JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 75–80

Received June 25, 2006
Accepted September 14, 2006
DOI 10.1211/jpp.59.1.0010
ISSN 0022-3573

Uptake characteristics of liposomes by rat alveolar macrophages: influence of particle size and surface mannose modification

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Abstract

The influence of particle size and surface mannose modification on the uptake of liposomes by alveolar macrophages (AMs) was investigated in-vitro and in-vivo. Non-modified liposomes of five different particle sizes (100, 200, 400, 1000 and 2000 nm) and mannosylated liposomes with 4-aminophenyl-α-D-mannopyranoside (particle size 1000 nm) were prepared, and the uptake characteristics by rat AMs in-vitro and in-vivo were examined. The uptake of non-modified liposomes by rat AMs in-vitro increased with an increase in particle size over the range of 100–1000 nm, and became constant at over 1000 nm. The uptake of non-modified liposomes by AMs after pulmonary administration to rats in-vivo increased with an increase in particle size in the range 100–2000 nm. The uptake of mannosylated liposomes (particle size 1000 nm) by rat AMs both in-vitro and in-vivo was significantly greater than that of non-modified liposomes (particle size 1000 nm). The results indicate that the uptake of liposomes by rat AMs is dependent on particle size and is increased by surface mannose modification.

Introduction

The alveolar macrophages (AMs) that are present in the alveolar epithelial lining fluid take up the spherical structure of phospholipids due to the surfactants secreted by type II alveolar epithelial cells, and are associated with surfactant metabolism (Fehrenbach 2001; Poelma et al 2002). Thus, liposomes that are spherical in structure formed from phospholipids may be useful as a drug carrier system targeting AMs for the treatment of respiratory intracellular parasite infections (Ellner et al 1991; Doganay 2003; Frieden et al 2003; Tarnvik & Berglund 2003; Nara et al 2004; Tsai 2005), pneumoconiosis (Nadif et al 2006) and alveolar proteinosis (Brasch et al 2004).

The uptake characteristics of liposomes by phagocytes such as peritoneal macrophages (Hsu & Juliano 1982; Nishikawa et al 1990; Huong et al 1998), Kupffer cells (Rahman et al 1982; Nag & Ghosh 1999; Kawakami et al 2000) and foam cells (Chono et al 2005, 2006) are well documented. However, there is little information about the uptake characteristics of liposomes by AMs. An evaluation of the uptake characteristics of liposomes by AMs is important to achieve efficient drug targeting to AMs. The uptake of liposomes by peritoneal macrophages (Hsu & Juliano 1982), Kupffer cells (Rahman et al 1982) and foam cells (Chono et al 2006) is particle size-dependent. Specific receptors such as mannose receptors (Lane et al 1998; Kudo et al 2004), surfactant protein receptors (Chroneos et al 1996; Crowther & Schlesinger 2006) and scavenger receptors (Gronlund et al 2000; Palecanda & Kobzik 2001) are expressed in AMs. Thus, the particle size and surface modification by specific ligands for these receptors may be key factors influencing the uptake of liposomes by AMs.

In the present study, non-modified liposomes (particle size: 100, 200, 400, 1000 and 2000 nm) and mannosylated liposomes with 4-aminophenyl-α-D-mannopyranoside (particle size: 1000 nm) were prepared, and the uptake characteristics by AMs were evaluated in-vitro and in-vivo to determine the influence of particle size and surface mannose modification.

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Acknowledgement and funding: The authors would like to thank Nikkiso Co., Ltd (Tokyo, Japan) for help with the zeta potential analysis. This work was supported in part by a Grant-in-Aid (No. 16590117) for Scientific Research provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported in part by the education and research fellowship (2005) provided by Hokkaido Pharmaceutical University.

Materials and Methods

Materials

Hydrogenated soybean phosphatidylcholine was purchased from NOF Co. (Tokyo, Japan); cholesterol was from Wako Pure Chemicals Co., Ltd (Osaka, Japan); dicetylphosphate and 4-aminophenyl-α-D-mannopyranoside were 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). All other reagents were commercially available and of analytical grade.

