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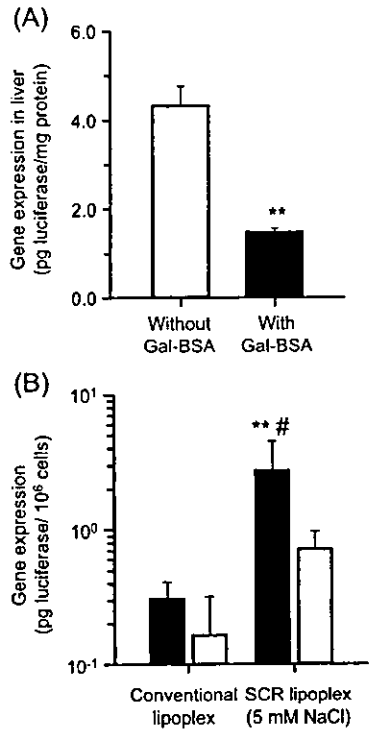

FIG. 4. Enhanced hepatocyte-selective gene transfer by galactosylated SCR lipoplexes after intraportal administration in mice. Each value represents the mean + SD of at least three experiments. (A) Inhibition of hepatic gene expression of galactosylated SCR lipoplexes (5 mM NaCl in lipoplex solution) by preadministration with or without galactosylated bovine serum albumin (Gal-BSA). Luciferase activity was determined 6 h post-injection of lipoplexes. Statistical comparisons were performed using an unpaired Student *t* test (**$P < 0.01$). (B) Intrahepatic gene expression of galactosylated SCR lipoplexes (5 mM NaCl in lipoplex solution). Luciferase activity was determined 6 h postinjection in the liver parenchymal cells (PC; filled bar) and nonparenchymal cells (NPC; open bar). Statistical comparisons were performed using Tukey's test (** indicates comparison with the PC group of galactosylated conventional lipoplexes, $P < 0.01$; # indicates comparison with the NPC group of galactosylated SCR lipoplexes, $P < 0.05$).

than that for the galactosylated conventional lipoplexes (PC/NPC ratio = 1.1) in our previous report [12]. These *in situ* experimental results partly support the belief that the *in vivo* hepatic transfection activity is enhanced by galactosylated SCR lipoplexes.

In Vitro Evaluation of Galactosylated SCR Lipoplexes

To assess whether galactosylated SCR lipoplexes exhibit superior transfection activity not only *in vivo* but also *in vitro*, we performed an *in vitro* experiment using the human hepatocarcinoma cell line HepG2, which expresses asialoglycoprotein receptors (Fig. 6). The transfection activity of the galactosylated SCR lipoplexes was significantly higher than that of the galactosylated conventional lipoplexes (Fig. 6A). In addition, these values were greatly higher than commercially available reagent Lipofectamine 2000, which enables one to transfect wide varieties of cells efficiently (0.32 ± 0.22 pg luciferase/mg protein). It is well known that pDNA released in the cytosol from the lipoplex is important for transgene expression [21–23]; therefore, we examined the intracellular localization of the galactosylated lipoplexes prepared from fluorescein-labeled pDNA and Rh-labeled liposomes by confocal laser scanning microscopy (Figs. 6B and 6C). The green, yellow, and red fluorescence indicated the emission from fluorescein–pDNA, lipoplexes, and Rh–liposomes, respectively. Rh-labeled liposomes diffused to the plasma membrane of HepG2 cells in both galactosylated SCR and conventional lipoplexes. The marked green fluorescence was more pronounced in the galactosylated SCR lipoplexes (Fig. 6C), suggesting that the galactosylated SCR lipoplexes release pDNA more easily in the cells.

DISCUSSION

Gene transfer to hepatocytes is of great therapeutic potential since hepatocytes are responsible for the synthesis of a wide variety of proteins that play important

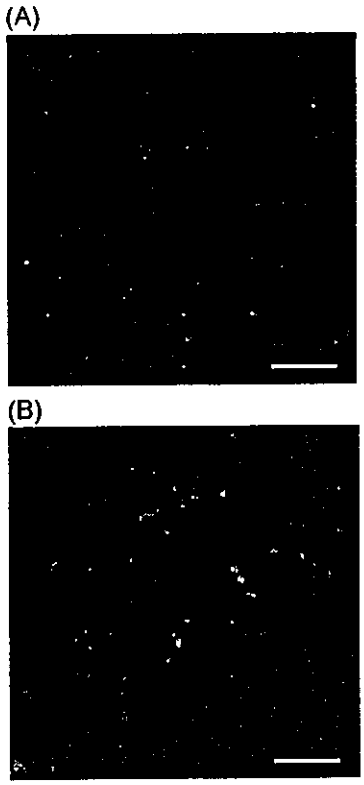

FIG. 5. Intrahepatic distribution of galactosylated (A) conventional and (B) SCR lipoplexes (5 mM NaCl in lipoplex solution) 2 h postinjection in mice. Thirty micrograms of pDNA containing 10% carboxyfluorescein-labeled pDNA complexed with galactosylated liposomes was injected intraportally. Frozen liver sections (5 μm thick) were examined by confocal laser scanning microscopy. Scale bars indicate 50 μm.

TABLE 1: Pharmacokinetic parameters of hepatic disposition of ^{32}P-labeled galactosylated SCR lipoplexes analyzed using a two-compartment dispersion model

	k_{12} (min^{-1})	k_{21} (min^{-1})	k_{12}/k_{21}	k_{int} (min^{-1})
Conventional lipoplex	30.0 ± 0.13	1.23 ± 0.23	24.9 ± 4.41	3.77 ± 0.46
SCR lipoplex (5 mM NaCl)	30.0 ± 0.08	0.22 ± 0.04[a]	136 ± 20.0[a]	3.81 ± 0.05

Results are expressed as the means ± SD of three experiments.
[a] Significant differences compared with conventional lipoplex group ($P < 0.01$).

physiological roles. There has been much interest in *in vivo* gene transfer to the liver as an alternative to *ex vivo* methods that require invasive surgery. So far, several methods involving the local administration of naked pDNA have been tested to achieve gene delivery targeted to the liver. Electroporation, the application of a controlled electric field to facilitate cell permeabilization, could enhance the transfection activity of pDNA administered via the intrahepatic, intraportal, and intravenous routes [24–26]. Similarly, instillation of pDNA onto the liver surface was recently demonstrated for liver-selective gene transfection [27]. Compared with these local applications to the liver, systemic application by vascular routes could transfect the gene to a large number of cells in the whole liver. Consequently, various nonviral vectors, including cationic liposomes and polymers, have been developed to enhance the *in vivo* gene expression. In most cases, however, the highest gene expression is observed in the lung after the intravenous administration of nonviral vectors because the lung capillaries are the first "traps" to be encountered [28,29]. In a previous study, we have demonstrated that galactosylation of the lipoplex (galactosylated conventional lipoplex) confers hepatocyte selectivity by asialoglycoprotein receptor-mediated endocytosis *in vivo* [9]. Transfection activity of galactosylated liposome complex was about 16, 22, and 50 times higher than those of naked plasmid DNA, DC–Chol liposome complex, and DOTMA–Chol liposome complex, respectively [9]. However, a number of possible barriers to a gene delivery system targeted to hepatocytes remain, e.g., (i) nonspecific interactions with erythrocytes, serum, and nontarget cells; (ii) aggregation of lipoplexes; and (iii) penetration of endothelial cells, etc., from the administration site to hepatocytes through gene expression [12,20,30–32]. Taking these into consideration, not only ligand modification (i.e., galactosylation) of the lipoplex but also controlling the stability of the ligand-modified lipoplex is important for achieving cell-selective gene transfection under *in vivo* conditions. In the present study, we demonstrated that the SCR lipoplex significantly delayed aggregation after exposure to saline compared with conventional lipoplex (Fig. 1B).

To date, lipoplexes for *in vivo* gene delivery are prepared in nonionic solution because the lipoplexes form aggregates in the presence of isotonic concentrations of ionic solutions such as saline. However, when systemically administered, the lipoplexes are exposed to the ionic solution and subsequently would aggregate as shown in the *in vitro* results (Fig. 1B). Thus, controlling the size of the lipoplex after exposure to the ionic solution is important for efficient gene delivery. We hypothesized that the presence of an essential amount of NaCl during lipoplex formation can regulate the repulsion between cationic liposomes and the fusion of the cationic liposomes in the lipoplex would be accelerated by partial neutralization of the positive charge on the surface of cationic liposomes. This hypothesis is confirmed, in part, by the results of the FRET analysis (Figs. 2A and 2B). Thus, moderate neutralization of the positive charge on the surface of cationic liposomes by a suitable concentration of NaCl can ensure sufficient repulsion of the lipoplex intermediates. However, excess neutralization of the charge by NaCl, e.g., saline (150 mM NaCl), might cause a lipoplex intermediate–intermediate interaction, followed by aggregation. The observation in the present study that the mean diameter of the lipoplex was increased by increasing NaCl concentration in lipoplex solution agrees with the recent findings by Wasan *et al.* [17]. Aggregated lipoplexes are not suitable for efficient *in vivo* hepatocyte-selective gene expression because the sinusoidal endothelium will block the penetration of such lipoplexes and, consequently, the lipoplexes would not be taken up by the hepatocytes. However, there is little concern with stabilization of lipoplex despite the fact that lipoplex aggregation must be controlled. In the present study, we demonstrated that the SCR lipoplex was shown to be relatively well protected from aggregation compared with conventional lipoplex after exposure to saline (Fig. 1B). This stabilizing effect after exposure of the SCR lipoplex to saline is also supported by the results of the FRET analysis (Fig. 2D). This effect may be partly explained by the fact that the fusion of each cationic liposome in the SCR lipoplex was enhanced (Fig. 2B); accordingly, cationic lipids may extensively cover pDNA in the SCR lipoplex. This stabilization effect on the galactosylated SCR lipoplex would be reflected in the extensive *in vivo* intrahepatic distribution after intraportal administration (Fig. 5).

