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Research paper

Subconjunctivally injected, liposome-encapsulated streptokinase enhances the absorption rate of subconjunctival hemorrhages in rabbits

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ABSTRACT

Liposome-encapsulated streptokinase (SK) was prepared with distearoylphosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}). In vitro release assay demonstrated over 81% of SK was released from liposomes at 48 h, and the effect of its subconjunctival injection on the absorption rate of induced subconjunctival hemorrhage (SH) in rabbits was evaluated. After 8 h of SH induction, eyes were randomly assigned to one of four subconjunctival injection groups (10 eyes each): group A: the free form of SK (1000 IU/mL); group B: liposome-encapsulated SK (1000 IU/mL); group C: 0.1 mL of liposomes; and group D: no injection. SHs were photographed at 8, 24, 48, 72, and 120 h after SH induction and their sizes were compared. Size decrease of the SH was faster in groups A and B than in groups C and D. Group B displayed significantly different absorption rates than group A at 24 and 48 h and with groups C and D at 24, 48, and 72 h, with the shortest mean elapsed time among all groups. The ocular absorption of SK was lower after the injection of the liposome-encapsulated SK than the free form. These results demonstrated that subconjunctival injection of liposome-encapsulated SK enhances the rate of SH absorption, especially in the early phases.

1. Introduction

Streptokinase (SK, m.w. 47 kDa) is one of the most widely used and least expensive fibrinolytic agents used for treating thromboembolic conditions [1], which counteracts with fibrin thrombus formation by converting proenzyme plasminogen into plasmin [2]. SK has a half-life of about 20–30 min after intravenous injection [3,4] due to its rapid clearance from circulation by antibodies and the reticuloendothelial system [4]. Liposomes have been used to improve the systemic delivery of drugs with short biological half-lives by protecting them from degradation in vivo [4], and SK-bearing liposome composed of cholesterol and distearoylphosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}) was shown to have 16-fold increased half-life of 5.4 h in rat plasma [4].

Subconjunctival hemorrhage (SH) is a common ophthalmic condition resulting in a troublesome cosmetic disfigurement. However, there has not been an approved therapeutic measure to enhance its absorption in ophthalmologic practice. There has been only one animal study reported from our laboratory with low-molecular-weight heparin (LMWH) [5], while few therapeutic trials have been attempted to enhance its absorption in the past [6].

Subconjunctival liposomes were reported to be associated with the sustained release and retentive effects of encapsulated drugs at the site of injection in animal models [7,8]: single subconjunctival injection of liposome-encapsulated antibiotics indeed demonstrated better effect than a higher dose of liposome-free antibiotics in a rabbit *Pseudomonas* keratitis model [7]. Subconjunctival injection might be the best way of local treatment for SH to increase the local concentration of the drug in the desired location while decreasing the systemic absorption and/or side effect of the drug, and sustained release of SK from liposomes may be helpful in facilitating the absorption of SH. Therefore, we planned to investigate the effect of subconjunctival injection of liposome-encapsulated SK on the absorption rate of induced SH in rabbits, and monitored the possible side effects of SK including systemic reaction and drug absorption into plasma and intraocular tissues at different time points.

2. Materials and methods

2.1. Materials and animals

Streptokinase (250,000 IU), cholesterol-3-sulfate (CHS), D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride (S-2251), soybean trypsin

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inhibitor (SBTI) and thrombin were purchased from Sigma (St. Louis, MO, USA). Distearolyphosphatidylcholine (DSPC), cholesterol (CH) and distearoylphosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Human plasminogen (120 U) was purchased from Merck (Darmstadt, Germany). Fifty New Zealand white rabbits, weighing 2.0 to 3.0 kg, were used as animal models. All experimental methods and animal care procedures were reviewed and approved by the institutional committee for animal studies at Kim's Eye Hospital.

2.2. Preparation of liposome-encapsulated SK

Liposome-encapsulated SK was prepared by the freeze-thawing method [9]. Briefly, lipid mixture (90 μmol total lipid) composed of DSPC, CH, CHS and DSPE-PEG_{2000} in a molar ratio of 1.85:0.85:0.15:0.15 was dissolved in the mixture of organic solvents ($CHCl_3$:MeOH = 2:1, v/v). The organic phase was removed using a rotary evaporator under a reduced pressure (360 mmHg) and liposomes were prepared by hydrating the lipid film with 2 mL of Tris–Cl buffer (pH 7.4, 0.05 M Tris, 0.01 M NaCl). The liposomes were subsequently frozen in liquid nitrogen, and thawed at room temperature with 100 μL of SK (100,000 IU/mL). After five freeze–thawing cycles, and the resulting liposomes were extruded 10 times through a 400 nm polycarbonate membrane (Whatman, Clifton, NJ, USA) under nitrogen pressure (300 kPa). The un-encapsulated SK was then separated by removing the supernatant after ultracentrifugation in a rotor (TLA 100.3; Beckman) at 150,000*g* for 1 h. Liposome-encapsulated SK was finally formed by resuspending liposome pellet, and the suspension was subsequently diluted in Tris–HCl buffer to make the final concentration of 1000 IU/mL and was stored at 4 °C.

2.3. Physical properties of SK-bearing liposomes

The mean particle size and polydispersity index (PI) of SK-encapsulating liposomes were determined by dynamic light scattering method using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at a fixed angle of 90° and at room temperature. Prior to measurement, liposomes were diluted with filtered deionized water. The system was used in the auto-measuring mode. The laser diffraction particle size analysis data were evaluated using volume distribution to detect even a few large particles. The PI is a measure of the distribution of particulate population. Small PI means the narrow distribution of liposomes.

The electrophoretic mobility of liposomes was determined using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan). The electrophoretic mobility was measured after the dilution of samples with filtered deionized water. The measured electrophoretic mobility data were converted into zeta potential using Helmholtz–Smoluchowski equation. The processing was done by the software included within the system.

Encapsulation efficiency of SK in liposomes was determined by measuring the SK activity after disrupting the liposomes with 0.1% Triton X-100. The SK activity was determined according to the previously reported method [10] with a slight modification. The determination of SK activity is based on the rate of amidolysis of the chromogenic substrate, S-2251, by the SK-plasminogen activator complex. The assay procedure was as follows: 470 μl of Tris–HCl buffer (pH 7.4, 0.05 M Tris, 0.01 M NaCl), 80 μl of SBTI (0.2 mg/mL), 100 μl of plasminogen (10 U/mL) and 50 μl of liposome solution pretreated with 0.1% Triton X-100 were mixed in a silanized glass tube at 37 °C. SK standard of 100 μl was added and the timer started. The concentration range of SK standard was between 20 and 1000 IU/mL. At 4 min after the addition of standard SK, 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. Then the mixture was shaken and the rate of amidolysis was recorded for 5 min. Maximum optical density change (ΔOD) at 405 nm was determined. The resulting maximal ΔOD was converted to SK activity in IU/mL according to the standard curve equation. Physical properties of liposome formulations are summarized in Table 1.

2.4. Evaluation of in vitro release of SK from liposomes

The SK-bearing liposomes were placed in a closed vial and immersed in shaking water bath at 37 °C. At 12, 24, 48, 72, 96, and 120 h after the preparation of SK-bearing liposomes, in vitro release rate of SK from liposomes was determined by separating liposomes from the suspension medium by ultracentrifugation at 150,000*g* for 1 h. The supernatant was removed and replaced with the same quantity of sterile saline for the subsequent analysis. SK content in the supernatant was determined by an assay for SK activity as described above [10]. The cumulative release fraction was defined as follows: $(C/T) \times 100$ (%), where C is the cumulative amount of SK detected only in the supernatant, and T is the total amount of SK in liposomes.

2.5. Induction of SHs and evaluation of the SH absorption rates

Animal experiments were conducted using the previously described rabbit SH model [5] with a slight modification. General anesthesia was achieved by intramuscular injection of 10–15 mg/kg of Zoletil® (5–7.5 mg/kg of tiletamine and 5–7.5 mg/kg of zolazepam: Virbac Laboratories, France) and 5–10 mg/kg of xylazine, and proparacaine hydrochloride was used for topical anesthesia. Autologous blood was sampled from the auricular veins and was injected subconjunctivally into both eyes of all rabbits (0.05 mL, 1–2 mm from the superior limbus).

Eight hours after the SH induction, the material to be injected for each eye of the rabbits was assigned by block randomization: group A (10 eyes): 0.1 mL of the free form of SK (1000 IU/mL); group B (10 eyes): 0.1 mL of liposome-encapsulated SK (1000 IU/mL); group C (10 eyes): 0.1 mL of liposomes; and group D (10 eyes): no injection. A preliminary experiment demonstrated no significant effects on the SH absorption rate in the fellow untreated eyes after the treatment of one eye with one of the regimens, thus allowing simultaneous use of both eyes. The participants, who performed block randomization, subconjunctival injection and measurement of the SH sizes, were blind to the types of the injected materials.

The image of SH was photographed with a digital camera (PowerShot® A85: Cannon, Tokyo, Japan) at 8, 24, 48, 72, 96, and 120 h after the hemorrhage induction with a translucent bar marked with 1-mm graduations as a reference for sizing. The sizes of digitalized SH images were measured using an image analyzer program (Image Pro-Plus, ver. 6.0; Media Cybernetics, Silver Spring,

Table 1
Physical properties and encapsulation efficiency of liposome formulations

	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
Liposome	236.1 ± 9.3	0.182 ± 0.021	−22.62 ± 4.34	–
Liposome-SK[a]	245.9 ± 13.3	0.190 ± 0.013	−26.99 ± 5.21	20.87 ± 2.72

The results are expressed as the mean ± SD (n = 5).
[a] Liposome-SK, liposome-encapsulated SK, SK-bearing liposomes.