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ORIGINAL ARTICLE

Effect of surface-mannose modification on aerosolized liposomal delivery to alveolar macrophages

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Abstract

Purpose: The effect of surface-mannose modification on aerosolized liposomal delivery to alveolar macrophages (AMs) was evaluated in vitro and in vivo. *Method*: 4-Aminophenyl-α-D-mannopyranoside (Man) was used for surface-mannose modification, and mannosylated liposomes with various mannosylation rates (particle size: 1000 nm) were prepared. *Results*: In the in vitro uptake experiments, the uptake of mannosylated liposomes by AMs was increased with the increase in the mannosylation rate over the range 2.4–9.1 mol% Man and became constant at over 9.1%. Thus, the most efficient mannosylation rate was 9.1 mol% Man. Furthermore, free mannose inhibited the uptake of mannosylated liposomes by AMs. This indicates that the uptake mechanism of mannosylated liposomes by AMs is mannose receptor-mediated endocytosis. In the in vivo animal experiments, the mannosylated liposomes (mannosylation rate, 9.1 mol% Man) were more efficiently delivered to AMs after pulmonary aerosolization to rats than nonmodified liposomes and did not harm lung tissues. *Conclusion*: These results indicate that surface-mannose modification is useful for efficient aerosolized liposomal delivery to AMs.

Key words: *Alveolar macrophages; liposomes; NR8383 cells; pulmonary aerosolization; surface mannose modification*

Introduction

The alveolar macrophages (AMs) that are present in the alveolar epithelial lining fluid (ELF) take up the spherical structure of phospholipids because of the surfactants secreted by type II alveolar epithelial cells and are associated with surfactant metabolism[1,2]. Thus, liposomes with a spherical structure formed from phospholipids are useful as drug carrier systems targeting AMs for the treatment of AM-associated diseases, such as respiratory intracellular parasite infections[3–8], pneumoconiosis[9], and alveolar proteinosis[10].

Recently, liposomes have attracted great interest as potential drug carriers for the treatment of many diseases, such as leishmaniasis[11], fungal infections[12,13], cancer[14,15], and atherosclerosis[16,17], because they are easy to prepare, their particle size can be altered easily and surface modifications are possible. However, until recently, there has been little detailed information published regarding the liposomal delivery to AMs. We have shown that the most effective particle size of liposomes for drug targeting to AMs following pulmonary aerosolization is 1000 nm and an aerosolized liposomal antibiotic formulation is an efficient therapeutic system for the treatment of respiratory infections compared with oral therapy[18–20].

Specific receptors, such as mannose receptors[21,22], surfactant protein (SP) receptors[23,24], and scavenger receptors[25,26], are expressed in AMs. Surface modification of liposomes by specific ligands for these receptors is a potentially useful pharmaceutical technique for achieving more efficient liposomal drug targeting to AMs. Mannose is a component of many foods and may be an excellent modification agent in terms of its availability and safety.

Initially, in this study, the uptake of mannosylated liposomes with various mannosylation rates (particle size: 1000 nm) by AMs was examined in vitro to select the

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optimal mannosylation rate for liposomes. Then, the delivery efficiency to AMs following pulmonary aerosolization of optimal mannosylated liposomes in rats was compared with nonmodified liposomes and their stability and cytotoxicity in the lung were examined to evaluate the efficacy of surface-mannose modification.

Materials and methods

Materials

Hydrogenated egg yolk phosphatidylcholine (HEPC) was purchased from NOF Co. (Tokyo, Japan), cholesterol (CH) was obtained from Wako Pure Chemicals Co. Ltd. (Osaka, Japan), dicetylphosphate (DCP) and 4-aminophenyl-α-D-mannopyranoside (Man) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]cholesterylhexadecylether ([^{3}H]CHE) was purchased from NEN Life Science Products, Inc. (Boston, MA, USA) and 5(6)-carboxyfluorescein (CF) was obtained from Kanto Kagaku (Tokyo, Japan). All other reagents were commercially available and of analytical grade.

Preparation of liposomes

Preparations of nonmodified and mannosylated liposomes with 0, 2.4, 4.8, 9.1, and 16.7 mol% Man were performed by the lipid film hydration method[27]. Briefly, HSPC, CH, DCP, and Man for mannose modification in a lipid molar ratio of 7/2/1/0, 7/2/1/0.25, 7/2/1/0.5, 7/2/1/1, or 7/2/1/2 were dissolved in chloroform/methanol (9/1), followed by evaporation to obtain a thin film. For the in vitro uptake and in vivo pharmacokinetic experiments, [^{3}H]CHE was used as a nonexchangeable lipid phase marker[28] to label the liposomes. The film was completely hydrated using phosphate-buffered saline (PBS, pH 7.4) to obtain the liposome suspension. The total lipid concentration in the liposome suspension was fixed at 18.2 μmol/mL. The particle size of the liposomes was adjusted to 1000 nm by the extrusion method using polycarbonate filters with pore sizes of 1000 nm (Nuclepore, Maidstone, UK). The particle size was determined by photon correlation spectroscopy using a Coulter N4 plus a submicron particle analyzer (Coulter Co., Miami, FL, USA). The particle size distributions of nonmodified and mannosylated liposomes with 0, 2.4, 4.8, 9.1, and 16.7 mol% Man were 1010 ± 110, 1013 ± 103, 1037 ± 113, 1024 ± 98, and 1027 ± 93 nm, respectively. The zeta potential of the liposomes was determined by a laser Doppler method using a zeta potential analyzer (Zeta Plus, Nikkiso Co. Ltd., Tokyo, Japan). The zeta potential was approximately −55 mV for all liposomes. For the in vitro stability testing, CF was used as an aqueous phase marker[29] to label the liposomes. Nonmodified and mannosylated liposomes with 0 and 9.1 mol% Man were prepared by the lipid film hydration method using 30 mM CF solution. The particle size of the liposomes was adjusted to 1000 nm by the extrusion method. After extrusion, the liposomal suspension was dialyzed in a cellulose dialysis tube against PBS for at least 3 days at 4°C with frequent changes of PBS to remove unencapsulated CF.

In vitro uptake experiments

NR8383 cells (American Type Culture Collection, Manassas, VA, USA) were used as cultured SD rat AMs. The cells were suspended at a concentration of 1×10^6 cells/mL in RPMI 1640 medium (Sigma Chemical Co). Aliquots of 1 mL of the cell suspension were then transferred to 24-well culture plates (Becton Dickinson, Lincoln Park, NJ, USA) and the plates were incubated for 90 minutes at 37°C with 5% CO_2 to develop cell monolayers. Then, liposomes labeled with [^{3}H]CHE (0.9 μmol lipids/mL in medium) were added to the cell monolayers, followed by incubation at 37°C for 2 hours. After incubation, the medium was removed and the cells were washed with PBS (pH 7.4). The cells were then extracted with 1 mL 0.1 M NaOH solution and the protein concentration in the cell extracts was determined using Coomassie Protein Assay reagent (Pierce Chemical Company, Rockford, IL, USA) with bovine serum albumin as a standard. The [^{3}H]CHE content in the cell extracts was assayed as follows. Eight hundred microliters of each cell extract and 7.2 mL Hionic-Fluor (Packard BioSci. Co., Meriden, CT, USA) were mixed and stored overnight. The [^{3}H]CHE radioactivity was determined by scintillation counting. In the uptake inhibition experiments, NR8383 cell monolayers were treated with liposomes labeled with [^{3}H]CHE (0.9 μmol lipids/mL in medium) in the presence of free mannose (20 mM in medium) at 37°C for 2 hours.

In vivo animal experiments

Male SD rats (190–220 g, Japan SLC, Inc., Hamamatsu, Japan) were used. The animal experimental plan was approved by the Committee of the Laboratory Animal Center (No. 07-011) and conforms to the Guiding Principles for the Care and Use of Experimental Animals in Hokkaido Pharmaceutical University. In the in vivo pharmacokinetic experiments, a liquid MicroSprayer™ (Model IA-1C, PennCentury, Inc., Philadelphia, PA, USA) was inserted into the trachea of rats under pentobarbital anesthesia. Then, aerosolized liposomes labeled with [^{3}H]CHE (4.6 μmol lipids/250 μL/kg) were sprayed into the lungs just before the bronchus. At indicated time points after administration, the trachea was immediately cannulated and the lungs were lavaged three times with 5 mL ice-cold PBS (pH 7.4)[30]. The