To evaluate the precise hepatic uptake characteristics, a pharmacokinetic analysis for *in situ* experiments with galactosylated SCR lipoplexes was also performed. As shown in Table 1, we confirmed that the galactosylated SCR lipoplexes exhibited a higher tissue binding affinity compared with the galactosylated conventional lipo-

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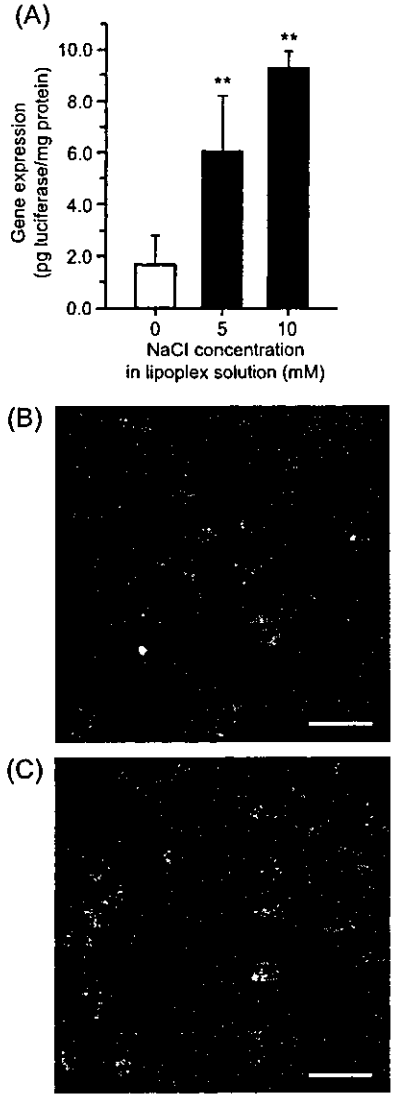

FIG. 6. (A) Transfection activity of galactosylated lipoplexes in HepG2 cells and (B, C) confocal microscopic images of galactosylated lipoplexes in HepG2 cells. (A) Luciferase activities were determined 24 h after transfection of galactosylated SCR lipoplexes. Each bar represents the mean value + SD of at least four experiments. Statistical comparisons with galactosylated conventional lipoplexes were performed using Dunnett's test at a significance level of 1% (**). (B, C) Intracellular localization of pDNA (green) and lipids (red) 4 h post-transfection of galactosylated (B) conventional and (C) SCR lipoplexes (5 mM NaCl in lipoplex solution). Scale bars indicate 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plexes, while the internalization rate of the galactosylated SCR lipoplexes was similar to that of the galactosylated conventional lipoplexes. However, the amount internalized is determined by the degree of tissue binding and the internalization rate, and the total amount of the galactosylated SCR lipoplexes internalized would be increased compared with that of the conventional lipoplexes. The high tissue binding affinity and degree of internalization of the galactosylated SCR lipoplexes found in the *in situ* experiments support the results of the higher gene expression in the liver (Fig. 3) and marked intensity of the fluorescein-labeled pDNA in the liver (Fig. 5).

It has been reported that the pDNA release from the complexes in the cell is important for gene expression [21–23]. As shown in Fig. 6A, the *in vitro* transfection efficacy of the galactosylated SCR lipoplexes was significantly higher than that of the galactosylated conventional lipoplexes. To analyze the mechanism of the transfection efficacy in the cell, the intracellular localization of pDNA and lipid components was examined by confocal microscopy. Enhanced pDNA release of galactosylated SCR lipoplexes in the cells compared with galactosylated conventional lipoplexes was observed (Figs. 6B and 6C). This result may be explained by the fact that the electrostatic interaction between pDNA and the cationic liposomes in the SCR lipoplexes was reduced (Fig. 2C); consequently the weaker interaction between pDNA and the cationic liposomes led to enhanced release of pDNA in the cells. The improvements in the intracellular distribution of galactosylated SCR lipoplexes also affected the enhanced *in vivo* gene expression in the liver.

In the present study, NaCl was selected for the ionic solution to prepare the SCR lipoplexes to control the positive charge of the cationic liposomes in the lipoplex. To evaluate the possibility of using other ionic solutions for SCR lipoplex formation, we also examined the role of disodium hydrogen phosphate and sodium dihydrogen phosphate solution and found that these salts also had a similar effect on the lipoplex formation and gene expression profiles. However, the enhanced transfection efficacy produced by NaCl was higher than that by disodium hydrogen phosphate and sodium dihydrogen phosphate (data not shown). The difference between NaCl and the other salts is still unclear; but these results also provide evidence that the theory of the SCR lipoplex is available for stabilization of the lipoplex as a novel approach.

In conclusion, we demonstrated the enhanced hepatic transfection activity by stabilized galactosylated lipoplexes using sodium chloride for complex formation. FRET analysis revealed that the NaCl solution in the lipoplex weakened the repulsion among cationic liposomes and enhanced the fusion of cationic liposomes in the lipoplex; consequently, the *in vivo* transfection in hepatocytes was greatly enhanced. Pharmacokinetic studies, both *in situ* and *in vivo*, demonstrated the higher tissue binding affinity and very marked intrahepatic distribution of the galactosylated SCR lipoplex. Moreover, enhanced transfection activity of the galactosylated SCR lipoplex was observed in HepG2 cells, and the confocal microscopic images showed that the release of pDNA in the cell was greatly accelerated. These characteristics partly explain the mechanism of enhanced in vivo

transfection efficacy by galactosylated SCR lipoplex. Hence, the information we obtained will be of value for the future use, design, and development of ligand-modified lipoplexes for *in vivo* applications.

MATERIALS AND METHODS

Materials. *N*-(4-Aminobutyl) carbamic acid *tert*-butyl ester and DOTMA were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Cholesterol was obtained from Nacalai Tesque, Inc. (Kyoto, Japan), and cholesteryl chloroformate was purchased from Sigma Chemicals, Inc. (St. Louis, MO, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-DOPE) were purchased from Avanti Polar Lipids, Inc. (AL, USA). PicoGreen dsDNA Quantitation Reagent was purchased from Molecular Probes, Inc. (Eugene, OR, USA). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham Co. (Tokyo, Japan). Gal-BSA as a ligand of asialoglycoprotein receptors was synthesized as described in our earlier study [33]. Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and Lipofectamine 2000 were purchased from Invitrogen Co. (Carlsbad, CA, USA). All other chemicals were of the highest purity available.

Construction and preparation of pDNA. pCMV-Luc was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from pGL3-control vector (Promega Co., Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen). pDNA was amplified in the *Escherichia coli* strain DH5α, isolated, and purified using a Qiagen Endofree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the pDNA concentration was measured by UV absorption at 260 nm. The pDNA for liver perfusion experiments was labeled with [α-^{32}P]dCTP by nick-translation [34]. Carboxyfluorescein labeling of pDNA for intrahepatic distribution and intracellular localization experiments was performed using the Label IT Fluorescein Nucleic Acid Labeling Kit (Mirus Co., Madison, WI, USA).

Synthesis of Gal-C4-Chol. Gal-C4-Chol was synthesized as reported previously [8]. Briefly, cholesteryl chloroformate and *N*-(4-aminobutyl)-carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. A solution of trifluoroacetic acid and chloroform was added dropwise and the mixture was stirred for 4 h at 4°C. The solvent was evaporated to obtain *N*-(4-aminobutyl) (cholesten-5-yloxyl)formamide, which was then combined with 2-imino-2-methoxyethyl-1-thiogalactoside [35] and the mixture was stirred for 24 h at 37°C. After evaporation, the resultant material was suspended in water, dialyzed against distilled water for 48 h (12-kDa cut-off dialysis tubing), and then lyophilized.

Preparation of galactosylated cationic liposomes. Mixtures of DOTMA, Chol, and Gal-C4-Chol were dissolved in chloroform at a molar ratio of 2:1:1 for galactosylated liposomes or 1:1:0 for nongalactosylated liposomes, vacuum-desiccated, and resuspended in sterile 5% dextrose at a concentration of 4 mg total lipids per milliliter. The suspension was sonicated for 3 min and the resulting liposomes were extruded 10 times through double-stacked 100-nm polycarbonate membrane filters.

Preparation of galactosylated conventional lipoplexes and SCR lipoplexes. For galactosylated conventional lipoplex preparation, 600 μl of 200 μg/ml pDNA in 5% dextrose was mixed with an equal volume of galactosylated cationic liposomes at 1160 μg/ml and incubated for 30 min. For galactosylated SCR lipoplex preparation, part of the 5% dextrose in pDNA solution was replaced with saline and adjusted to the final NaCl concentration as indicated before being mixed with liposome solution. The mixing ratio of liposomes and pDNA was expressed as a +/− charge ratio, which is the molar ratio of cationic lipids to pDNA phosphate residue [30]. The charge ratio of unity was 2.52 μg total lipid/μg pDNA for DOTMA/Chol/Gal-C4-Chol liposomes. According to our previous report, the charge ratio was adjusted to 2.3:1.0, which exhibits the highest and most selective gene expression in the liver [9]. The particle size of the lipoplex was measured using a dynamic light scattering spectrophotometer (LS-900; Otsuka Electronics, Osaka, Japan).

FRET analysis. FRET analysis was previously applied for assessment of the stability of nonviral gene vectors by Itaka et al. [36]. Liposomes were labeled with two types of fluorescent lipid, NBD-DOPE or Rh-DOPE, at 2% (mol/mol) total lipid. pDNA was labeled with PicoGreen prior to being mixed with liposomes. The fluorescence intensity spectra were measured using a spectrofluorophotometer (RF540; Shimadzu Co., Kyoto, Japan). The excitation wavelengths were 460, 550, and 480 nm for NBD-DOPE, Rh-DOPE, and PicoGreen, respectively. The FRET from NBD–liposomes to Rh–liposomes was measured as a liposome–liposome interaction. Fusion of liposomes in the lipoplex was measured by FRET reduction caused by membrane mixing between NBD-DOPE/Rh-DOPE double-labeled liposomes and unlabeled liposomes during complex formation. FRET from PicoGreen in pDNA to Rh–liposome was measured as the pDNA–liposome interaction. FRET from NBD–lipoplex to Rh–lipoplex was measured as the lipoplex aggregation after mixing with saline.

