Enhanced Gene Transfection in Macrophages by Histidine-Conjugated Mannosylated Cationic Liposomes

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Targeted gene delivery to macrophages is important for the treatments of various immune diseases. Since macrophages express mannose receptors, development of efficient mannosylated non-viral carriers is an effective approach to macrophages-selective *in vivo* gene transfection. In this study, a pH-sensitive mannosylated cholesterol derivative, Man-His-C4-Chol, which possesses histidine (His) residues, containing lipoplexes (Man-His-lipoplexes) was characterized for transfection both *in vitro* and *in vivo*. In primary cultured macrophages, both Man-His-lipoplexes and mannosylated (Man)-lipoplexes showed significantly higher cellular uptake than bare-lipoplexes and there was no significant difference between Man-His-lipoplexes and Man-lipoplexes at 37 °C but the cellular uptake of these three lipoplexes was reduced at 4 °C. Similarly, the transfection efficacy of Man-His-lipoplexes showed significantly higher gene expression than bare-lipoplexes and Man-lipoplexes. After intraperitoneal administration to mice, Man-His-lipoplexes showed higher gene expression in peritoneally exuded cells (PECs) which contained macrophages than Man-lipoplexes and bare-lipoplexes at 3, 6, and 24 h. In addition, Man-His-lipoplexes showed higher gene expression than Gal-His-lipoplexes in PECs, suggesting that Man-His-lipoplexes are taken up by macrophages *via* mannose receptor-mediated endocytosis. These results suggest that Man-His-lipoplexes have superior transfection activity to Man-lipoplexes in macrophages.

Key words gene delivery; macrophage; mannosylated liposome; transfection

The use of non-viral carriers has attracted great interest for *in vivo* gene delivery since they are free from some of the risks inherent in these systems compared with viral vectors. Furthermore, non-viral carriers are easier to prepare and to modify by chemical approaches. To achieve cell-selective targeted *in vivo* gene transfection, a number of receptor-mediated non-viral carriers have been developed.[1)] Antigen presenting cells (APCs) such as macrophages and dendritic cells are important targets for DNA vaccine therapy.[2,3)] It is known that the mannose receptor is a 175 kDa protein expressed on the surface of APCs and it recognizes the terminal of mannose groups. To date, mannose modified non-viral vectors, including cationic liposomes,[4,5)] polyethyleneimine,[6,7)] dendrimers,[8)] and chitosan[9,10)] have been reported to achieve targeted gene delivery into APCs *via* mannose receptor-mediated endocytosis.

Recently, we reported that mannosylated cationic liposomes modified with cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)butyl)formamide (Man-C4-Chol) enhanced DNA vaccine potency through efficiently targeted gene delivery to APCs in mice.[11,12)] However, the degradation of plasmid DNA in the lysosomes after their incorporation into cells is a rate-limiting step for transfection in macrophages.[13,14)] Therefore, an escape path from the endosomes needs to be introduced into our mannosylated cationic liposomes for more efficient gene transfer in macrophages. Histidine is known to have pH-buffering capacity under the slightly acidic conditions such as are found in lysosomes and, accordingly, it could enhance the cytoplasmic distribution of plasmid DNA before degradation in lysosomes.[15)] More recently, we have demonstrated that histidine-conjugated galactosylated liposomes, which possess pH-buffering capacity, can provide efficient hepatocyte-selective gene transfer in human hepatoma HepG2 cells.[16)]

In this study, we extended our earlier investigations[5,16)] and a pH-sensitive mannosylated cholesterol derivative, Man-His-C4-Chol (Chart 1), which possesses histidine residues, containing lipoplexes (Man-His-lipoplexes) was characterized for transfection both *in vitro* and *in vivo*. Man-His-lipoplexes possess multi-functional properties for efficient transfection into macrophages, *i.e.*, i) cholesterol for their stable incorporation into cationic liposomes, ii) a mannose group for recognition by mannose receptors in macrophages, iii) an imino group for binding plasmid DNA without loss of the cationic nature of the cationic liposomes, iv) a histidine group to allow an endosomal escape by the pH-buffering capacity. Since the physicochemical properties of mannosylated lipoplexes are also important to achieve cell-selective gene transfection,[1)] the His group was directly introduced into our previously synthesized mannosylated cholesterol derivative, Man-C4-Chol; consequently, a pH-buffering capacity could be introduced in the formulation without any significant change into the optimal physicochemical properties.