Preparation of liposomes

Non-modified and mannosylated liposomes were prepared by the lipid film hydration method (Bangham et al 1965). Briefly, hydrogenated soybean phosphatidylcholine, cholesterol, dicetylphosphate and 4-aminophenyl-α-D-mannopyranoside for mannose modification in a lipid molar ratio of 7/2/1/0 or 7/2/1/1 were dissolved in chloroform/methanol (9/1), followed by evaporation to obtain a thin film. The film was completely hydrated using phosphate-buffered saline (pH 7.4) to obtain the liposomes. The particle size of the liposomes was adjusted by the extrusion method using polycarbonate filters (Chono et al 2006). The particle size was determined by photon correlation spectroscopy using a Coulter N4 plus a submicron particle analyser (Coulter Co., Miami, FL, USA). The distributions of the particle sizes of non-modified liposomes were (mean ± s.d.): 2059 ± 268, 1015 ± 123, 422 ± 44, 207 ± 21 and 97 ± 10 nm. In the case of the mannosylated liposomes, the distribution of particle size (means ± s.d.) was 987 ± 107 nm. The zeta potential of the liposomes was determined by a laser Doppler method using a zeta potential analyser (Zeta Plus; Nikkiso Co., Ltd, Tokyo, Japan). The zeta potential was approximately –70 mV for all liposomes. [^{3}H]CHE was used as a non-exchangeable lipid phase marker (Derksen et al 1987) to label the liposomes.

In-vitro uptake experiments

NR8383 cells (American Type Culture Collection, Manassas, VA, USA) were used as cultured SD rat AMs for the in-vitro uptake experiments. The cells were suspended at a concentration of 10^6 cells mL^{-1} in RPMI 1640 medium (Sigma Chemical Co.) containing 10% fetal bovine serum (Sigma Chemical Co.). Aliquots (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 min at 37°C with 5% CO_2 to develop NR8383 cell monolayers. After incubation, non-adherent cells were removed and then RPMI 1640 medium containing 1% fetal bovine serum was added to the monolayers. Liposomes were added to the NR8383 cell monolayers, and then the cells were incubated at 37°C with 5% CO_2. The concentration of liposomes in the medium was 1 mmol total lipid mL^{-1}. At 2 h after incubation, the medium was removed and the cells were washed with RPMI 1640 medium. 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 (Bradford 1976). The [^{3}H]CHE content in the cell extracts was assayed as follows. A fraction (700 μL) of each cell extract and 6.3 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-vivo uptake experiments

The animal experimental plan was approved by the Committee of the Laboratory Animal Center (No. 06-004), and conforms to the Guiding Principles for the Care and Use of Experimental Animals in Hokkaido Pharmaceutical University. Male SD rats (200–250 g; Japan SLC, Inc., Hamamatsu, Japan) were used for the in-vivo uptake experiments. Liposomes were administered to the lungs of rats via the nasal cavity using Liquid MicroSprayers (Model IA-1C; PennCentury, Inc., Philadelphia, PA, USA) under pentobarbital anaesthesia. The dosage volume was 250 μL kg^{-1} with a dose corresponding to 5 μmol total lipid kg^{-1}. At indicated time points after administration, the trachea was immediately cannulated and the lungs were lavaged three times with 5 mL ice-cold phosphate-buffered saline (pH 7.4) (Antonini & Reasor 1991). The bronchoalveolar lavage fluid was immediately centrifuged at 4°C (650 *g* for 10 min) to separate the AMs. 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.

The uptake profiles of non-modified and mannosylated liposomes by AMs following pulmonary administration to rats were analysed according to Equation 1 with the non-linear least-squares program MULTI (Yamaoka et al 1981). The Damping Gauss Newton method was chosen as an algorithm.

$$X = K\,(\exp(-k_{el}\,t) - \exp(-k_{up}\,t)) \quad (1)$$

k_{up} and k_{el} are the uptake and elimination rate constants, respectively. It was assumed that there was no flip-flop phenomenon. K is the corresponding zero-time intercept, and t is time.

The pharmacokinetic parameters were calculated as follows:

$$AUC = K\,(1/k_{el} - 1/k_{up}) \quad (2)$$

$$MRT = 1/k_{el} + 1/k_{up} \quad (3)$$

AUC and MRT are the area under the uptake amount–time curve and the mean residence time, respectively.

Statistics

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

Results

Influence of particle size on uptake of liposomes by rat AMs in-vitro and in-vivo

The uptake characteristics of non-modified liposomes by NR8383 cells in-vitro and AMs following pulmonary administration were examined. The uptake amounts of non-modified liposomes (particle size: 100–2000 nm) by NR8383 cells at 2 h after application and AMs at 2 h after pulmonary administration to rats are shown in Figure 1. The uptake of non-modified liposomes by NR8383 cells increased with an increase in the particle size over the range of 100–1000 nm and became constant at over 1000 nm (Figure 1A). The uptake of non-modified liposomes by AMs increased with an increase in the particle size over the range of 100–2000 nm (Figure 1B).

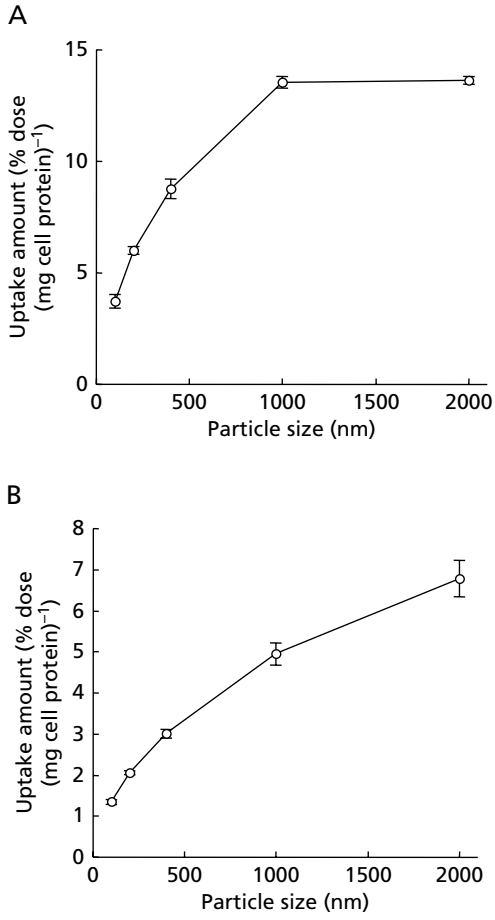

Figure 1 Uptake of non-modified liposomes by NR8383 cells in-vitro (A) and alveolar macrophages in-vivo (B). A. Non-modified liposomes (particle size: 100, 200, 400, 1000 and 2000 nm) were added to NR8383 cells followed by incubation at 37°C for 2 h. B. Non-modified liposomes (particle size: 100, 200, 400, 1000 and 2000 nm) were administered to rat lungs. At 2 h after administration, alveolar macrophages were collected by the bronchoalveolar lavage method. Each value represents the mean ± s.e., n = 4.

Influence of surface mannose modification on uptake of liposomes by rat AMs in-vitro and in-vivo

The uptake characteristics of mannosylated liposomes by NR8383 cells in-vitro and AMs following pulmonary administration were examined. The uptake amounts of non-modified and mannosylated liposomes (particle size: 1000 nm) by NR8383 cells at 2 h after application and AMs until 24 h after pulmonary administration to rats are shown in Figure 2. A particle size of 1000 nm was chosen because the uptake of non-modified liposomes by NR8383 cells was the most

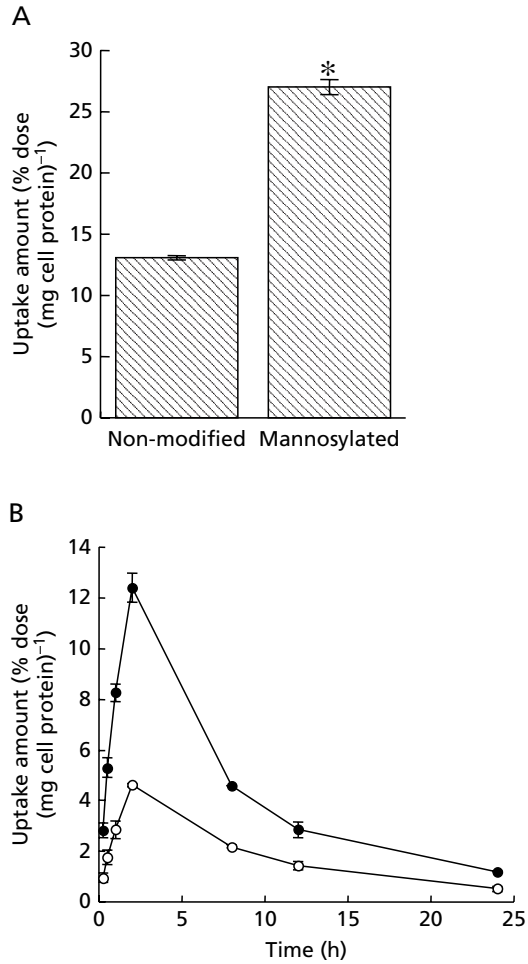

Figure 2 The uptake of mannosylated liposomes by NR8383 cells in-vitro (A) and alveolar macrophages in-vivo (B). A. Non-modified or mannosylated liposomes (particle size: 1000 nm) were added to NR8383 cells followed by incubation at 37°C for 2 h. Each value represents the mean ± s.e., n = 4. *$P < 0.05$, significant difference (Student's *t*-test) compared with non-modifed liposomes. B. Non-modified (○) or mannosylated (●) liposomes (particle size: 1000 nm) were administered to rat lungs. At each time point after administration, the alveolar macrophages were collected by the bronchoalveolar lavage method. Each value represents the mean ± s.e., n = 4. A significant difference ($P < 0.05$) was found by the Mann–Whitney *U*-test for each time point.

Table 1 Pharmacokinetic parameters of non-modified and mannosylated liposomes (particle size: 1000 nm) in alveolar macrophages after pulmonary administration to rats

Pharmacokinetic parameters	Non-modified liposomes	Mannosylated liposomes
k_{up} (h^{-1}) (mean ± s.d.)	1.022 ± 0.206	1.446 ± 0.473
k_{el} (h^{-1}) (mean ± s.d.)	0.098 ± 0.011	0.099 ± 0.017
AUC (% of dose h (mg cell protein)$^{-1}$) (mean)	47.2	104.7
MRT (h) (mean)	11.2	10.7

Non-modified and mannosylated liposomes were administered to rat lungs. At each time point after administration, the alveolar macrophages were collected and the radioactivity in the alveolar macrophages was determined. AUC, area under the uptake amount–time curve; MRT, mean residence time.

efficient. The uptake of mannosylated liposomes by NR8383 cells was significantly greater than that of non-modified liposomes (Figure 2A). The uptake of mannosylated liposomes by AMs was markedly greater than that of non-modified liposomes at each time point (Figure 2B). The pharmacokinetic parameters of non-modified and mannosylated liposomes (particle size: 1000 nm) in AMs following pulmonary administration are summarized in Table 1. The k_{up} and AUC values of mannosylated liposomes were 1.4- and 2.2-fold greater than those of non-modified liposomes, respectively (Table 1). The k_{el} and MRT values were similar in both types of liposomes (Table 1).