In vivo gene expression experiments. Female 5-week-old ICR mice (20–23 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University. Mice were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). An incision was made in the abdomen, and the portal vein was exposed. pDNA complexed with cationic liposomes was administered to the portal vein (30 μg pDNA/300 μl), and the abdomen was closed by wound clips. For the *in vivo* transfection experiments, the liver and lung were excised at 6 h after injection. Each sample was homogenized with lysis buffer (0.1 M Tris/HCl containing 0.05% Triton X-100 and 2 mM EDTA (pH 7.8)). After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000 g for 10 min at 4°C. Twenty microliters of each supernatant was mixed with 100 μl of luciferase assay solution (Picagene; Toyo Ink Mfg. Co., Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507; Berthold Technologies GmbH, Bad Wildbad, Germany). The gene expression is indicated as the luciferase amount per milligram of protein converted from relative number of light units using purified enzyme. The protein content of the samples was determined using a protein quantification kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA). For evaluation of the intrahepatic localization of gene expression, the luciferase activities in the liver PC and NPC were independently determined after centrifugal separation of PC and NPC in collagenase-digested liver as previously described [9].

For the intrahepatic distribution study, at 2 h after injection of lipoplexes containing 10% fluorescein-labeled pDNA, the mice were killed and the livers were fixed by infusing cold 4% paraformaldehyde dissolved in PBS (pH. 7.4), followed by immersion in the fixative on ice for 2 h. The livers were subsequently embedded in Tissue-Tek OCT compound (Miles, Inc., Elkhat, IN, USA) and frozen in cold isopentane. Frozen sections, 5 μm thick, were prepared on a Cryostat (Leica Microsystems AG, Wetzlar, Germany). The liver sections were mounted on glass slides, covered by slips with 50% glycerol, and observed by confocal laser scanning microscope (MRC 1024; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Liver perfusion experiments and pharmacokinetic analysis. Male Wistar rats (170–210 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals. *In situ* liver perfusion studies were carried out as reported previously [12,20]. Briefly, the portal vein was

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catheterized with a polyether nylon catheter (SURFLO iv catheter, 16 G'2"; Terumo Co., Tokyo, Japan) and immediately perfused with Krebs–Ringer bicarbonate buffer supplemented with 10 mM glucose (oxygenated with 95% O_2–5% CO_2, adjusted to pH 7.4 at 37°C). The perfusate was circulated using a peristaltic pump (SJ-1211; ATTO Co., Tokyo, Japan) at a flow rate of 13 ml/min. After a stabilization period for 25 min, liposome/[^{32}P]pDNA complexes (30 μg pDNA/300 μl) were administered via the portal vein using a six-position rotary valve injector (Type 50 Teflon rotary valves; Rheodyne, Inc., Cotati, CA, USA). After addition of 5 ml Clear-Sol I, the radioactivity of the effluent perfusate was measured in a scintillation counter (LSA-500; Beckman Coulter, Inc., Fullerton, CA, USA). The outflow patterns were analyzed based on a two-compartment dispersion model involving sinusoidal and binding compartments [12,20]. The mass balance equations involving the axial dispersion in the sinusoidal space are given as

$$\frac{\partial C_S(t,z)}{\partial t} + v\frac{\partial C_S(t,z)}{\partial z} = D\frac{\partial^2 C_S(t,z)}{\partial z^2} - k_{12}C_S(t,z) + k_{21}C_B(t,z), \quad (1)$$

$$\frac{\partial C_B(t,z)}{\partial t} = \frac{1}{\varepsilon}k_{12}C_S(t,z) - k_{21}C_B(t,z) - k_{int}C_B(t,z), \quad (2)$$

where $C_S(t, z)$ and $C_B(t, z)$ are the concentrations of drug in the sinusoidal space and binding compartment, respectively; D is the dispersion coefficient; ε is the volume ratio of the binding compartment to the sinusoidal space in the liver; k_{12} and k_{21} are the forward and backward partition rate constants between the sinusoidal space and the binding compartment; k_{int} is the first-order internalization rate constant from the binding compartment to the intracellular space; v is the linear flow velocity of the perfusate; t is time; and z is the axial coordinate in the liver. The initial and boundary conditions are given as

$$C_S(t,0) = M/Q \cdot f_I(t), C_S(0,z) = 0, C_S(t,\infty) = 0, C_B(t,0) = 0, C_B(0,z) = 0,$$

where M is the amount of drug injected into the liver, Q is the flow rate of the perfusate, and $f_I(t)$ has the dimension of the reciprocal of time. Taking the Laplace transform with respect to t, rearranging, substituting the length of the sinusoidal space L with z and introducing the cross-sectional area of the sinusoidal space A, we obtain the image equation

$$\bar{C}_S(s) = \frac{M}{Q}\bar{f}_I(s)\cdot\exp\left[\left\{\frac{Q}{2D_C} - \sqrt{\left(\frac{Q}{2D_C}\right)^2 + \frac{1}{D_C}\left\{s + k_{12} - \frac{k_{12}\cdot k_{21}}{s + k_{21} + k_{int}}\right\}}\right\}V_S\right], \quad (3)$$

where $C_S(s)$ and $f_I(s)$ denote the Laplace transform of the concentration in the venous outflow and input function $f_I(t)$, respectively. D_C is the corrected dispersion coefficient ($D_C = D \cdot A^2$), V_S is the sinusoidal volume ($= L \cdot A$), and the flow rate Q is equal to $A \cdot v$.

Each parameter (D_C, k_{12}, k_{21}, k_{int}, and V_S) was calculated by curve fitting of the Laplace-transformed equation to the experimental venous outflow pattern using a nonlinear least-squares program with a fast inverse Laplace transform algorithm, MULTI (FILT) [37]. The damping Gauss Newton method with no constraint was used for curve fitting with the MULTI algorithm. Here, $f_I(t)$ was assumed to be a δ function, since the lipoplexes were rapidly injected using a six-rotary valve injector.

For evaluation of the intrahepatic localization of uptake amounts, 30 min after injection of ^{32}P-labeled lipoplexes into the isolated perfused liver, radioactivities in the liver PC and NPC were separately determined after centrifugal separation of PC and NPC in collagenase-digested liver as previously described [9].

In vitro gene expression and intracellular localization experiments. HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS at 37°C under an atmosphere of 5% CO_2 in air. The cells were plated on a 12-well cluster dish at a density of 8.0×10^4 cells/3.8 cm^2 and cultivated in 1 ml DMEM supplemented with 10% FBS. All lipoplexes were prepared under the same conditions for the *in vivo* experiment to assess the mechanism of enhancing *in vivo* transfection activity of galactosylated SCR lipoplexes. For transfection experiments, the medium was replaced with 1 ml DMEM supplemented with 10% FBS containing 0.5 μg/ml pDNA complexed with liposomes after 24 h in culture. Twenty-four hours later, the cells were scraped and suspended in 200 μl PBS. Cell suspensions were subjected to luciferase assay. For intracellular localization experiments, cells were cultured on sterilized coverslips for confocal microscopy. After 24 h in culture, the medium was replaced with 1 ml DMEM supplemented with 10% FBS containing 0.5 μg/ml fluorescein-labeled pDNA complexed with Rh–liposomes. Four hours later, the cells on the coverslips were washed twice with PBS and fixed with 4% paraformaldehyde on ice for 2 h. The coverslips were mounted on glass slides with 50% glycerol and subsequently examined by confocal laser microscopy.

Statistical analysis. Statistical comparisons were performed using unpaired Student's t test for two groups (Figs. 1B, 3C, and 4A and Table 1), Tukey–Kramer's test for multiple groups (Figs. 3B and 4b) or Dunnett's test for comparison with a control group (other figures).

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GENE DELIVERY

Tissue and intrahepatic distribution and subcellular localization of a mannosylated lipoplex after intravenous administration in mice

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Abstract

We have previously reported that, unlike a lipoplex and mannosylated (Man) lipoplex underwent gene transfer to liver nonparenchymal cells (NPC) that possess mannose receptors after intravenous administration in mice. In this study, the tissue, intrahepatic distribution, and subcellular localization of the lipoplex after intravenous administration were investigated. DC-Chol liposome was selected as a cationic liposomes. After administration of lipoplex and Man lipoplex, the high gene expression was observed in the lung and liver, respectively. After administration of [^{32}P]Man lipoplex, about 80% of [^{32}P]plasmid DNA (pDNA) was accumulated in the liver. As for the intrahepatic distribution, the NPC/parenchymal cells (PC) ratio of [^{32}P]Man lipoplex was 9.64, whereas the NPC/PC ratio of [^{32}P]lipoplex was 1.93. The radioactivity in the cytosolic fraction of liver homogenate of [^{111}In]Man lipoplex was two-fold higher than that of [^{111}In]lipoplex, indicating that Man liposomes facilitate the release of pDNA into the cytosolic space. However, a rapid sorting of the radioactivity from endosomes to lysosomes was observed with the [^{111}In]Man lipoplex. Also, amplification of pDNA by PCR suggested that the Man lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex. These results suggested that modulation of its intracellular sorting could improve the transfection efficiency of Man lipoplex.

Keywords: Gene delivery; Mannose receptor; Mannosylated liposomes; Cationic liposomes; Plasmid DNA

1. Introduction

The success of in vivo gene therapy relies on the development of a vector that achieves target cell-specific, efficient, and prolonged transgene expression following its application. Nonviral vectors are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, attractive vectors for clinical application. One of the most promising class of nonviral vectors developed so far is the cationic liposome-based transfection system. The lipoplex formation via electrostatic interaction of cationic liposomes and plasmid DNA (pDNA) facilitates the interaction of pDNA with cell membranes, leading to transgene expression in the cells [1]. In an attempt to increase the efficiency of transgene expression as well as to reduce cytotox-

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icity, several kinds of cationic lipids, such as quaternary ammonium detergents, cationic derivatives of cholesterol [2], diacylglycerol [1,3], and alkyl derivative of polyamines [4] have been developed. Among them, some lipoplex have been used in clinical trials for the treatment of cancer and cystic fibrosis [5,6].

The lipoplex is a useful nonviral vector, but it lacks specificity in delivery and transfection after systemic administration. Although the levels of gene expression vary from study to study, the lung invariably shows the highest gene expression. The attachment of a ligand that can be recognized by a specific mechanism would endow a vector with the ability to target a specific population of cells. In the search for macromolecule-based nonviral vectors, several ligands including galactose [7,8], mannose [9,10], transferrin [11], and antibodies [12] have been used to improve the delivery of pDNA to target cells. Therefore, the incorporation of such ligands into cationic liposomes would improve the cell specificity of in vivo gene transfer by lipoplex.