First, we evaluated the cellular uptake and transfection characteristics of Man-His-lipoplexes using primary cultured mouse peritoneal macrophages. Then, the transfection activities of peritoneally exuded cells (PECs) following intraperitoneal administration were evaluated in mice. As a control

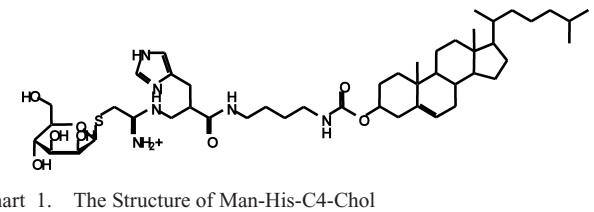

Chart 1. The Structure of Man-His-C4-Chol

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formulation, mannosylated (Man)-lipoplexes composed of cytomegalovirus (pCMV)-luc and Man-liposomes, composed of *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)/Chol/Man-C4-Chol (2 : 1 : 1), were selected to evaluate the function of the Man-His-lipoplexes because of their high transfection efficacy after intraperitoneal administration.[5)]

MATERIALS AND METHODS

Animals Male ICR (4-week-old) mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

Chemicals Cholesteryl chloroformate and 1,4-diazabicyclo-[2,2,2]octane (DABCO) were obtained form Sigma Chemicals Inc. (St. Louis, MO, U.S.A.). DOTMA and *N*-(4-aminobutyl) carbamic acid *tert*-butyl ester were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). *tert*-Butoxycarbonyl (Boc)-His(Boc)-OH·dicyclohexylammonium (DCHA) was obtained from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Picagene® was obtained from Toyo Ink Co., Ltd. (Tokyo, Japan). The Protein Quantification Kit was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All other chemicals were of the highest purity available.

Synthesis of Man-His-C4-Chol and Gal-His-C4-Chol Man-His-C4-Chol and Gal-His-C4-Chol were synthesized according to the method reported previously.[16)] Briefly, cholesteryl chloroformate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. After completion of the reaction, trifluoroacetic acid (TFA) was added and the mixture was stirred for 4 h at 4 °C. The solvent was evaporated to obtain *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide. This was coupled with Boc-His(Boc)-OH·DCHA in *N*,*N*-dimethylformamide (DMF) by the *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU)-*N*-hydroxybenzotriazole (HOBt) method, and then deprotection was performed by TFA treatment. The product was reacted with 2-imino-2-methoxyethyl-1-thiomannoside for Man-His-C4-Chol or 2-imino-2-methoxyethyl-1-thiogalactoside for Gal-His-C4-Chol in pyridine for 24 h at room temperature. After evaporation, the resultant material was suspended in water, dialyzed against distilled water for 48 h, and then lyophilized.

Preparation of Liposomes and Lipoplexes To prepare cationic liposomes, Man-liposomes, Man-His-liposomes, and Gal-His-liposomes, DOTMA, cholesterol, Man-C4-Chol, Man-His-C4-Chol, and Gal-His-C4-Chol were mixed in chloroform at a molar ratio of 2 : 2 : 0 : 0 : 0, 2 : 1 : 1 : 0 : 0, 2 : 1 : 0 : 1 : 0, and 2 : 1 : 0 : 0 : 1, respectively. The mixture was dried, vacuum desiccated, and resuspended in sterile 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer or 5% dextrose solution for experiments conducted *in vitro* and *in vivo*, respectively. The suspension was sonicated and then sterilized by passing it through a 0.45 μm filter. Lipoplexes were formed by mixing equal volumes of pDNA solution and liposome solution in Opti-MEM I® for *in vitro* experiments or 5% dextrose for *in vivo* experiments and then left at room temperature for 30 min. The particle size and the zeta potential of the lipoplexes in 5% dextrose were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., U.K.).