bronchoalveolar lavaged fluid was immediately centrifuged at 4°C (650 × *g* for 10 minutes) to separate the AMs from the diluted ELF. The AMs were then extracted with 1 mL 0.1 M NaOH solution for scintillation analysis. The cell protein concentration and the [^{3}H]CHE content in the cell extract were determined as described above. To calculate the concentration of liposomes in AMs, the intracellular volume of the AMs was determined by a velocity-gradient centrifugation technique using ^{3}H-water[31] and was estimated to have a mean value of 4.2 μL/mg cell protein. In the cytotoxic testing, aerosolized liposomes (4.6 μmol lipids/kg) were sprayed into the lungs as described above. PBS (pH 7.4) and Triton X-100 [0.25%, in PBS (pH 7.4)] were used as negative and positive controls, respectively. The dosage volume was 250 μL/kg in each case. After 24 hours, the diluted ELF was collected as described above. The lactate dehydrogenase (LDH) level in the diluted ELF was determined using an LDH-Cytotoxic test kit (Wako Pure Chemicals, Osaka, Japan) with LDH from chicken heart as a standard. To calculate the LDH level in ELF, the apparent volume of ELF was estimated using urea as an endogenous marker of ELF dilution[32]. The mean value estimated was 398.3 μL/whole rat lung.

In vitro stability testing

A 2.5-mL aliquot of ELF dilution collected from rats as described above was pre-incubated for 15 minutes in a cell placed in a fluoro-spectrophotometer (F-2000, Hitachi Co., Ltd., Tokyo, Japan) under a constant temperature at 37°C. A 5-μL aliquot of liposome suspension (18.2 μmol lipids/mL) was added to the diluted ELF and the mixture was incubated for 120 minutes with stirring. The fluorescence intensity (Ex 495 nm; Em 515 nm) produced by the CF released from liposomes was recorded continuously ($FI_{S,\ ELF}$). After the final recording, 500 μL ethanol was added to the incubation mixture and the total fluorescence intensity was measured ($FI_{T,\ ELF}$). A control study was also carried out with PBS (pH 7.4) instead of diluted ELF and the fluorescence intensity was measured with or without ethanol ($FI_{S,\ PBS}$ and $FI_{T,\ PBS}$, respectively). As shown in Equation (1), the degradation of liposomes in ELF was evaluated by the percentage of CF that had leaked from the liposomes corrected by the control value, which was measured by incubating liposomes with PBS (pH 7.4):

$$\text{Degradation}(\%) = (FI_{S,ELF} / FI_{T,ELF} - FI_{S,PBS} / FI_{T,PBS}) \times 100. \quad (1)$$

The latency of the liposomes was used as an index of liposomal stability in ELF and was expressed as follows:

$$\text{Latency}\ (\%) = 100 - \text{degradation}(\%). \quad (2)$$

Statistics

Statistical analysis was performed by Student's *t*-test and the Tukey–Kramer test using Stat View Software (Abacus Concepts Inc., Berkeley, CA, USA).

Results and discussion

Uptake of liposomes by rat AMs in vitro

The uptake of liposomes by NR8383 was examined. The uptake of nonmodified and mannosylated liposomes with 0, 2.4, 4.8, 9.1, and 16.7 mol% Man by NR8383 at 2 hours after application is shown in Figure 1A. The

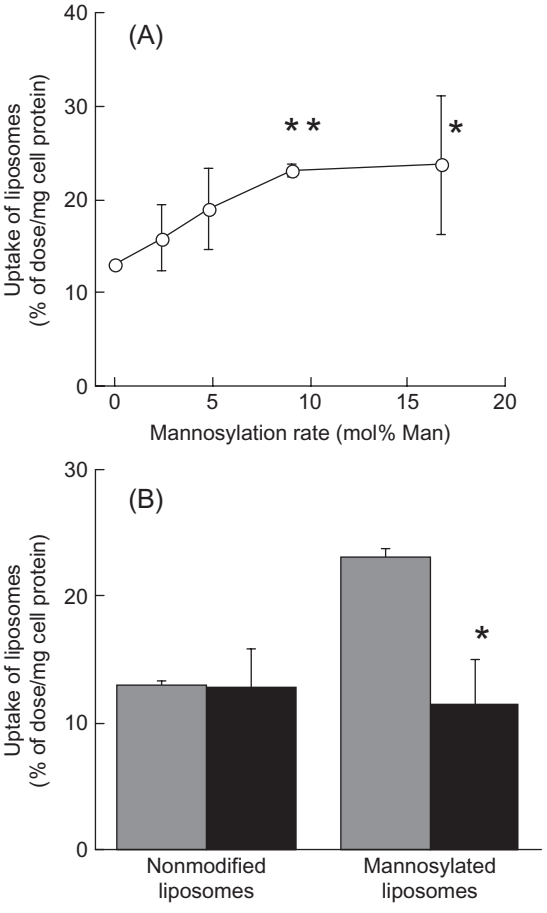

Figure 1. The uptake of nonmodified and mannosylated liposomes by NR8383 (A) and the effect of free mannose on their uptake (B). (A) Nonmodified and mannosylated liposomes with 0, 2.4, 4.8, 9.1, or 16.7 mol% Man were added to NR8383, followed by incubation at 37°C for 2 hours. *$P < 0.05$, **$P < 0.01$, significant difference compared with nonmodified liposomes (0 mol% Man) in the Tukey–Kramer test. (B) NR8383 were treated with nonmodified or mannosylated liposomes with 0 or 9.1 mol% Man in the absence or presence of free mannose at 37°C for 2 hours. Columns: gray column, without free mannose; black column, with free mannose. *$P < 0.01$, significant difference compared with the treatment without free mannose in Student's *t*-test. In both panels, each value represents the mean ± SD (n = 3–4).

uptake of mannosylated liposomes was increased with the increase in the mannosylation rate over the range 2.4–9.1 mol% Man and became constant at over 9.1%. This indicates that the uptake capacity of mannosylated liposomes is saturated at 9.1 mol% Man. Because the most efficient mannosylation rate was 9.1 mol% Man, mannosylated liposomes with 9.1 mol% Man were used for the subsequent examinations. The effect of free mannose on the uptake of liposomes by NR8383 was also examined to clarify the uptake mechanism. The uptake of nonmodified and mannosylated liposomes by NR8383 at 2 hours after application in the presence of free mannose is shown in Figure 1B. Free mannose significantly inhibited the uptake of mannosylated liposomes. This indicates that the uptake mechanism of mannosylated liposomes by AMs is mannose receptor-mediated endocytosis.

Aerosolized liposomal delivery to AMs in rats

The aerosolized liposomal delivery to AMs was examined. The concentration of nonmodified and mannosylated liposomes in AMs after pulmonary aerosolization to rats is shown in Figure 2. The concentration of mannosylated liposomes in AMs was markedly higher than that of nonmodified liposomes at each time point. This indicates that mannosylated liposomes were more efficiently delivered to AMs than nonmodified liposomes, similarly to peritoneal macrophages[33] and Kupffer cells[34]. Thus, surface mannose modification is a rational technique for increasing the delivery of liposomes to AMs.

The alveolar SP opsonizes pathogens and enhances phagocytosis by AMs[35–38]. Mannose receptors are expressed in AMs[21,22] and SP in ELF upregulates the activity of the mannose receptors of AMs[39]. The uptake of liposomes by phagocytes, such as peritoneal macrophages and Kupffer cells, is accelerated by opsonization based on complement activation[40–42]. The SP-A and SP-D bind to mannose receptors as well as SP receptors of AMs[43]. The ultrastructures of SP-A and SP-D are similar to mannose-binding protein[44], thus, SP-A and SP-D have a high affinity for mannose[45]. These reports and the results of the in vitro uptake inhibition experiments (Figure 1B) suggest that unopsonized mannosylated liposomes are taken up by AMs via upregulated mannose receptors, and mannosylated liposomes opsonized by SP-A and SP-D in ELF are taken up via upregulated mannose receptors as well as SP receptors. These different uptake routes may account for the enhanced uptake of mannosylated liposomes.

Pulmonary cytotoxicity of liposomes in rats

The pulmonary cytotoxicity of liposomes was examined by the measurement of the LDH released from lung tissues into ELF. The LDH levels in ELF at 24 hours after pulmonary aerosolization of liposomes, PBS as a negative control or Triton X-100 solution as a positive control, in rats are shown in Figure 3. The LDH levels after treatment of mannosylated liposomes were similar to that after PBS treatment. This indicates that the mannosylated liposomes do not injure lung tissues and

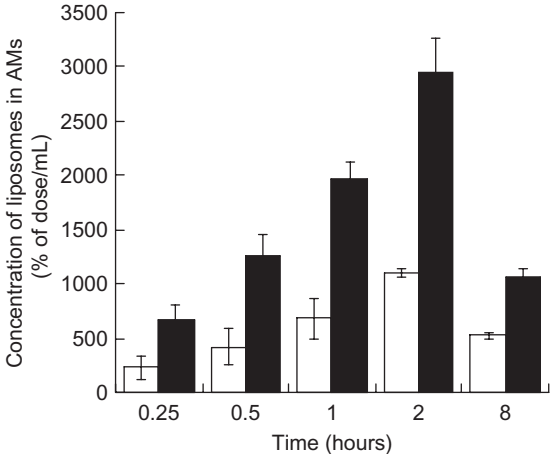

Figure 2. The delivery of nonmodified and mannosylated liposomes to AMs after pulmonary aerosolization in rats. Nonmodified or mannosylated liposomes with 0 or 9.1 mol% Man were sprayed into rat lungs. At each time point, the AMs were collected and the concentration of liposomes was determined. Symbols: open column, nonmodified liposomes; closed column, mannosylated liposomes. A significant difference was shown by the Mann–Whitney U-test ($P < 0.05$) for each time point. Each value represents the mean ± SD ($n = 3$–4).

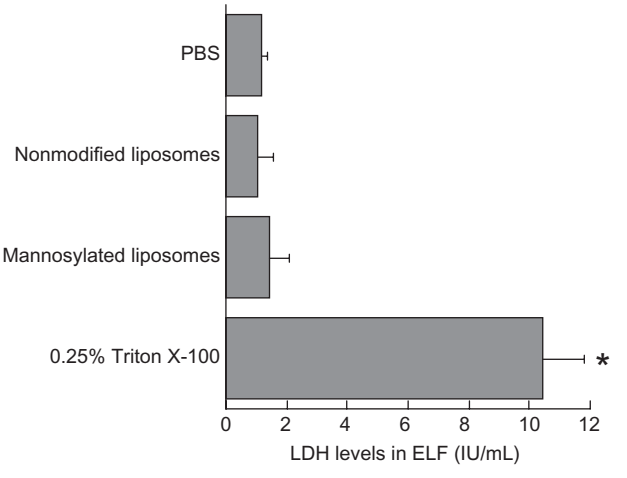

Figure 3. Pulmonary cytotoxicity of nonmodified and mannosylated liposomes after pulmonary aerosolization in rats. Nonmodified or mannosylated liposomes with 0 or 9.1 mol% Man were sprayed into rat lungs. PBS and 0.25% Triton X-100 solution were used as negative and positive controls, respectively. After 24 hours, ELF was collected and LDH levels in ELF were determined. Each value represents the mean ± SD ($n = 3$). *$P < 0.01$, significant difference compared to the treatment with PBS in Dunnett's test.

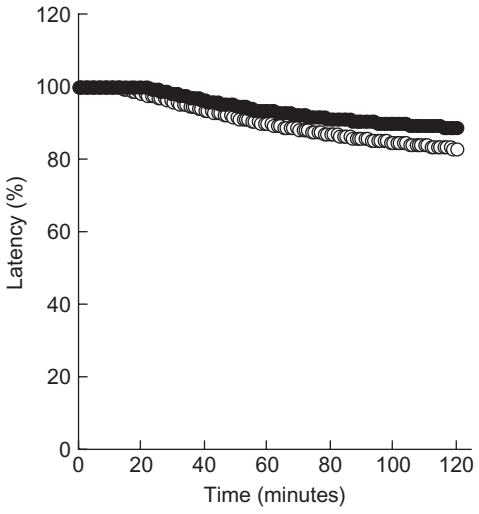

Figure 4. Stability of nonmodified and mannosylated liposomes in ELF. The latency of nonmodified and mannosylated liposomes with 0 or 9.1 mol% Man as the index of liposomal stability in ELF was calculated from in vitro degradation profiles. Symbols: open circle, nonmodified liposomes; closed square, mannosylated liposomes.

are biocompatible in lung, at least at the dose used in this study. The major components of mannosylated liposomes are phospholipid and cholesterol. These are also components of ELF[46,47]. Again, mannose used for surface modification is biocompatible and biodegradable[48]. These observations explain the pulmonary compatibility of mannosylated liposomes.

Stability of liposomes in ELF

The stability of liposomes in ELF was examined. The latency profiles of nonmodified and mannosylated liposomes in ELF are shown in Figure 4. The latency of mannosylated liposomes after 2 hours of incubation was more than 90%, thus, mannosylated liposomes were stable in ELF. This indicates that mannosylated liposomes, initially particulate and undegraded, are stably taken up by AMs in ELF after pulmonary aerosolization.

Conclusion

In conclusion, this study examined the effect of surface mannose modification on aerosolized liposomal delivery to AMs. We have shown that the delivery of liposomes to AMs following pulmonary aerosolization is enhanced by surface mannose modification. The mannosylated liposomes were found to be compatible in pulmonary tissue. These findings suggest that mannosylated liposomes are useful as drug carrier systems to target AMs.

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Declaration of interest

The authors report no conflicts of interest.

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