Mannose receptor-mediated targeting is a promising approach to achieve cell-specific delivery after systemic administration because (i) the expression of mannose receptors is restricted to the liver NPC and other macrophages, (ii) a complex entering the systemic circulation has easy access to the liver NPC, and (iii) the liver has a high blood flow. These physical and biological features give a mannosylated vector an opportunity to deliver pDNA to the liver NPC via mannose receptor-mediated endocytosis. Liver nonparenchymal cells (NPC), including sinusoidal endothelial cells and Kupffer cells, can be the targets for gene therapy because they have been implicated in a wide variety of diseases [13,14].

In a previous study, we developed a novel mannosylated derivative of cholesterol, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino) butyl)formamide (Man-C4-Chol), and used it to prepare a cationic liposome formulation (Man liposome) [10]. Man-C4-Chol possesses multi-functional properties, that is, (i) a lipophilic anchor moiety (cholesterol) for stable incorporation into liposomes, (ii) a mannose moiety for recognition by the mannose receptors, and (iii) an imino group for binding to pDNA via electrostatic interaction [15]. Furthermore, low-molecular-weight glycolipids are more promising due to their low immunogenicity, high reproducibility, and ease of mass production. Although, a high gene expression in the liver and spleen after intravenous injection was observed for Man lipoplex via mannose receptor-mediated endocytosis compared with the lung, its transfection efficiency was relatively low and, consequently, further improvements in the efficiency of transgene expression are required.

In order to obtain a theoretical strategy to develop an efficiently targetable gene carrier to the liver by mannosylation, therefore, detailed information on the distribution of a Man lipoplex needs to be obtained. In the present study, we studied the tissue, intrahepatic distribution, and subcellular localization of a [^{32}P]- or [^{111}In]-labeled Man lipoplex after intravenous administration. The results were compared with those for a 3β[*N*′,*N*′,*N*′-dimethylaminoethane]-carbamoyl]cholesterol liposomes (DC liposome), which is a cationic cholesterol derivative, based lipoplex [2].

2. Materials and methods

2.1. Chemicals

N-(4-Aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate was obtained from Sigma (St. Louis, MO, USA), dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar-Lipids (Alabaster, AL, USA). [α-^{32}P]dCTP was obtained from Amersham (Tokyo, Japan). ^{111}Indium chloride ([^{111}In]$InCl_3$) was supplied by Nihon Medi-Physics (Hyogo, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride and 4-[*p*-azidosalicylamido]butylamine (ASBA) were purchased from Dojindo (Kumamoto, Japan) and Pierce Biotechnology (Rockford, IL, USA). Man-C4-Chol [10] and DC-Chol [2] were synthesized as reported previously. Mannosylated bovine serum albumin (Man-BSA) was synthesized and radiolabeled with [^{111}In]$InCl_3$ as reported previously [16]. All other chemicals were obtained commercially as reagent-grade products.

2.2. Construction and preparation of pDNA

pCMV-Luc was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from

pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the E coli strain DH5α, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN, Hilden, Germany). Purity was confirmed by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

2.3. Animals

Female ICR mice (5-week-old, 20–25 g) were obtained from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and with the Guidelines for Animal Experiments of Kyoto University.

2.4. Preparation of liposomes

Man-C4-Chol (8.1 μmol) or DC-Chol (10.0 μmol) was mixed with DOPE (5.4 μmol for Man-C4-Chol and 6.7 μmol for DC-Chol) in chloroform (5.0 ml) and evaporated to dryness in a round-bottomed flask. Then, the lipid film was vacuum desiccated to remove any residual organic solvent and resuspended in 5% (w/v) dextrose (2.5 ml). After hydration, the dispersion was sonicated for 5–10 min in a bath sonicator to produce liposomes. The liposome formulations were passed through a polycarbonate membrane filter (0.22 μm) for sterilization. The liposomal lipid concentration was determined by phosphorous analysis [17] and was adjusted to 3 mg/ml.

2.5. Lipoplex formation

The mixing ratio of liposomes with pDNA was expressed as a charge ratio, which is the molar ratio of the cationic lipids to the pDNA phosphate residues [18]. The charge ratio (+: −) of these liposomes and pDNA complex was adjusted to 2.3:1.0 for cell-selective gene transfection [19,20]. A solution of cationic liposomes (0.2 ml) was added to an equal volume of pDNA solution (0.2 ml and pDNA concentration ≅0.33 μg/μl) in a polyethylene tube. Then, the lipoplex was agitated rapidly by pipetting it up and down twice using a micropipette (PIPETMAN®, GILSON, Villier-le Bel, France) and left to stand for 30 min. The particle size and zeta potential of the lipoplex were measured using a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan) and a laser electrophoresis zeta-potential analyzer (LEZA-500T, Otsuka Electronics), respectively.

2.6. pDNA radiolabeling methods

pDNA was radiolabeled with [α-^{32}P]dCTP by nick translation [21]. In a separate preparation, pDNA was radiolabeled with ^{111}In using a newly developed method [22]. Briefly, a dimethylsulfoxide solution of ASBA was added to DTPA anhydride under darkroom conditions, and the mixture was incubated at room temperature for 1 h. Then, pDNA solution was added to the mixture, and the mixture was immediately irradiated under an UV lamp at room temperature for 15 min to obtain DTPA-ASBA coupled pDNA (DTPA-ASBA-pDNA). The product was purified by precipitation twice with ethanol, and labeled with [^{111}In]$InCl_3$. The purity of each pDNA was checked by Sephadex G-25 column chromatography and 1% (w/v) agarose gel electrophoresis.

2.7. Gene expression experiments

Five-week-old ICR mice were injected intravenously with 300 μl of lipoplex using a 30-gauge syringe needle. Three or six hours after injection, mice were killed and lung, liver, kidney, spleen, and heart were removed and assayed for gene expression. The organs were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). The lysis buffer was added in a weight ratio of 5 μl/mg for liver samples or 4 μl/mg for other organ samples. After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C and 20 μl supernatant was analyzed to determine the luciferase activity using a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany). The protein

concentration of each tissue extract was determined by the modified Lowry method. Luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

2.8. Tissue distribution of the [^{32}P]lipoplex

The [^{32}P]lipoplex was intravenously injected into mice. At predetermined time periods after injection, the blood was collected from the vena cava under ether anesthesia. The mice were then killed and the liver, spleen, kidney, lung, and heart were excised, rinsed with saline, and weighed. Tissue samples were digested in 0.7 ml Soluene-350 (Packard, Merdin, CT, USA) by overnight incubation at 45 °C. Then, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5 M HCl, and, finally, 5 ml Clear-Sol I (Nacalai Tesque, Kyoto, Japan) were added to the digested samples. The radioactivity of the samples was assayed in a Beckman Model LS5000TA liquid scintillation counter (Beckman, Tokyo, Japan).

2.9. Intrahepatic distribution of the [^{32}P]lipoplex

Ten minutes after intravenous injection of the [^{32}P]lipoplex, the liver of each mouse was perfused with a buffer [16] and the liver cells were separated into parenchymal cells (PC) and nonparenchymal cells (NPC) by differential centrifugation. Then, the ^{32}P radioactivity of the cell suspensions was assayed as above.

2.10. Fractionation of liver homogenate

After intravenous injection of [^{111}In]Man-BSA, naked [^{111}In]pDNA or [^{111}In]both lipoplex into mice, the liver was sampled at intervals. HEPES–sucrose buffer (250 mM sucrose, 20 mM HEPES, 2 mM EDTA, pH 7.2) was added to the liver in a ratio of 3 μl buffer/mg tissue, then the liver was homogenized in a Potter-type homogenizer. The homogenate was centrifuged at 4 °C for 10 min at 800 × *g* to remove nuclei and debris. The supernatant was subsequently centrifuged at 4 °C for 30 min at 100,000 × *g* and the resulting supernatant was collected as the cytosol fraction. The pellet obtained was resuspended with 0.5 ml HEPES–sucrose buffer using a syringe and needle. Part of the suspension was mixed with HEPES–sucrose buffer and Percoll (Amersham Biosciences, Piscataway, NJ, USA) in a centrifuge tube. The final concentration of Percoll was adjusted to 35% (v/v). The sucrose solution (65% (w/v), 0.5 ml) was layered with a syringe and needle at the bottom of the centrifuge tube. The gradient was centrifuged at 4 °C for 25 min at 50,000 × *g*. Fractions (approximately 0.4 ml each) were collected from the bottom of the tube using a peristaltic pump and the radioactivity or enzyme activity of each fraction was measured.

2.11. Organelle marker detection

The activity of a fluid phase endocytosis marker, horseradish peroxidase (HRP), was detected using its substrate *o*-phenylenediamine dihydrochloride to identify the endosome fractions. Five minutes after injection of HRP, the liver was taken and fractionated by Percoll density gradient centrifugation as described above. Twenty microliters of each fraction was added to 150 μl 0.04% (w/v) *o*-phenylenediamine dihydrochloride, 0.012% (w/v) hydrogen peroxide in 0.1 M citrate–phosphate buffer (pH 5.0). The reaction was stopped by the addition of 20% (w/v) H_2SO_4 (50 μl). The activity of a lysosomal marker enzyme, β-hexosaminidase, was determined with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. Twenty microliters of each fraction was added to 50 μl 1.2 mM 4-methylumbelliferyl-β-D-galactoside solution and subsequently incubated at 37 °C for 30 min. The reaction was stopped by the addition of 150 μl 0.5 M glycine in 0.5 M carbonate buffer (pH 10.0). The fluorescence intensity of each sample was measured (excitation wavelength 365 nm; emission 460 nm).

2.12. PCR amplification of pDNA

pDNA in the subcellular fractions of the liver was purified by GenElute Plasmid Miniprep Kit (Sigma). Then, aregion of about 2.8 kbp of the pDNA containing the luciferase gene was amplified by PCR using a forward (5′-GTATCTGCTCCCTGCTTGTG-3′) and reverse (5′-TCCGCCTCAGAAGCCATAGA-3′) primer under standard conditions for 20 cycles.

