the surface of liposome due to its large size. Thus, it might partially inhibit the fusion process during the cycles (Takashi *et al.*, 1985). Resulting liposomes had mean diameters less than 250 nm and the incorporation efficiency of streptokinase in DSPC-PEG liposome was 15~25%. This liposome was also physically stable for at least 30 days at 4°C.

Assay of streptokinase in rat plasma

Within the range of streptokinase concentrations in plasma between 50 and 800 IU/ml, the amidolysis rates were linear regardless of the added amount of human plasminogen from 1.75 to 14 U/ml. Thus, 10 U/ml of human plasminogen was used for the assay of streptokinase in the plasma. The intraday covariance of the assay was within 10% and the assay was reliable within a day. In determination of the activity of streptokinase incorporated in liposomes, plasma samples were incubated with 0.1% Triton X-100 at 37°C for 5 min before the assay. Although the detergents are known to interfere the activities of proteins, we confirmed that the activity of streptokinase was not significantly affected by 0.1% Triton X-100 under current experimental condition.

Pharmacokinetics

The mean amidolytic activity-time curves of streptokinase after i.v. administration of streptokinase alone, streptokinase incorporated in DSPC-PEG liposome, and the mixture of streptokinase and blank liposomes to rats are shown Fig. 2. Almost all the activities of streptokinase disappeared from the blood stream at 1.5 h after the dose of streptokinase alone. On the other hand, streptokinase incorporated in DSPC-PEG-liposome showed the prolonged amidolytic activity in blood compared with streptokinase alone. This might be due to the prolonged circulation of sterically stabilized liposome as a streptokinase carrier. The liposomes did not alter the pharmacokinetic behavior of streptokinase in rats after the co-administration of streptokinase and blank liposome.

The pharmacokinetic parameters of streptokinase after i.v. administration of streptokinase alone and streptokinase incorporated in DSPC-PEG-liposome are listed in Table I. The $T_{1/2}$, AUC_{∞} and MRT of streptokinase incorporated in DSPC-PEG-liposome increased 16.3-, 6.1- and 12.6-fold compared with those of stre-

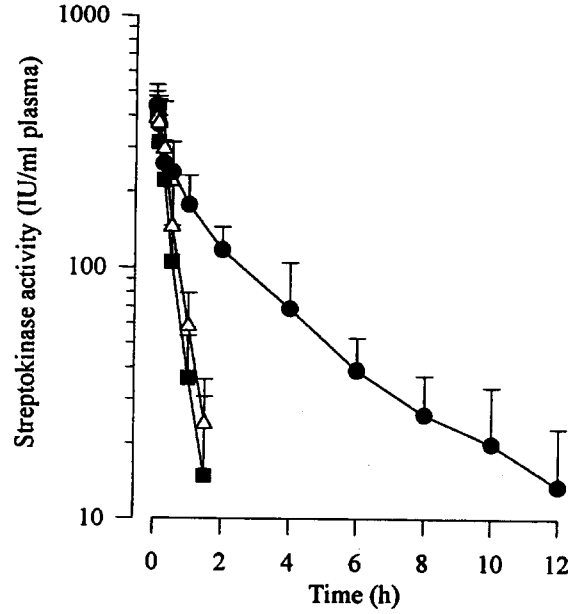

Fig. 2. Plasma amidolytic activity-time curve of streptokinase after i.v. administration of streptokinase alone (■), streptokinase incorporated in DSPC-PEG-liposome (●), and the physical mixture of streptokinase and blank DSPC-PEG-liposome (△) (15000 IU/kg) to rats. Each result represents the mean±S.D. (n=6)

Table I. Pharmacokinetic parameters of streptokinase after i. v. administration of streptokinase alone and streptokinase incorporated in DSPC-PEG-liposome to rats (15000 IU/kg)

Parameter \ Formulation	Streptokinase alone	Liposomal streptokinase DSPC/CH/CS/PEG-PE
$T_{1/2}$ (h)	0.33±0.06	5.37±0.84[a]
AUC_∞ (IU·h·ml^{-1})	158.51±45.84	963.85±253.21[a]
MRT (h)	0.43±0.05	5.42±0.70[a]
CL (L·h^{-1}·kg)	103±37	17±5[a]
Vdss (L·kg^{-1})	44±18	91±31[b]

[a]Significantly different at $p<0.01$ by the Student's t-test when compared to streptokinase alone.
[b]Significantly different at $p<0.05$ by the Student's t-test when compared to streptokinase.

ptokinase alone (5.37±0.84 versus 0.33±0.06 h, 963.85 ±253.21 versus 158.51±45.84 IU·h·ml^{-1}, 5.42±0.70 versus 0.43±0.05 h), respectively. The total body clearance (CL) of streptokinase incorporated in DSPC-PEG-liposome decreased 1/6-fold compared with that of streptokinase alone (17±5 versus 103±37 L·h^{-1}·kg).

Liposomes are known to protect the incorporated materials from enzymatic degradation and act as microreservoirs *in vivo* (Gao *et al.*, 1994). Pharmacokinetic parameters in Table I indicated that streptokinase incorporated in liposome could be protected from neutralization by anti-streptokinase antibodies in the body which are known for the primary mechanism of the elimination of streptokinase from circulation at the initial phase after the dose (Fletcher *et al.*, 1959). Moreover, it is thought that PEG-lipid derivatives incorporated in liposomal bilayer could enhance the duration of liposome itself in the circulation (Maruyama *et al.*, 1992). Therefore, streptokinase incorporated in DSPC-PEG-liposome could also remain in the blood stream much longer than streptokinase alone.

CONCLUSION

Being incorporated into DSPC-PEG-liposome, streptokinase could remain much longer than streptokinase alone in the circulatory system. Since the streptokinase is a thrombolytic agent of which target site is a clot in the blood stream, the prolonged circulation of streptokinase in blood may provide the more opportunity of drug to meet the clot. Thus, the prolonged duration of streptokinase by means of incorporating into long circulating liposomes here has a meaning of prolonged targeting to the acting site of streptokinase.

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