Discussion

The uptake characteristics of liposomes by AMs were evaluated in-vitro and in-vivo to determine the influence of particle size and surface mannose modification. The influence of particle size on the uptake of liposomes by rat AMs was examined in-vitro and in-vivo (Figure 1). The uptake of liposomes by phagocytes such as peritoneal macrophages (Hsu & Juliano 1982), Kupffer cells (Rahman et al 1982) and foam cells (Chono et al 2006) is particle size-dependent. The uptake of non-modified liposomes by NR8383 cells in-vitro was also particle size-dependent in the range 100–1000 nm (Figure 1A). These findings indicate that the uptake of liposomes by NR8383 cells is similar to the uptake by phagocytes as described above. The uptake of non-modified liposomes became constant at particle sizes over 1000 nm (Figure 1A). This result indicates that the uptake capacity of liposomes is saturated at a particle size of 1000 nm. The reason for the saturation at 1000 nm is not clear and so further investigation is required. We previously reported the uptake of non-modified liposomes by peritoneal macrophages in-vitro (Chono et al 2006). The uptake by peritoneal macrophages was greater than the uptake by NR8383 cells at each particle size. NR8383 cells are a cell line, whereas peritoneal macrophages are primary culture cells. Thus, the activity of peritoneal macrophages may be greater than NR8383 cells. It is thought that the difference in uptake of liposomes by peritoneal macrophages and NR8383 cells is based on cell activity. The uptake of non-modified liposomes by NR8383 cells in-vitro was size-dependent over the range of 100–1000 nm (Figure 1A), whereas the uptake by AMs after pulmonary administration was size-dependent over the range of 100–2000 nm (Figure 1B). The uptake of non-modified liposomes at a particle size of 2000 nm by AMs was significantly greater than that of 1000-nm particles ($P < 0.05$) (Figure 1B). The alveolar surfactant proteins opsonize bacteria and enhance phagocytosis by AMs (Benne et al 1997; Holmskov et al 1999; Schagat et al 2001). Also, the uptake of liposomes by phagocytes is induced by opsonization based on complement activation (Harashima et al 1996; Huong et al 1998, 1999). Thus, opsonization of liposomes by alveolar surfactant proteins may influence the uptake of liposomes by AMs. The curvature of non-modified liposomes at a particle size of 2000 nm is less than that of 1000-nm particles, and complement binds readily to large-sized particles having a low curvature (Harashima et al 1996). Consequently, opsonization of non-modified liposomes at a particle size of 2000 nm by alveolar surfactant proteins may be greater than in the case of 1000-nm particles. Therefore, the uptake of non-modified liposomes with a particle size of 2000 nm by AMs may be greater than that of 1000-nm particles (Figure 1B). The uptake amount of liposomes by NR8383 cells in-vitro was greater than the uptake of liposomes of the same size by AMs following pulmonary administration (Figure 1). Under in-vitro experimental conditions, excessive liposomes are present around the NR8383 cells, whereas under the in-vivo experimental conditions, there is no evidence that administered liposomes are always present around AMs. This may explain the difference in uptake amount of liposomes by rat AMs in-vitro and in-vivo. Although the uptake mechanism of non-modified liposomes by AMs was not investigated in the present study, according to a previous report (Harashima et al 1994), it is suggested that the uptake of liposomes is mediated by phagocytosis (200–2000 nm) and pinocytosis (100 nm).

The influence of surface mannose modification on uptake of liposomes by rat AMs was examined in-vitro and in-vivo (Figure 2). The uptake of liposomes by peritoneal macrophages (Barratt et al 1986) and Kupffer cells (Kawakami et al 2000) is enhanced by surface mannose modification. The uptake of mannosylated liposomes with a particle size of 1000 nm by NR8383 cells was significantly greater than that of non-modified liposomes of the same particle size (Figure 2A). This finding indicates that the uptake capacity of liposomes by NR8383 cells is enhanced by surface mannose modification, similar to peritoneal macrophages and Kupffer cells. The uptake of mannosylated liposomes by AMs after pulmonary administration was markedly greater than that of non-modified liposomes at each time point (Figure 2B), and the AUC of mannosylated liposomes was 2.2-fold greater than that of non-modified liposomes (Table 1). The concentration of mannosylated liposomes in the alveolar epithelial lining fluid was similar to that of non-modified liposomes (data not shown). In the case of both types of liposomes, the uptake amount of liposomes for a given dose was remarkably small and most of the

administered liposomes were distributed in the epithelial lining fluid. These results indicate that the uptake of liposomes by AMs following pulmonary administration does not influence the concentration in the epithelial lining fluid. The k_{up} of mannosylated liposomes was 1.4-fold greater than that of non-modified liposomes (Table 1). This indicates that the uptake rate of liposomes is enhanced by surface mannose modification. The k_{el} and MRT values were similar for both types of liposomes (Table 1). These pharmacokinetic parameters indicate that the elimination rate of liposomes from AMs is not influenced by surface mannose modification. Mannose receptors are expressed in AMs (Lane et al 1998; Kudo et al 2004) and, thus, it is thought that the uptake mechanism of mannosylated liposomes by AMs is mannose receptor-mediated endocytosis. Pulmonary surfactant protein in epithelial lining fluid up-regulates the activity of the mannose receptors of AMs (Beharka et al 2002). Thus, a surface mannose modification is thought to be a rational technique for increasing the uptake of liposomes by AMs. The influence of the density of surface mannose modification on the uptake of liposomes by AMs should be investigated in the future.

Conclusion

We examined the influence of particle size and surface mannose modification on the uptake of liposomes by AMs. We have shown that the uptake of liposomes by AMs is particle size-dependent and is enhanced by surface mannose modification. These findings suggest that liposomes may be useful as drug carriers targeting AMs. Further evaluation of the influence of lipid formulations, electron charge density, the nature of the ligands and the density of surface modification on the uptake of liposomes by AMs should provide useful information for the development of drug delivery systems to target AMs.

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