3. Results

3.1. Physicochemical properties and gene expression of the lipoplex

The zeta potential of lipoplex and Man lipoplex was 9.78 ± 3.5 ($n=3$) and 12.5 ± 4.11 ($n=3$), respectively. The mean particle size of lipoplex and Man lipoplex was 287.2 ± 1.2 nm ($n=3$) and 285.4 ± 18.3 nm ($n=3$), respectively. Thus, physicochemical properties of both lipoplex were almost the same.

Fig. 1A demonstrates the gene expression after intravenous administration of Man lipoplex at 3 and 6 h. High gene expression was observed in the liver and spleen, which is expressed mannose receptor on cell surface. Fig. 1B shows the gene expression after intravenous administration of lipoplex at 6 h. Among these organs, the highest gene expression was observed in the lung.

3.2. Tissue distribution of the [^{32}P]lipoplex

Fig. 2 shows that the tissue radioactivity after intravenous administration of [^{32}P]lipoplex and Man lipoplex up to 6 h. Both [^{32}P]lipoplex rapidly disappeared from the blood circulation. [^{32}P]Lipoplex accumulated in the lung and liver, whereas [^{32}P]lipoplex accumulated largely in the liver.

3.3. Intrahepatic distribution of the [^{32}P]lipoplex between liver PC and NPC

After intravenous administration of the [^{32}P]Man lipoplex, the radioactivity in the liver was preferentially recovered from the NPC fractions, with the radioactivity ratio of NPC to PC (NPC/PC ratio on a cell-number basis) in the liver being approximately 9.6 (Fig. 3). On the other hand, the [^{32}P]lipoplex had an NPC/PC ratio of 1.9.

3.4. Subcellular localization of the [^{111}In]lipoplex

Fig. 4 shows the radioactivity of the cytosolic fractions in liver homogenate after intravenous administration of the [^{111}In]lipoplex and Man lipoplex up to 60 min. In both cases, the radioactivity gradually increased with time. Thirty and sixty minutes after intravenous administration, however, the cytosolic amount of the [^{111}In]Man lipoplex was always greater than that of the [^{111}In]lipoplex, suggesting that Man liposomes facilitate the release of pDNA from intracellular vehicles into the cytosol.

To characterize the gradient of liver homogenate using Percoll-gradient centrifugation, the enzymatic activity of β-hexosaminidase, a lysosome marker, was used for the determination of the endosomal fractions. As shown in Fig. 5A, β-hexosaminidase activity was predominantly detected in the lower

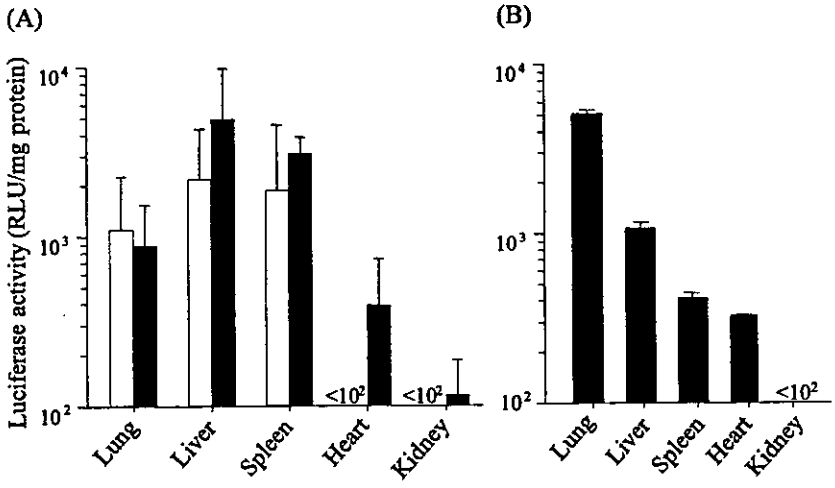

Fig. 1. Gene expression after intravenous administration at 3 (□) and 6 h (■) of Man lipoplex (A) and at 6 h of lipoplex (B) in mice. Each value represents the mean + S.D. values of three experiments.

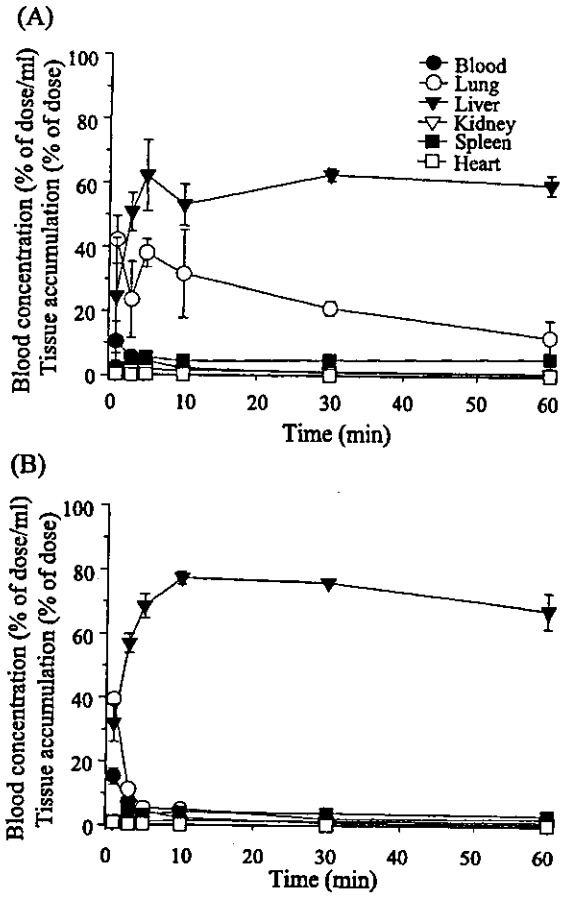

Fig. 2. Radioactivity in blood and tissues after intravenous administration of [^{32}P]lipoplex (A) or Man lipoplex (B) in mice. Each value represents the mean ± S.D. values of three experiments.

seven fractions. In a similar manner, the activity of horseradish peroxidase (HRP) injected intravenously into mice was used to confirm the lysosomal fractions. HRP was predominantly recovered in the upper seven fractions (data not shown). Therefore, the upper seven fractions and the lower seven fractions were identified as the endosomal and lysosomal fractions, respectively. To validate this system for the subcellular sorting of externally internalized compounds, the localization of [^{111}In]Man-BSA, a well-known ligand for mannose receptors [23], was then examined. At 15 min after intravenous administration, the radioactivity was largely recovered in the endosomal fractions, then the majority was transferred to the lysosomal fractions with time (Fig. 5B). These

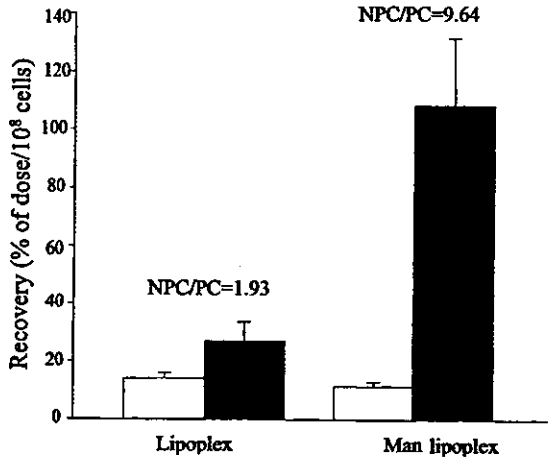

Fig. 3. Cellular localization of radioactivity between PC (□) and NPC (■) 30 min after intravenous administration of [^{32}P]lipoplex and Man lipoplex in mice. Each value represents the mean + S.D. values of three experiments.

results for [^{111}In]Man-BSA were in good agreement with previously reported characteristics of these ligands as far as mannose receptors [24] were concerned, indicating the validity of the detection method.

Fig. 6 shows the endosomal and lysosomal localization of radioactivity after intravenous admin-

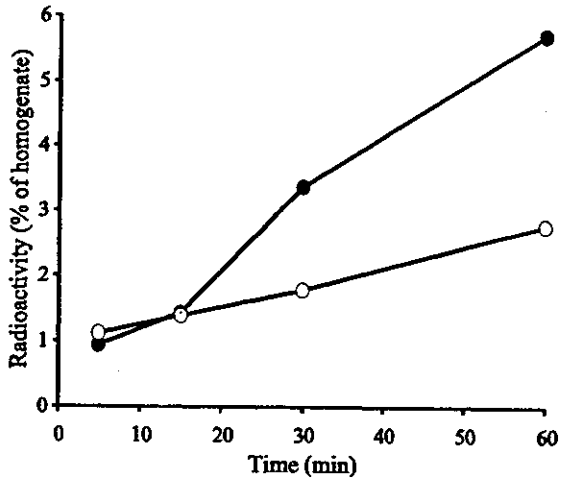

Fig. 4. Radioactivity in cytoplasmic fraction of mouse liver homogenates 5, 15, 30, and 60 min after intravenous administration of [^{111}In]lipoplex (○) or Man lipoplex (●) in mice. Similar results were obtained in two other independent runs.