Harvesting and Culture of Mouse Peritoneal Macrophages Elicited macrophages were harvested from ICR mice 4 d after intraperitoneal administration of 1 ml 2.9% thioglycolate medium (Nissui, Tokyo, Japan). The cells were suspended in RPMI 1640 medium and then seeded on 12-well plates at a density of 5.0×10^5 cells/3.8 cm^2. After incubation for 6 h at 37 °C in 5% CO_2, non-adherent cells were washed off, and cells were cultivated for 96 h.

Cellular Association Study in Cultured Macrophages Macrophages were seeded on 12-well plates at a density of 5.0×10^5 cells/3.8 cm^2. After 4 d in culture, the culture medium was replaced with Hanks' balanced salt solutions (HBSS) containing [^{32}P]-labeled pDNA complexed with liposomes. After incubation, the cells were solubilized with 0.3 M NaOH solution with 10% Triton X-100. The radioactivity was measured in a liquid scintillation counter (LSC-500, Beckman, Tokyo, Japan). The radioactivity data were normalized with respect to the protein contents of the cells.

In Vitro* Transfection Study in Cultured Macrophages** Macrophages were seeded on 12-well plates at a density of 5.0×10^5 cells/3.8 cm^2. After 4 d in culture, the culture medium was replaced with Opti-MEM I® containing 2.0 μg/ml pDNA complexed with liposomes. Six hours later, the incubation medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and incubated for 18 h. Then, the cells were scraped off and suspended in 200 μl of lysis buffer (0.05% Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 M Tris, pH 7.8), and centrifuged at 8000 ***g for 8 min. Twenty microliters of supernatant was analyzed for luciferase activity with 100 μl of Picagene® using a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany). The protein content of the cell suspension was determined with a Protein Quantification Kit. Luciferase activity was indicated as the relative light units per milligram of protein.

In Vivo* Transfection Study in Mice** The lipoplexes were prepared at a charge ratio (−:+) of 1.0 : 2.3 and administered intraperitoneally to 4-week-old ICR mice at a dose of 50 μg of pDNA. At predetermined times, mice were sacrificed, and the peritoneal cavity was washed with 1 ml of saline to collect the peritoneal exuded cells (PECs). PECs were resuspended in lysis buffer (400 μl) after centrifugation (4000 ***g for 5 min at 4 °C). The homogenates were centrifuged at 13000 ***g*** for 8 min at 4 °C and then a 20 μl supernatant was used to determine the luciferase activity using a luminometer (Lumat LB9507; EG&G Berthold). The protein concentration of each tissue extract was determined using a Protein Quantification Kit. Luciferase activity was indicated as the relative light units per milligram of protein.

Statistical Analysis Statistical analysis was performed using the Tukey–Kramer test for multiple comparisons between groups. $p<0.05$ was considered statistically significant.

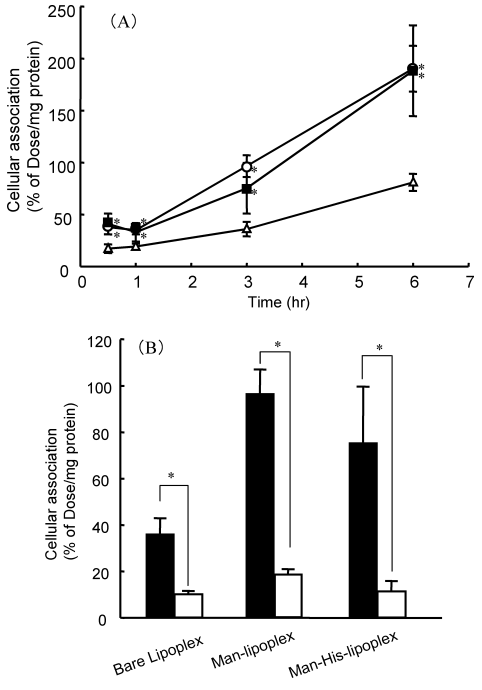

Fig. 1. Cellular Association of Lipoplexes in Cultured Mouse Peritoneal Macrophages

(A) Cellular association time–course of ^{32}P-labeled bare lipoplexes (△), Man-lipoplexes (○), and Man-His-lipoplexes (■) at 37 °C. ＊Statistically significant difference compared with the bare lipoplex-treatment group ($p<0.05$) (B) Cellular association of ^{32}P-labeled lipoplexes at 37 °C (solid bars), and at 4 °C (open bars) at 3 h. Each value represents the mean±S.D. ($n=3$). ＊Statistically significant difference compared with the 4 °C group ($p<0.05$).

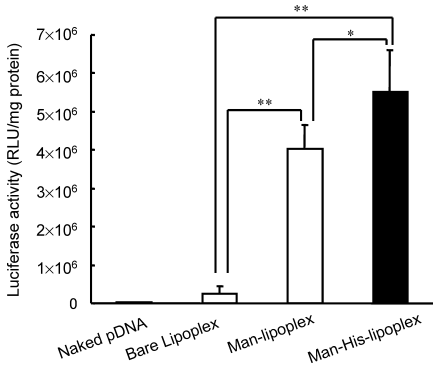

Fig. 2. Transfection Activity of Lipoplexes in Cultured Mouse Peritoneal Macrophages

Luciferase activity is indicated as the relative light units per milligram of protein. Each value represents the mean±S.D. ($n=5, 6$). ＊$p<0.05$, ＊＊$p<0.01$.

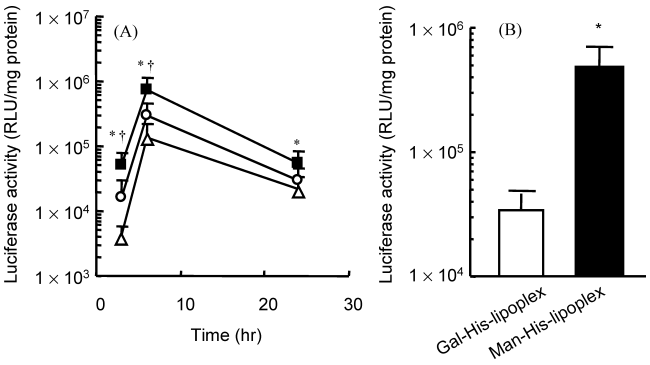

Fig. 3. Transfection Activity of Lipoplexes in PECs after Intraperitoneal Administration

(A) Transfection activity time–course of bare lipoplexes (△), Man-lipoplexes (○), and Man-His-lipoplexes (■) in PECs after intraperitoneal administration. Luciferase activity is indicated as the relative light units per milligram of protein. Each value represents the mean±S.D. ($n=3$). ＊Statistically significant difference compared with the bare lipoplex-treated group ($p<0.05$), †statistically significant difference compared with the Man-lipoplex-treated group ($p<0.05$). (B) Transfection activity of Man-His-lipoplexes and Gal-His-lipoplexes in peritoneal macrophages after intraperitoneal administration. Transfection activity was determined 6 h after intraperitoneal administration. Each value represents the mean±S.D. ($n=3$). ＊$p<0.05$.

RESULTS AND DISCUSSION

Since mannose receptors are expressed on the macrophages, development of an efficient mannosylated non-viral carrier is an effective approach for macrophages-selective *in vivo* gene transfection. In this study, enhanced gene transfection in macrophages by histidine-conjugated mannosylated cationic liposomes was evaluated both *in vitro* and *in vivo*.

To evaluate the suitability of Man-His-lipoplexes, both the uptake and transfection characteristics of Man-His-lipoplexes were investigated in primary cultured mouse peritoneal macrophages. In our previous study, we showed that this optimized Man-lipoplex formulation was efficiently taken up by mannose receptor-mediated endocytosis in primary cultured mouse peritoneal macrophages.[5] Both the Man-His-lipoplexes and Man-lipoplexes showed significantly higher cellular uptake than bare lipoplexes and there was no significant difference between Man-His-lipoplexes and Man-lipoplexes at 37 °C (Fig. 1A); in addition, the cellular uptake of these three lipoplexes was reduced at 4 °C (Fig. 1B). These results suggest that endocytosis are involved in the uptake process and introduction of histidine residues does not influence the cellular uptake by primary cultured macrophages. In contrast, the transfection activity of Man-His-lipoplexes was significantly higher than that of naked pDNA, bare lipoplexes, and Man-lipoplexes (Fig. 2). These uptake and transfection characteristics of Man-His-lipoplexes and Man-lipoplexes suggest the efficient endosomal escape of pDNA by Man-His-lipoplexes. This observation is corresponding with those obtained in the previous study about histidine-conjugated galactosylated liposomes in HepG2 cells.[16] Therefore, this histidine conjugation method may be efficient for enhanced gene transfection in cultured cells.

For cell-selective *in vivo* gene transfection, consideration of the relationship between the administration route and physicochemical properties such as particle sizes and zeta potentials of lipoplexes are important. Recently, we have reported that the intraperitoneal administration of Man-lipoplexes at a charge ratio (−：+) of 1.0：2.3 showed higher macrophages-selective *in vivo* gene transfection activity in PECs, spleen, and liver, which contain many macrophages.[5] Based on this finding, Man-His-lipoplexes for transfection were prepared in this study. The mean particle sizes of the bare-lipoplexes, Man-lipoplexes, and Man-His-lipoplexes were 118±14.3, 102±11.2, and 116±15.8 nm ($n=3$), respectively, while the zeta potentials of the bare-lipoplexes, Man-lipoplexes, and Man-His-lipoplexes were 66.4±1.05, 61.3±5.32, and 60.6±1.82 mV ($n=3$), respectively. Since these physicochemical properties were similar at the same charge ratio, the His group in Man-His-C4-Chol would not be affected by the physicochemical properties of Man-His-lipoplexes. This observation is corresponding with our previous report.[16] As shown in Fig. 3A, Man-His-lipoplexes

showed higher gene expression in PECs than Man-lipoplexes, at all time points, and also bare-lipoplexes. These *in vivo* transfection results support those obtained in the *in vitro* study (Fig. 2). As far as the possibility of mannose receptor-mediated uptake is concerned, the transfection activity between Gal-His-lipoplexes, prepared from Gal-His-C4-Chol which allows transfection into hepatocytes *via* asialoglycoprotein receptor-mediated endocytosis.[16] Although these glycosylated lipoplexes possess similar physicochemical properties, Gal-His-lipoplexes are not recognized by mannose receptors in macrophages. After intraperitoneal administration, Man-His-lipoplexes showed significantly higher gene expression than Gal-His-lipoplexes in PECs (Fig. 3B). This result may indicate that Man-His-lipoplexes are taken up *via* mannose receptor-mediated endocytosis by macrophages after intraperitoneal administration.

CONCLUSION

It is suggested that Man-His-lipoplexes have superior transfection activity to Man-lipoplexes in an optimized formulation previously reported[5,11,12] in macrophages both *in vitro* and *in vivo*. Their enhancing transfection effects may involve not only mannose receptor-mediated endocytosis but also endosomal escape of pDNA in the macrophages. Although further studies are needed to investigate this mechanism, the information obtained in the present study will be valuable for the development and rational design of a non-viral gene carrier for transfection into APCs.

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