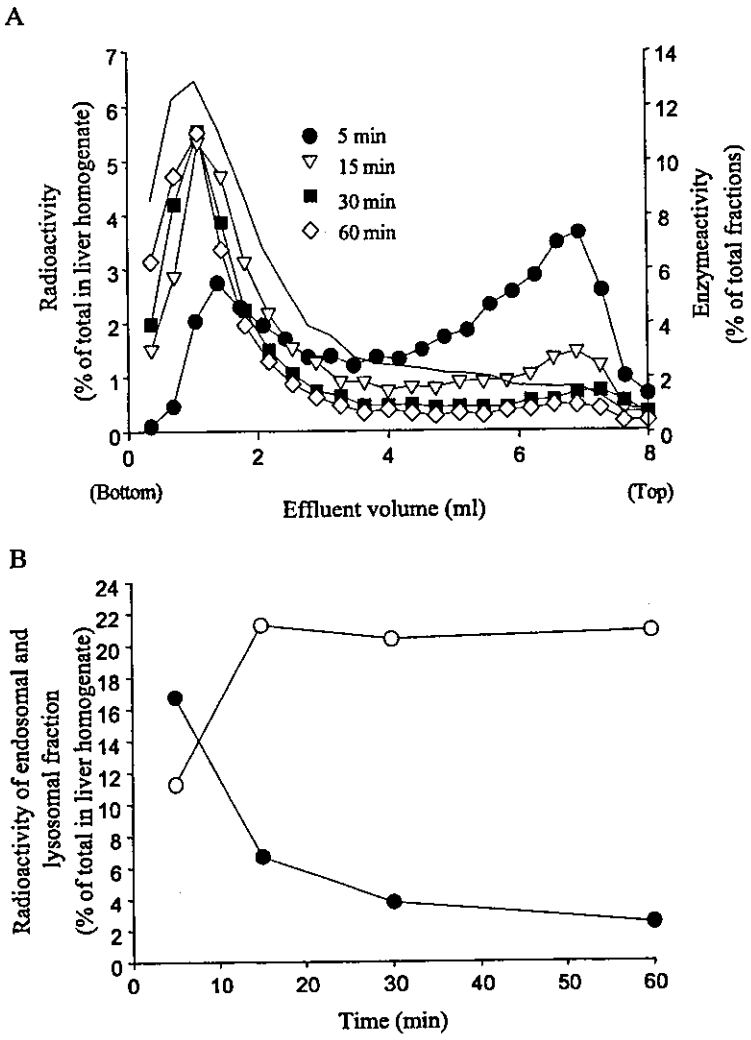

Fig. 5. Each fraction (A) and calculated endosomal (●) or lysosomal (○) localization (B) of radioactivity 5, 15, 30, and 60 min after intravenous administration of [^{111}In]mannosylated bovine serum albumin in mice. Liver homogenate was separated by Percoll density gradient centrifugation. Solid line in A represents the localization of β-hexosamidase activity. Similar results were obtained in two other independent runs.

istration of [^{111}In]lipoplex (B) or Man lipoplex (D). Sixty minutes after intravenous administration, the [^{111}In]Man lipoplex showed a larger distribution to the lysosome fractions than the [^{111}In]lipoplex. For comparison, naked [^{111}In]pDNA, which is extensively accumulated in the liver after intravenous administration but exhibits little gene expression [25], was also subjected to this assay (Fig. 6F). Naked [^{111}In]pDNA showed faster transfer to the lysosome fractions than both the [^{111}In]lipoplex and Man lipoplex.

3.5. PCR amplification

To determine whether pDNA within the subcellular fractions retained its structure, the luciferase sequence of pDNA was amplified by PCR (Fig. 7). When the Man lipoplex was intravenously adminis-

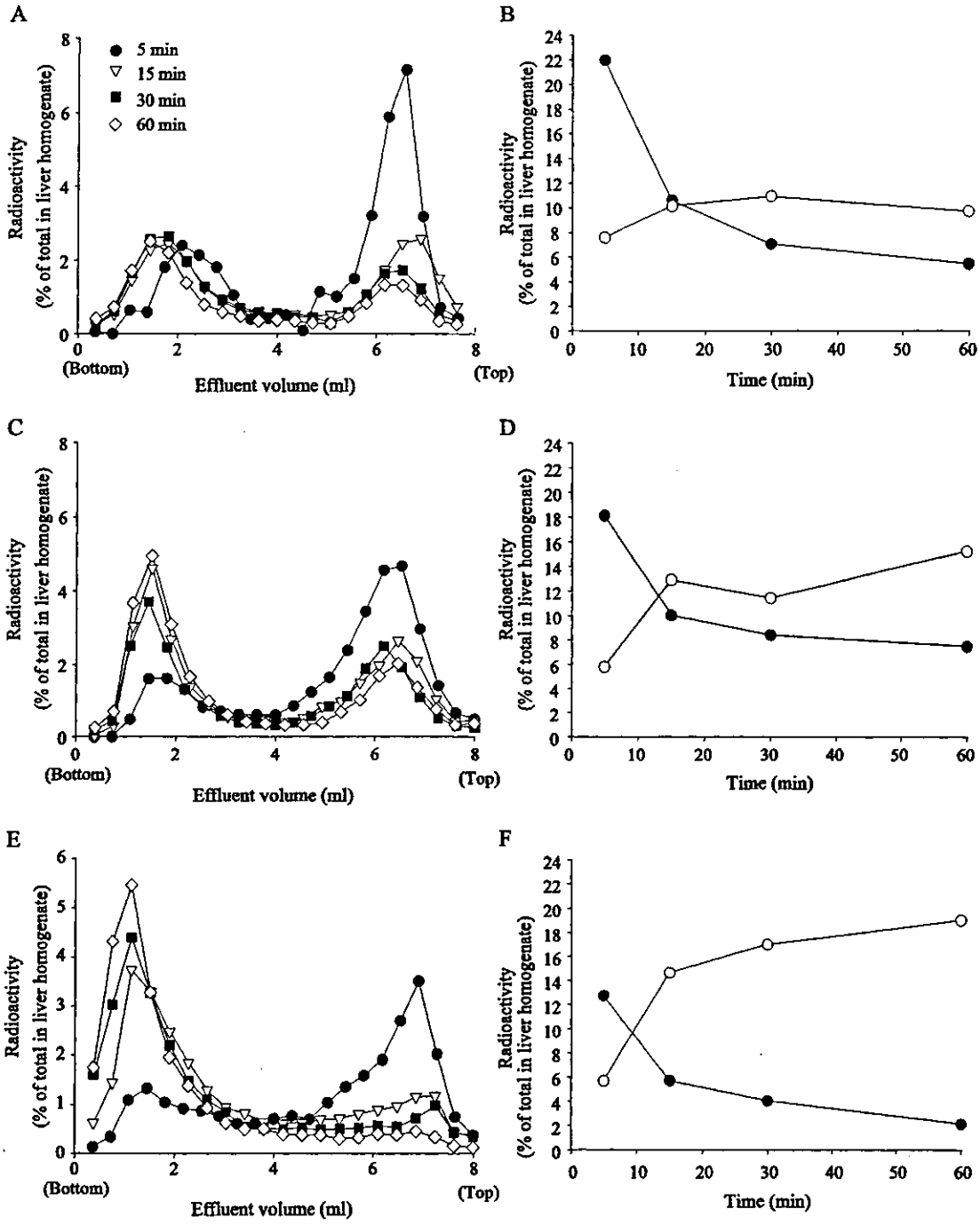

Fig. 6. Each fraction (A, C, and E) and calculated endosomal (●) and lysosomal (○) localization (B, D, and F) of radioactivity 5, 15, 30, and 60 min after intravenous administration of [^{111}In]lipoplex (A, B), Man lipoplex (C, D), or naked [^{111}In]pDNA (E, F) in mice. Liver homogenate was separated by Percoll density gradient centrifugation. Similar results were obtained in two other independent runs.

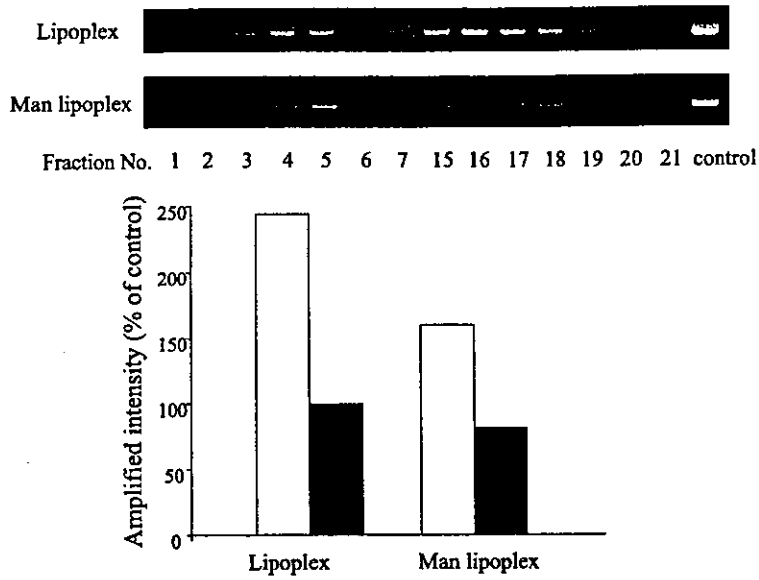

Fig. 7. Amplification of pDNA in endosomal (□) and lysosomal (■) fractions 30 min after intravenous administration of lipoplex and Man lipoplex in mice. Liver homogenate was separated by Percoll density gradient centrifugation. pDNA (1 ng/μl) was used as a control. Similar results were obtained in two other independent runs.

tered, the amounts of DNA amplified from the endosome and lysosome fractions were smaller than those after administration of the lipoplex.

4. Discussion

Transgene expression in target cells after intravenous administration of the Man lipoplex involves a number of distribution processes for pDNA: delivery to the target cells (tissue distribution), internalization, intracellular sorting, and nuclear entry [26]. In particular, the data presented in this study show the importance of intracellular sorting for efficient gene transfection of the Man lipoplex.

Since lipoplex was taken up the cell by the mechanism of endocytosis, pDNA needs to avoid degradation in lysosomes for improving the transfection efficiency. Thus, understanding of the intracellular fate of pDNA will help in the development of better transfection carrier systems. However, there are only a few studies that quantitatively investigate the intracellular fate of pDNA under in vivo conditions. Although so far several tracing methods of pDNA have been used such as ^{32}P-label by nick translation, however, radioactive metabolite, which are generated before and after the cellular uptake of radiolabeled pDNA, often make it extremely difficult to quantitative analyze the tissue distribution and pharmacokinetics of pDNA. In our preliminary experiment, when the fate of internalized [^{32}P]pDNA in liver homogenate was investigated, we found that [^{32}P]pDNA was not suitable for subcellular distribution studies because the radioactivity derived from [^{32}P]pDNA rapidly diminishes due to degradation during the preparation of subcellular fractions. More recently, we have developed [^{111}In]pDNA, an alternative radiolabeling method for pDNA [22], in a similar manner to the preparation of ^{111}In-labeled proteins [27,28], and demonstrated that the radioactivity of [^{111}In]pDNA is slowly released from cells after internalization. Therefore, [^{111}In]pDNA is considered the suitable method for analyzing the tissue and intrahepatic distribution of Man lipoplex. In fact, we observed that both [^{32}P]pDNA and [^{111}In]pDNA mainly accumulated in the liver after intravenous administration, but the radioactivity of ^{32}P gradually decreased (Fig. 2). In contrast, ^{111}In remained at a high level for 2 h after administration (data not shown). Such differences were explained by the poorer membrane permeability

of radioactive metabolites due to the attachment of DTPA for chelation of ^{111}In. Taking this into consideration, [^{111}In]pDNA was effective rebelling method for the subcellular distribution study of lipoplex.

As shown in Fig. 4, the cytoplasmic radioactivity was increased with time after intravenous injection of the [^{111}In]lipoplex and Man lipoplex; accordingly, pDNA is considered to be efficiently released into cytoplasm. On the other hand, the shift of radioactivity from the endosomal to the lysosomal fractions after intravenous administration of the [^{111}In]lipoplex and Man lipoplex suggests that both DC and Man liposomes promote pDNA transfer to lysosomes in the cell (Fig. 6). We previously reported that mannosylated proteins are internalized faster than cationic proteins, which are internalized by the liver via adsorptive endocytosis [16]. Receptor-mediated uptake of the Man lipoplex would explain its faster transport to the lysosome fractions. When naked [^{111}In]pDNA was injected intravenously, however, it showed more rapid transfer to lysosomes than both pDNA complexes. The lysosomes are where internalized substances are degraded, and it can be considered that lysosomal delivery is not suitable for transgene expression. Amplification of pDNA by PCR supported that the Man lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex (Fig. 7). Therefore, these results suggested that modulation of its intracellular sorting could improve the transfection efficiency of Man lipoplex.

After administration into the blood circulation, the lipoplex interacts with various cells and molecules, such as serum proteins and erythrocytes [29,30]. The cationic nature of the lipoplex attracts negatively charged cells and molecules, which eventually leads to an alteration in the physicochemical properties of the complex. Generally, cellular uptake of a lipoplex is considered to be a nonspecific process based on the interaction of its excess positive charge and the negatively charged cell membrane. Thus, high accumulation of radioactivity was observed both in lung and liver after intravenous administration of the [^{32}P]lipoplex (Fig. 2). On the other hand, the [^{32}P]Man lipoplex did not accumulate in the lung to any great extent compared with the [^{32}P]lipoplex, suggesting that nonspecific interaction could be reduced by mannosylation of cationic liposomes. This distribution study may be partly supported by the fact that our previous observation involved the liver NPC selective gene transfection after intravenous administration of Man lipoplex [10].

In conclusion, the Man lipoplex showed specific accumulation in NPC and achieved higher gene expression than the lipoplex after intravenous administration. It was shown that pDNA delivered by Man liposomes, which is taken up by the mannose receptor, was more susceptible to intracellular degradation than that delivered by conventional cationic liposomes, and this would impair higher gene expression. Also, this observation leads us to believe that further carrier development studies are needed for improving the intracellular sorting of pDNA to avoid degradation.

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Effect of cationic charge on receptor-mediated transfection using mannosylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice

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The purpose of this study was to evaluate the effect of cationic charge of complexes after intravenous administration of cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiomannosyl-ethyl)amino]butyl}formamide (Man-C4-Chol) containing cationic liposomes/pDNA complexes in mice. Transfection efficiency after intravenous administration of complex at a charge ratio (− : +) of 1.0 : 2.3 and/or 1.0 : 3.1 in liver and spleen expressing a mannose receptor on the cell surface were higher than those in lung. When complexes were formed at a charge ratio (− : +) of 1.0 : 4.7, on the other hand, transfection efficiency in the lung was highest, suggesting a non-specific interaction. Although asialoglycoprotein receptors are expressed on hepatocytes, a liver-selective gene transfection was not achieved by the intravenous administration of pDNA complexed with cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl)-amino]butyl}formamide (Gal-C4-Chol)/DOPE liposomes at a charge ratio (− : +) of 1.0 : 2.3. This information supports the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery after intravenous administration.

1. Introduction

The success of *in vivo* gene therapy relies on the development of a vector that achieves target cell-specific, efficient, and prolonged transgene expression following its application. Non-viral vectors are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, attractive for clinical application. Although the cationic liposome/plasmid DNA (pDNA) complex is a useful non-viral vector, it lacks specificity in delivery and transfection. Intravenous administration of pDNA/cationic liposome complexes expressed genes into various tissues, with highest gene expression in the lung (Templeton et al. 1997; Li et al. 1997). The attachment of a ligand that can be recognized by a specific mechanism would endow a vector with the ability to target a specific population of cells. In the search for cationic liposomes-based non-viral vectors, several ligands including asialofetuin (Hara et al. 1995), galactose (Kawakami et al. 1998; 2000a; Fumoto 2003), and mannose (Kawakami et al. 2000b; 2001; Sato et al. 2001) have been used to improve the delivery of pDNA to target cells. Therefore, the incorporation of such ligands into cationic liposomes would improve the cell-specificity of *in vivo* gene transfer by cationic liposome/pDNA complexes.

The mannose receptor is expressed on Kupffer cells, splenic, alveolar, peritoneal macrophages, monocyte-derived dendritic cells, and subsets of vascular and lymphatic endothelial cells (Weis et al. 1998). Recently, we designed cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiomannosyl-ethyl)-amino]butyl}formamide (Man-C4-Chol) to prepare mannosylated cationic liposomes for mannose receptor-mediated gene delivery (Kawakami et al. 2000b). In the previous study, we have reported that the highest gene expression in the liver and spleen after intravenous injection was observed for Man-C4-Chol/dioleoylphosphatidylethanolamine (DOPE) liposome/pDNA complexes via mannose receptor-mediated endocytosis (Kawakami et al. 2000b). However, not only the nature of the ligands grafted to carriers but also the overall physicochemical properties of the complexes need to be optimized for the successful delivery of pDNA under systemic injection (Mahato et al. 1997; Kawakami et al. 2002). Although it is well known that a high cationic charge of cationic liposome/pDNA complexes enhances the transfection efficiency in the lung after intravenous injection because of the non-specific interaction between lung and complex (Templeton et al. 1997; Li et al. 1997), the effect of a cationic charge of ligands grafted cationic liposomes/pDNA complex on cell-selective transfection efficiency is not clear. Once the *in vivo* gene expression is linked with its physicochemical properties, it is then possible to design liposomes or a pDNA/liposome complex to enable cell-specific *in vivo* gene delivery.

In the present study, we tried to elucidate the effect of a cationic charge of complexes after intravenous injection of Man-C4-Chol containing a cationic liposome/pDNA complex in mice. Also, we evaluated the transfection efficiency of Man-C4-Chol containing a cationic liposome/pDNA complex using primary cultured mouse peritoneal

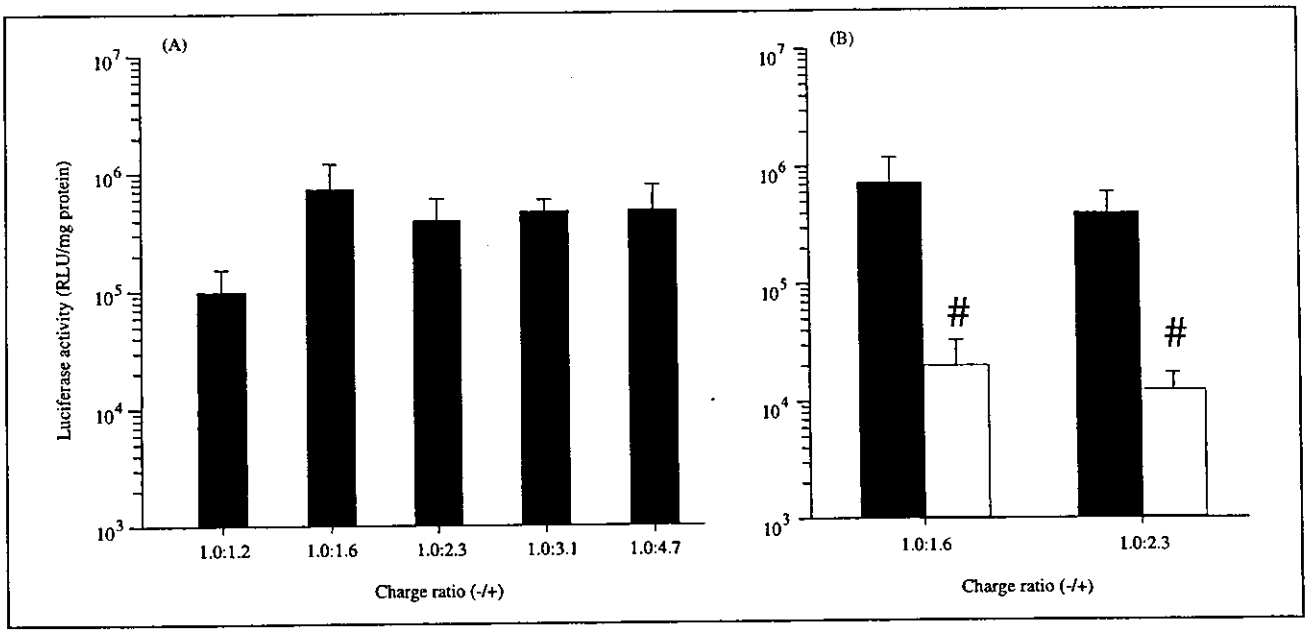

Fig. 1: Transfection activity at various charge ratio (− : +) (A) and the absence (■) or presence (□) of 1mg/ml mannan on transfection activity (B) of pDNA complexed with Man-C4-Chol/DOPE liposomes in cultured mouse peritoneal macrophages. pDNA concentration was fixed at 0.5 μg/ml in all experiments. Each value represents the mean ±S.D. values (n = 3). Stastical analysis was performed by analysis of variance ($^{\#}P < 0.05$)

macrophages which express the mannose receptor, to compare the results with *in vivo* application. pCMV-Luc was selected as a model pDNA because transfected luciferase is easy to detect by fluorescence.

2. Investigations, results and discussion

Complex formation between pDNA and Man-C4-Chol/DOPE complex at a charge ratio (− : +) of 1.0 : 1.2, 1.0 : 1.6, 1.0 : 2.3, 1.0 : 3.1, and 1.0 : 4.7 was determined by agarose gel electrophoresis, followed by gel staining with ethidium bromide and photography under UV light. The addition of any Man-C4-Chol/DOPE liposome to pDNA at these charge ratios resulted in the formation of complexes that did not move towards the positive pole (data not shown).

First, we evaluated the transfection efficiency and its gene expression mechanism on transfection of pDNA complexed with Man-C4-Chol/DOPE liposome at various charge ratios (− : +) in cultured mouse peritoneal macrophages. As shown in Fig. 1, the transfection efficiency was almost the same at charge ratios (− : +) from 1.0 : 1.2 to 1.0 : 4.7. In the presence of 1 mg/ml mannan, the transfection efficiency of pDNA complexed with Man-C4-Chol/DOPE liposomes was significantly reduced. This result suggested that the mannose receptor-mediated endocytosis is involved in the transfection mechanism of pDNA complexed with Man-C4-Chol/DOPE liposomes.

Table: Mean particle sizes of liposome/pDNA (50 μg) complexes in 5% dextrose for *in vivo* experiments

Charge ratio (− : +)	Particle size (nm)
1.0 : 1.2	Aggregated
1.0 : 1.6	Aggregated
1.0 : 2.3	178.1 ± 13.1
1.0 : 3.1	118.6 ± 3.7
1.0 : 4.7	121.3 ± 8.5

Each value represents the mean ± S.D. values (n = 3).

Ogris et al. reported that mixing complexes at low ion strength prevents aggregation, although, large complexes resulting from aggregation showed high transfection efficiency *in vitro* in the case of DNA/transferrin-PEI complexes (Ogris et al. 1998). Referring to this report, the complexes were prepared with 5% dextrose solution. The Table 1 summarizes the particle sizes of Man-C4-Chol/DOPE liposome/pDNA complexes in 5% dextrose solution. When 50 μg of pDNA was mixed with Man-C4-Chol/DOPE liposomes, complexes at a charge ratio (− : +) of 1.0 : 1.2 and 1.0 : 1.6 were aggregated. In contrast, complexes at a charge ratio (− : +) from 1.0 : 2.3 to 1.0 : 4.7 can be prepared and their size is approximately 120–180 nm. The gene expression in mice following the intravenous injection with pDNA complexed with Man-C4-Chol/DOPE liposomes at charge ratio (− : +) of 1.0 : 2.3, 1.0 : 3.1, and 1.0 : 4.7 is shown in Fig. 2. The transfection efficiency after intravenous administration of

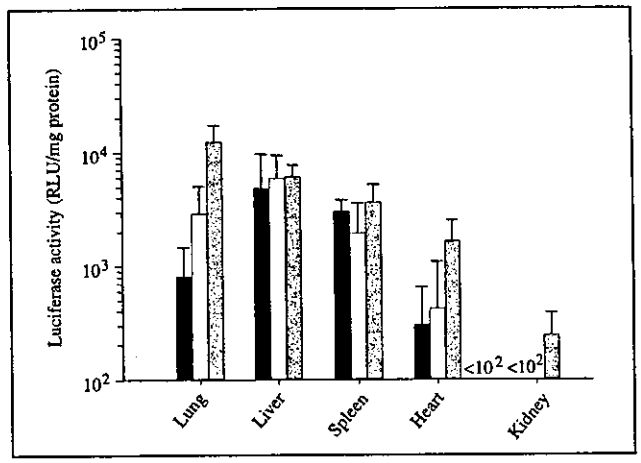

Fig. 2: Transfection activity of pDNA complexed with Man-C4-Chol/DOPE liposomes after intravenous administration in mice. pDNA (50 μg) was complexed with cationic lipids at a charge ratio (− : +) of 1.0 : 2.3 (■), 1.0 : 3.1 (□), and 1.0 : 4.7 (▨). Luciferase activity was determined 6 h post-injection in the lung, liver, spleen, heart, and kidney. Each value represents the mean ±S.D. values (n = 3)

complexes at charge ratios (− : +) of 1.0 : 2.3 and 1.0 : 3.1 in liver and spleen, expressing mannose receptors on the cell surface, were higher than those in lung. When the complex was formed at a charge ratio (− : +) of 1.0 : 4.7, on the other hand, transfection efficiency in the lung was highest, suggesting a non-specific interaction. Also, these results are well congruent with our previous report concerning the asialoglycoprotein receptor-mediated gene transfection following the intraportal administration of pDNA complexed with DOTMA/Chol/cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl)amino]butyl}formamide (Gal-C4-Chol) liposomes in mice (Kawakami et al. 2000a).

Intravenous gene delivery to the lung via pDNA/cationic liposome complexes has been reported. Several parameters have been identified to be important for achieving a high level of gene expression (Huang and Li 1997). Among them, a high cationic charge between a pDNA and cationic lipid is important for efficient intravenous gene delivery; however, these strategies of gene transfection include non-specific adsorption mediated endocytosis. In the present study, we demonstrated that liver and spleen selective gene transfection using mannose receptor mediated endocytosis is achieved by complexes prepared at a charge ratio (− : +) of 1.0 : 2.3 and/or 1.0 : 3.1. An excess cationic charge of complexes resulted in enhanced gene expression in the lung with non-specific interaction.

It has been reported that hepatocytes exclusively express large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior. Referring to the results presented in Fig. 2, pDNA/galactosylated liposome complexes were prepared at a charge ratio (− : +) of 1.0 : 2.3. Sinusoids in the liver lobules are invested with a unique type of endothelial lining consisting of endothelial cells with flattened processes perforated by small fenestrae of about 200 nm in size. Therefore, pDNA/liposome complexes with a diameter less than this can readily pass through the fenestration into the Disse space and we prepared pDNA complexed with Gal-C4-Chol/DOPE liposomes having a size of approximately 150 nm in diameter for free to access to hepatocytes. Although the size of the liposomes was approximately 150 nm, selective gene targeting to the liver was not achieved (Fig. 3). For the target cells of mannosylated liposome/pDNA complexes, there is not need to pass through fenestrae; therefore, selective gene targeting in liver and spleen on macrophages may be achieved by intravenous administration.

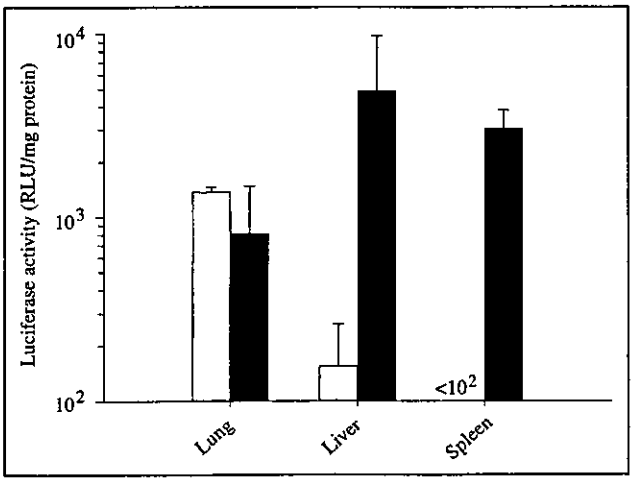

Fig. 3: Transfection activity of pDNA complexed with Gal-C4-Chol/DOPE liposomes (□) or Man-C4-Chol/DOPE liposomes (■) after intravenous administration in mice. pDNA (50 μg) was complexed with cationic lipids at a charge ratio (− : +) of 1.0 : 2.3. Luciferase activity was determined 6 h post-injection in the lung, liver, and spleen. Each value represents the mean ±S.D. values (n = 3)

The difference of transfection between galactosylated liposomes and mannosylated liposomes suggested that the size of complexes at a charge ratio (− : +) of 1.0 : 2.3 might be increased over 200 nm after intravenous administration. Thus, not only the introduction of a ligand to the cationic liposomes but also a controlled size of complexes is important for an efficient targeted gene delivery. We previously reported that the pDNA/cationic liposome complexes are interacting with erythrocytes after intravenous administration (Sakurai et al. 2001). Recently, Eliyahu et al. (2002) also characterized the interaction of blood components with pDNA/cationic liposome complexes under conditions relevant to *in vivo* intravenous administration. In their report, the selection of a medium (i.e. plasma and serum) and/or modification of the cationic liposomes with 1% polyethyleneglycol lipids reduced the aggregation of pDNA/cationic liposome complexes in the presence of erythrocytes; accordingly, such approaches may enhance the cell-specificity of pDNA complexed with galactosylated and mannosylated cationic liposomes. Further studies on the interaction with blood components and/or the synthesis of polyethyleneglycol-grafted glycosylated lipids for cell-selective gene delivery are required. These information supports the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery under *in vivo* conditions.

3. Experimental

3.1. Materials

N-(4-Aminoethyl) carbamic acid *tert*-butyl ester and *N*-(4-aminobutyl) carbamic acid tert-butyl ester, *N*-(4-aminohexyl) carbamic acid tert-butyl ester were obtained from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate and DOPE were obtained from Sigma Chemicals (St. Louis, MO) and Avanti Polar-Lipids (Alabaster, AL), respectively. Fetal bovine serum (FBS) was obtained from Biowhittaker (Walkersville, MD). Opti-MEM I and other culture reagents were obtained from Gibco BRL (Grand Island, NY). All other chemicals were of the highest purity available.

3.2. Construction and preparation of pDNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA). pDNA was amplified in the E. coli strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

3.3. Synthesis of Man-C4-Chol and Gal-C4-Chol

As reported previously (Kawakami et al. 2000c), *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide was obtained from cholestery chloroformate and *N*-(4-aminobuthyl) carbamic acid tert-butyl ester. The product was reacted with 5 equivalents of 2-imino-2-methoxyethyl-1-thiomannoside or 2-imino-2-methoxyethyl-1-thiogalactoside (Lee et al. 1976) in pyridine containing 1.1 equivalents of triethylamine for 24 h. After evaporation of the reaction mixture *in vacuo*, the resultant material was suspended in water and dialyzed against water for 48 h. Then, the compounds were lyophilized.

3.4. Particle size measurements

The particle sizes of liposome/pDNA complexes were measured in a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan).

3.5. Harvesting and culture of macrophages

Male ICR mice weighing 20–25 g were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. Elicited macrophages were harvested from mice 4 days after intraperitoneal injection of 1 ml 2.9% thioglycolate medium (Nissui Pharmaceutical Co. LTD., Tokyo, Japan). The washed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum