In Vivo Gene Delivery to the Liver Using Novel Galactosylated Cationic Liposomes

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Purpose. The purpose of this study is to elucidate the *in vivo* gene transfer for galactosylated liposomes containing cholesten-5-yloxy-N-(4-((1-imino-2-β-D-thiogalactosylethyl)amino)butyl)formamide(Gal-C4-Chol) in relation to lipid composition and charge ratio.

Methods. Galactosylated cationic liposomes containing N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride(DOTMA), Gal-C4-Chol and cholesterol(Chol), and similar liposomes were prepared. Plasmid DNA complexed with a galactosylated liposome preparation was injected intraportally into mice. The mice were sacrificed after 6 hours. The tissues were subjected to luciferase assay.

Results. A markedly higher gene expression in the liver following injection of plasmid DNA that has been complexed with DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) and DOTMA/Gal-C4-Chol(1:1) liposomes was observed. The effect was one order of magnitude higher than naked DNA and DOTMA/Chol(1:1) liposomes. Pre-exposing with galactosylated bovine serum albumin significantly reduced the hepatic gene expression. By comparison, the gene expression for galactosylated cationic liposomes containing 3β[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol, Gal-C4-Chol and dioleoylphosphatidylethanolamine was 10 times lower. As far as the charge ratio of DOTMA/Chol/Gal-CA-Chol(1:0.5:0.5) liposomes to plasmid DNA(1.6–7.0) was concerned, complexes with charge ratios of 2.3–3.1 produced maximal gene expression in the liver. Whereas, higher ratios resulted in enhanced expression in the lung.

Conclusions. By optimizing lipid composition and charge ratio, galactosylated liposome/DNA complexes allow superior *in vivo* gene transfection in the liver via asialoglycoprotein receptor-mediated endocytosis.

KEY WORDS: cationic liposome; gene delivery; targeting; asialoglycoprotein receptor; *in vivo*.

INTRODUCTION

A number of receptor-mediated gene delivery systems have been developed to introduce foreign DNA into specific cell types *in vivo*. Ligands currently being investigated include asialoglycoproteins (1–3), lactose (4), transferrin (5–6), epidermal growth factor (7), and antibodies (8). In a series of investigations, we have developed galactosylated poly(L-lysine) to deliver plasmid DNA to hepatocytes *in vivo* (9–10). In addition, we have confirmed that 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 hepatic targeting of plasmid DNA (9–10). This is closely connected to the findings from our studies on the relationship between the *in vivo* disposition of drug-macromolecular conjugates and chemically modified proteins and their physicochemical properties (11).

Besides macromolecular carrier systems, we have also studied liposomal gene carrier systems (12). To this end, we developed novel galactosylated cholesterol derivatives, cholesten-5-yloxy-N-(4-((1-imino-2-β-D-thiogalactosylethyl)amino) alkyl) formamide, for hepatocyte-directed gene carriers, demonstrating that the liposomes containing this galactolipid showed higher transfection activities than 3β[N′,N′,N′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) liposomes (13) in human hepatoma cells (HepG2) (12).

In this study, we investigated the *in vivo* transfection activities of novel galactosylated liposome/plasmid DNA complexes in mice to examine the effect of the lipid composition of the liposomes and their charge ratios. Results are compared with those of N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA)/cholesterol (Chol) liposomes and DC-Chol/dioleoylphosphatidylcholine (DOPE) liposomes, which are reported to have a high potential for *in vivo* and *in vitro* gene transfection, respectively (13).

EXPERIMENTAL METHODS

Materials

N-(4-aminoethyl) carbamic acid *tert*-butyl ester, N-(4-aminobutyl) carbamic acid tert-butyl ester, N-(4-aminohexyl) carbamic acid tert-butyl ester and DOTMA were obtained from Tokyo Chemical Industry (Tokyo, Japan). [α-^{32}P]-dCTP (3000 Ci/mmol) was obtained from Amersham (Tokyo, Japan). Clearsol I and Soluene 350 were obtained from Nacalai Tesque (Kyoto, Japan) and Packard (Groningen, Netherlands), respectively. Cholesteryl chloroformate and DOPE were obtained from Sigma Chemicals (St. Louis, MO) and Avanti Polar-Lipids (Alabaster, AL), respectively. DC-Chol was synthesized according to the published method (12). Galactosylated bovine serum albumin (Gal-BSA) was synthesized as reported previously (14). Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). 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.

Construction and Preparation of Plasmid DNA (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). Plasmid DNA was amplified in the E coli strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration

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ABBREVIATIONS: pCMV-Luc, plasmid DNA encoding firefly luciferase under the control of cytomegalovirus promoter.

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was measured by UV absorption at 260 nm. The plasmid was labeled with [α-^{32}P]-dCTP by nick translation (15)

Synthesis of Cholesten-5-yloxy-N-(4-((1-imino-2-β-Dthiogalactosylethyl)amino)butyl)formamide (Gal-C4-Chol)

Gal-C4-Chol was prepared according to the method reported previously (11). Cholesteryl chloroformate and N-(4-aminobutyl) carbamic acid tert-butyl ester were reacted in chloroform for 24 h at room temperature. After the reaction mixture was incubated with trifluoroacetic acid for 4 h at 4°C, the solvent was evaporated in vacuo to yield N-(4-aminobutyl)-(cholesten-5-yloxyl)formamide. This was reacted with an excess amount of 2-imino-2-methoxyethyl-1-thiogalactoside (14) in pyridine containing triethylamine for 24 h at room temperature. After the reaction mixture was evaporated in vacuo, the resultant material was suspended in water, and dialyzed against distilled water for 48 h using a dialysis membrane (12 kDa cut-off). After the dialyzate was lyophilized, the crude product was purified by recrystalization three times in ethyl acetate.

Preparation of Liposomes

Mixtures of Gal-C4-Chol, DC-Chol, and DOPE or those of Gal-C4-Chol, DOTMA and Chol were dissolved in chloroform, vacuum-desiccated, and resuspended in sterile 5% dextrose. The suspension was sonicated on ice for 3 min and the resulting liposomes were extruded 10 times through double-stacked 100 nm polycarbonate membrane filters. The particle size of the liposome/plasmid DNA complexes was measured in a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan).

In Vitro Transfection Experiment

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) 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 6-well cluster dish at a density of 2×10^5 cells/10.5 cm^2 and cultivated in 2 ml DMEM supplemented with 10% FBS. After 24 h in culture, the medium was replaced with Opti-MEM I containing 0.5 μg/ml plasmid DNA complexed with liposomes. Six hours later, the incubation medium was replaced again with DMEM supplemented with 10% FBS and incubated for another 42 h. The cells were scraped and suspended in 200 μl pH 7.4 phosphate-buffered saline (PBS). One hundred microliters cell suspension was subjected to three cycles of freezing (liquid N_2 for 3 min) and thawing (37°C for 3 min), followed by centrifugation at 10,000 × *g* for 3 min. The supernatants were stored at −20°C until the luciferase assays were performed. Ten microliters supernatant was mixed with 100 μl luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The protein content of the cell suspension in PBS was determined by a modified Lowry method using BSA as a standard (16). The luciferase activity is indicated as the relative number of light units per mg protein.

In Vivo Gene Expression

Five-week-old ICR (18.5∼22.0 g) mice were intravenously injected with 300 μl of a suspension of liposome/plasmid DNA complexes in 5% dextrose at a plasmid DNA of 30 μg. Similarly, five-week-old ICR mice (18.5∼22.0 g) anesthetized with ether were intraportaly injected with 300 μl liposome/plasmid DNA complexes at a plasmid DNA of 30∼80 μg. Some mice were intravenously injected with 20 mg/kg galactosylated bovine serum albumin (Gal-BSA) (15) prior to intraportal injection of the complexes. Six hours later, mice were sacrificed, and lung, liver, kidney, spleen and heart were removed. The organs were washed twice with ice-cold saline and homogenized with a lysis buffer. The lysis buffer consisted of 0.1M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA. The volumes of the lysis buffer added were 5 μl/mg for liver and 4 μl/mg for other organs. 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 subjected to the luciferase assay. The protein contents of each supernatant was also determined.

In Vivo Distribution

Five-week-old ICR mice (18.5∼22.0 g) anesthetized with ether were intraportally injected with 17 kBq [^{32}P]plasmid DNA and 30 μg cold plasmid DNA complexed with liposomes in 300 μl 5% dextrose solution. Blood was collected from the vena cava at 1, 3, 5, 10, 30, 60 min, and mice were killed at each collection time point. Liver, kidney, spleen, heart, and lung were removed, washed with saline, blotted dry, and weighed. Immediately prior to blood collection, urine was also collected directly from the urinary bladder. Ten microliters blood and 200 μl urine, and a small amount of each tissue were digested with Soluene-350 (0.7 ml for blood, urine and tissues) by incubation overnight at 45°C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydroperoxide, 0.1 ml 5 N HCl, and 5.0 ml Clear-Sol I were added. The samples were stored overnight, and radioacivity was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan).

Cellular Localization of Luciferase Activity in Liver

Six hour after intraportal injection of plasmid DNA/liposome complexes, each mouse was anesthetized with pentobarbital sodium (40–60 mg/kg) and the liver was perfused with perfusion buffer (Ca^{2+}, Mg^{2+}-free HEPES solution, pH 7.2) for 10 min and then with HEPES solution containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I) (pH 7.5) for 10 min. Immediately after the start of perfusion, the vena cava and aorta were cut and the perfusion rate maintained at 3–4 min/min. Following discontinuation of the perfusion, the liver was excised and the capsule membranes removed. The cells were dispersed in ice-cold Hank's-HEPES buffer containing 0.1% BSA by gentle stirring. The dispersed cells were filtered through the cotton mesh sieves, followed by centrifugation at 50 g for 1 min. The pellets containing PC were washed twice with Hank's-HEPES buffer by centrifuging at 50 g for 1 min. The supernatant containing NPC was similarly centrifuged twice more. The resulting supernatant was then centrifuged twice at 200 g for 2 min. PC and NPC were resuspended seperately in ice-cold Hank's-HEPES buffer (2 mL for PC and

Table 1. Lipid Composition of Liposomes and the Mean Particle Size of Various Liposome/Plasmid DNA Complexes in 5% Dextrose

Lipid composition of liposomes (molar ratio)	Particle size of complexes (nm)[a]
DC-Chol/DOPE(6:4)	152.1 ± 8.7
Gal-C4-Chol/DOPE(6:4)	150.1 ± 17.1
Gal-C4-Chol/DC-Chol/DOPE(3:3:4)	145.1 ± 7.5
DOTMA/Chol(1:1)	137.7 ± 25.7
DOTMA/Gal-C4-Chol(1:1)	141.1 ± 5.5
DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5)	141.2 ± 6.5

[a] Thirty μg pCMV-Luc plasmid DNA and various liposomes were mixed at a charge ratio of 2.3. The mean particle size of the complexes was measured using a laser light scattering particle size analyzer. Results are expressed as the mean ± S.D. of three experiments.

2 mL for NPC). The cell numbers and viability were determined by the trypan blue exclusion method. After three cycles of freezing and thawing, the homogenates were centrifuged at 10000 g for 10 min at 4°C and 20 μl supernatant was analyzed for luciferase activity using a luminometer.

RESULTS

Particle Sizes of Liposome/DNA Complexes

Table 1 summarizes the particle sizes of liposome/DNA complexes in 5% dextrose when the cationic liposomes were complexed with plasmid DNA at a charge ratio of 2.3. The theoretical charge ratio of DNA/cationic lipid was calculated as a molar ratio of DC-Chol (monovalent), DOTMA (monovalent), and Gal-C4-Chol (monovalent) to a nucleotide unit (average molecular weight 330). The liposome/plasmid DNA complexes prepared were very similar in particle size (130~150 nm).

Gene Expression in HepG2 Cells *In Vitro*

Figure 1 shows the expression of luciferase gene in HepG2 cells transfected with various liposome/DNA complexes. Gal-C4-Chol/DC-Chol/DOPE-based liposome/DNA complexes exhibited greater transfection activity than DOTMA/Chol/Gal-C4-Chol-based liposome/DNA complexes *at the charge ratio of 2.3* (Fig. 1(A)(B)). The presence of 20 mM galactose inhibited the gene expression of plasmid DNA complexed with Gal-C4-Chol-containing liposomes, but not that with either DC-Chol/DOPE(6:4) or DOTMA/Chol(1:1) liposomes.

In Vivo Gene Expression

The gene expression in mice following intravenous injection with plasmid DNA (30 μg) complexed with liposomes is shown in Fig. 2. In the case of DOTMA/Chol(1:1) liposomes, the highest gene expression was observed in the lung while

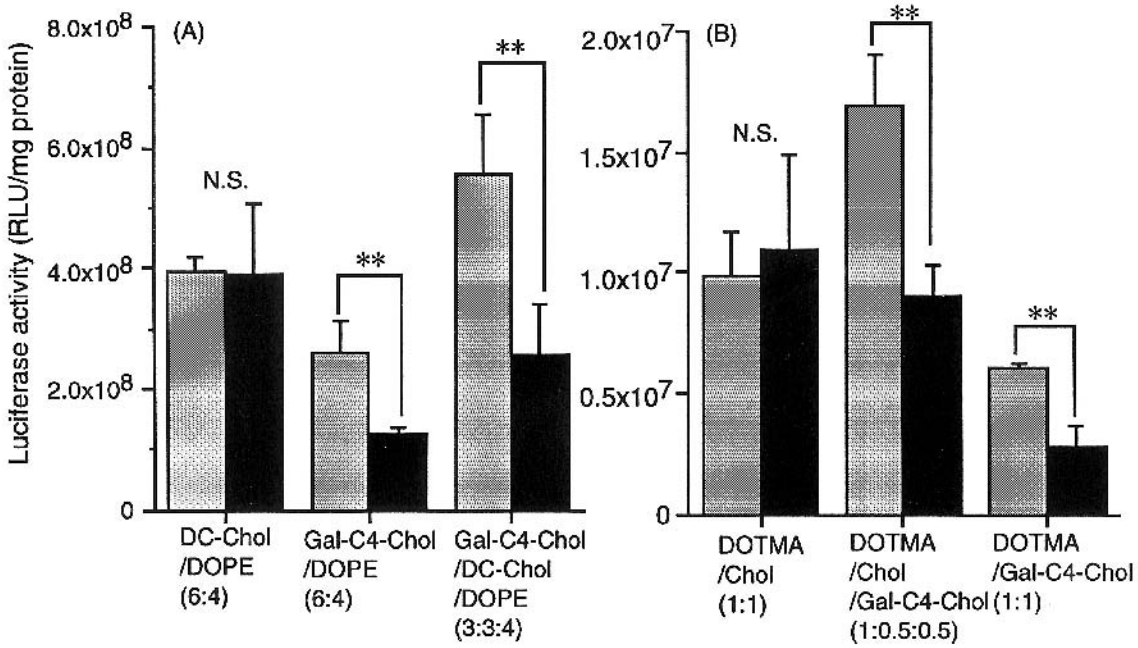

Fig. 1. *In vitro* transfection activity of DNA/liposome complexes at a charge ratio of 2.3 in HepG2 cells. Cells were transfected with DNA/liposome complexes in the absence (□) and presence (■) of 20 mM galactose. DOPE containing liposome/DNA complexes (A) and DOPE non-containing liposome/DNA complexes (B) were applied to the cells. DNA concentration was fixed at 0.5 μg/ml in all experiments. Statistical analysis was performed by analysis of variance (** indicated $P < 0.01$; N.S., not significant). Each value represents the mean ± S.D. values ($n = 3$).

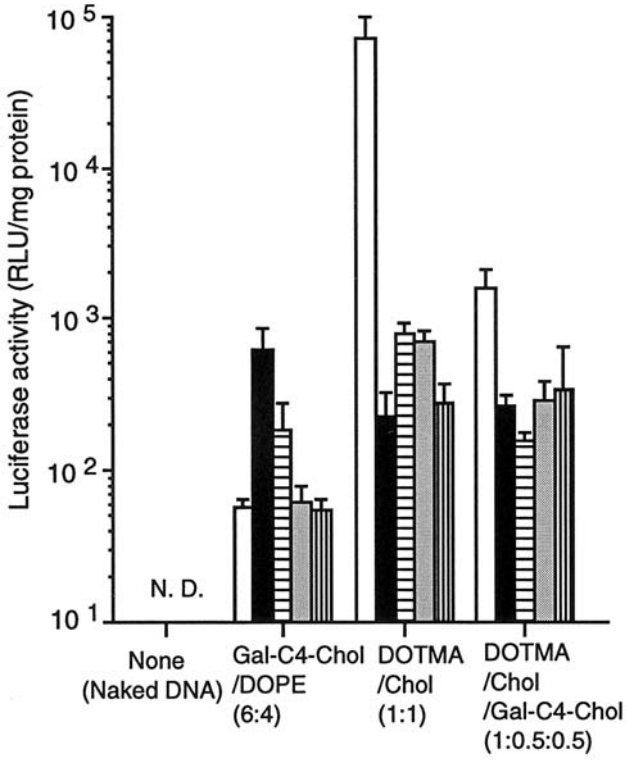

Fig. 2. Transfection activity of liposome/plasmid DNA complexes after intravenous injection in mice. Plasmid DNA (30 μg) was complexed with cationic lipids at a charge ratio of 2.3. Luciferase activity was determined 6 h post-injection in the lung (□), liver (■), kidney (▤), spleen (▩), and heart (▥). N.D. means not detected. Each value represents the mean ± S.D. values (n = 3).

the gene expression in the liver was much lower in all the formulations tested. Figure 3 shows the luciferase activity in several organs after intraportal administration of 30 μg pCMV-Luc plasmid DNA complexed with various liposomes. Unlike the case of *in vitro* gene transfection, the DOPE-containing liposome/plasmid DNA complexes were not as effective *in vivo* at the same charge ratio of *in vitro* experiments (Fig. 3(A)). On the other hand, more than a 10-fold greater gene expression was observed in the liver when plasmid DNA was complexed with DOTMA/Chol/Gal-C4-Chol (1:0.5:0.5) liposomes and DOTMA/Gal-C4-Chol (1:1) liposomes (Fig. 3(B)). Interestingly, the lung exhibited the greatest gene expression with DOTMA/Chol(1:1) liposome/DNA complexes, in spite of intraportal injection.

Figure 4(A) shows the gene expression of liver parenchymal cells (PC) and liver non-parenchymal cells (NPC) after intraportal injection of liposomes/DNA complexes. The gene expression of PC of DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposomes was significantly higher than that of NPC. On the other hand, the gene expression of PC and NPC of DOTMA/Chol (1:1) liposomes was almost same extent. Another experiment revealed that gene expression was increased by increasing the dose of DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes (30, 50 and 80 μg DNA) (Fig. 4(B)). The gene expression in the liver is higher than that of other organs over the dose range 30 to 80 μg plasmid DNA.

When an excess amount of Gal-BSA was intravenously injected 5 min prior to intraportal injection of DNA complexed with DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposomes, gene expression in the liver was significantly reduced (Fig. 5). In contrast, Gal-BSA did not affect the transfection activity of DOTMA/Chol(1:1) liposomes/DNA complexes.

The effect of the liposome/DNA charge ratio on the gene expression in mice following intraportal injection is shown in Fig. 6. As far as the DOT/Chol(1:1) liposome/DNA complexes were concerned, gene expression in the lung increased with an increase in the charge ratio, whereas the charge ratio had no obvious effect on gene expression in the liver. On the other hand, the DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes exhibited an optimal charge ratio of 2.3∼3.1 for gene expression in the liver. In a similar manner to DOTMA/Chol(1:1) liposomes, gene expression in the lung was greater at higher charge ratios. At a charge ratio of 7.0, gene expression in the lung exceeded that in the liver.

Biodistribution of Liposome/DNA Complexes

Figure 7 shows the time-courses of the radioactivity in blood, kidney, spleen, liver, and lung after intraportal administration of [^{32}P]DNA complexed with DOTMA/Chol(1:1) and DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposomes. Radioactivity from the DOTMA/Chol(1:1) liposome/[^{32}P]DNA complexes was taken up mainly by liver, with the peak being observed at 30 min. On the other hand, the radioactivity in the liver from the DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complex was about 75% of the dose, even 1 min after intraportal injection. The radioactivities in the liver of DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposomes at 1, 3 and 60 min were significantly higher than that of DOTMA/Chol(1:1) liposomes.

DISCUSSION

Hara *et al.* (3) reported that asialofetuin-labeled liposome encapsulating plasmid DNA is effective in gene expression after intraportal injection with a preload of EDTA. However, introduction of asialoglycoproteins to the carrier is rather complicated, so that the carrier may cause some problems as far as reproducibility, stability and immunogenicity are concerned. In a previous study, we developed galactosylated lipids by introducing a sugar moiety to Chol and reported the *in vitro* activity of galactosylated cationic liposomes for asialoglycoprotein receptor-mediated gene transfer to HepG2 cells (12). Now, in the present study, we have investigated their *in vivo* potential. Partial or complete replacement of Chol with Gal-C4-Chol in DOTMA/Chol(1:1) liposomes markedly increased the gene expression of DNA in the liver (Fig. 3), while the original formulation is known to be one of the most potent non-vital vectors for *in vivo* gene delivery (17,18). The hepatic cellular gene expression was preferentially observed in PC after intraportal injection of galactosylated liposome/DNA complexes (Fig. 4 (A)). Moreover, the gene expression with galactosylated liposome/DNA complexes was significantly inhibited by preloading Gal-BSA, which is a substrate of asialoglycoprotein receptors (Fig. 5). These results suggested that plasmid DNA complexed with Gal-C4-Chol-containing liposomes might be taken up by the hepatocytes via asialoglycoprotein receptor-mediated endocytosis in *in vivo* conditions.

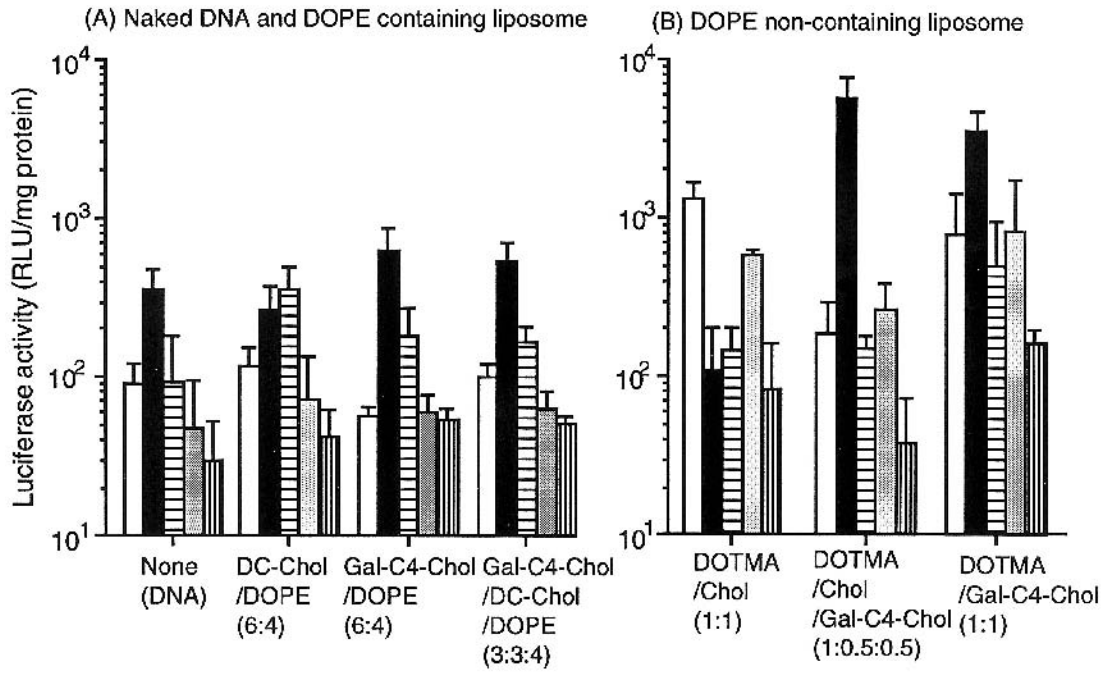

Fig. 3. Transfection activity of liposome/plasmid DNA complexes after intraportal injection in mice. Plasmid DNA (30 μg) was complexed with DOPE containing liposome/DNA complexes (A) and DOPE non-containing liposome/DNA complexes (B) at a charge ratio of 2.3. Luciferase activity was determined 6 h post-injection in the lung (□), liver (■), kidney (▤), spleen (▩), and heart (▥). Each value represents the mean ± S.D. values (n = 3).

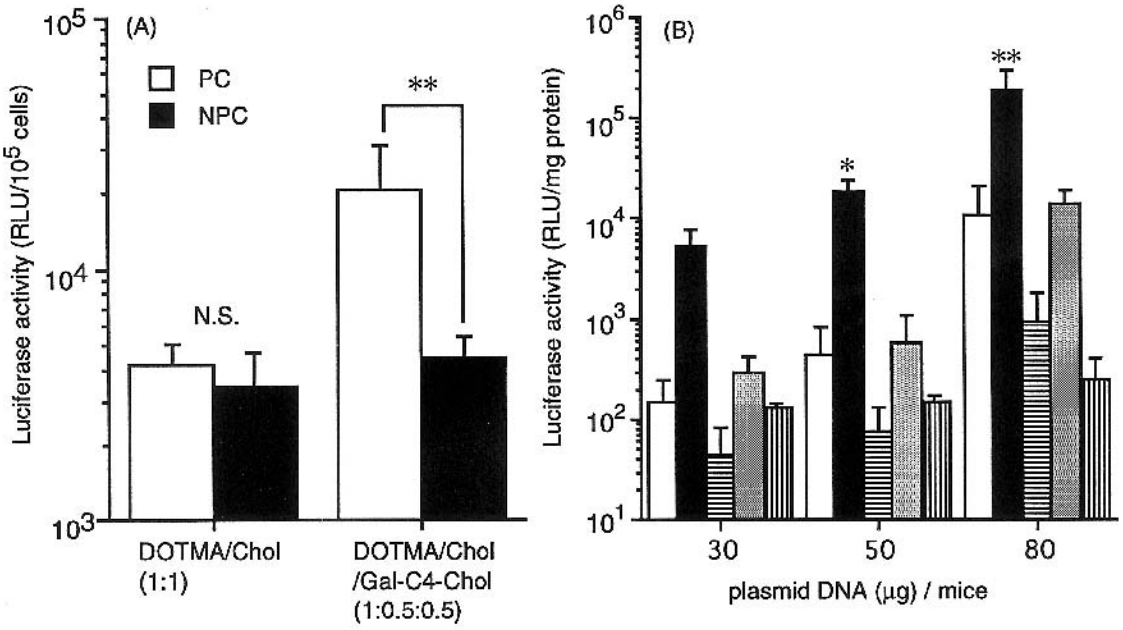

Fig. 4. (A) Intrahepatic gene expression of liposome/plasmid DNA complexes after intraportal injection in mice. Plasmid DNA (50 μg) was complexed with cationic lipids at a charge ratio of 2.3. Luciferase activity was determined 6 h post-injection in the PC (□), NPC (■). Statistical analysis was performed by analysis of variance (** indicated $P < 0.01$; N.S., not significant). Each value represents the mean ± S.D. values (n = 3). (B) Effect of dose of plasmid DNA on transfection activity of DOTMA/Chol/Gal-C4-Chol (1:0.5:0.5) liposome/plasmid DNA complexes after intraportal injection in mice. Plasmid DNA (30, 50, 80 μg) was complexed with cationic lipids at a charge ratio of 2.3. Luciferase activity was determined 6 h post-injection in the lung (□), liver (■), kidney (▤), spleen (▩), and heart (▥), respectively. Statistical analysis was performed by analysis of variance (* indicated $P < 0.05$; ** indicated $P < 0.01$). Each value represents the mean ± S.D. values (n = 3).

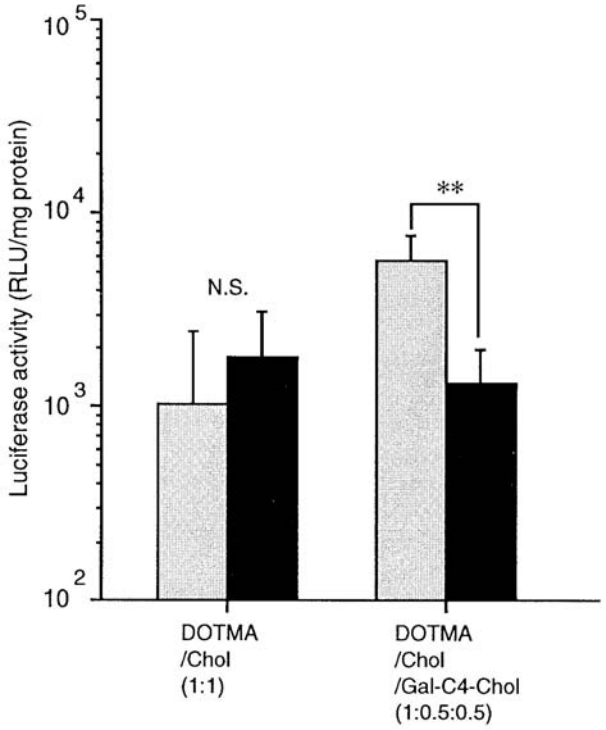

Fig. 5. Inhibition of hepatic gene expression of DNA/liposome complexes by co-administration with galactosylated BSA in mice. Plasmid DNA (30 μg) was complexed with cationic lipids at a charge ratio of 2.3. Luciferase activity was determined at 6 h. Liposome/plasmid DNA complexes were injected with (■) or without (□) galactosylated BSA. Statistical analysis was performed by analysis of variance (** indicated $P < 0.01$; N.S., not significant). Each value represents the mean ± S.D. values ($n = 3$).

As far as DOTMA/Chol(1:1) liposome/DNA complexes are concerned, the lung exhibited greater gene expression than the liver, in spite of intraportal injection (Fig. 3). Similar results have been reported with 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP)/protamine/DNA complexes (19). They defined this phenomenon as "first-passage effect" because lung is first capillary bed the liposome/DNA complexes encounters after intravenous injection. Greater gene expression in the lung is not necessarily related to the biodistribution profile since the radioactivity of DOTMA/Chol(1:1) liposome/[^{32}P]DNA complexes accumulated mainly in the liver as did DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/[^{32}P]DNA (Fig. 7). In the latter case, however, intraportal injection resulted in greater gene expression in the liver than in the lung (Fig. 3).

A marked difference in biodistribution was found between DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes and DOTMA/Chol(1:1) liposome/DNA complexes during the ininial distribution phase. Specifically, more than 75% of DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes was taken up by the liver at 1 min after injection (Fig. 7). The superior hepatic gene expression with DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA should be the result of asialoglycoprotein receptor-mediated endocytosis during the first passage through the PC (Fig. 4(A)).

In this study, we have investigated the effects of the charge ratio and lipid composition of liposome/DNA complexes on their *in vivo* gene transfection. Yang *et al.* reported that the neutralization of excess positive charge in the liposome/DNA complex by negatively charged serum proteins is likely to decreased transfection efficiency in *in vitro* experiments (20). They also demonstrated that it can be overcome by increasing charge ratio of cationic liposome/DNA complexes and its optimal charge ratio was 4 for efficient cationic liposome mediated gene transfer (20). Recently, it was also demonstrated that

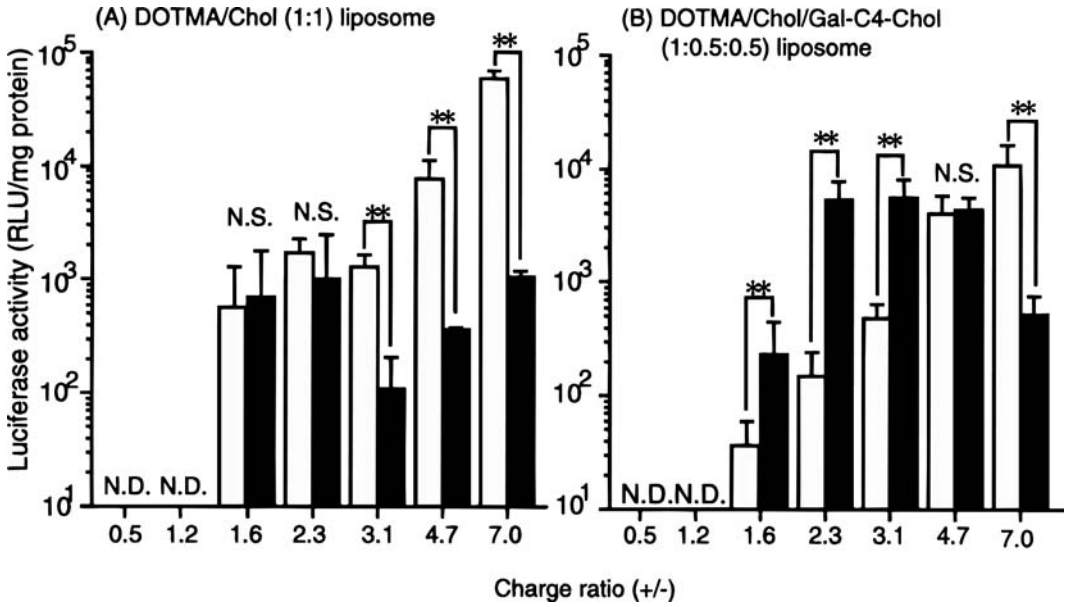

Fig. 6. Effect of charge ratio on the transfection activity of liposome/plasmid DNA complexes *in vivo*. Plasmid DNA (30 μg) was complexed with cationic liposomes at charge ratios of 1.6, 2.3, 3.1, 4.7 and 7.0. Luciferase activities were determined in the lung (□) and liver (■) at 6 h after intraportal injection. N.D. means not detected. Statistical analysis was performed by analysis of variance (** indicated $P < 0.01$; N.S., not significant). Each value represents the mean ± S.D. values ($n = 3$).

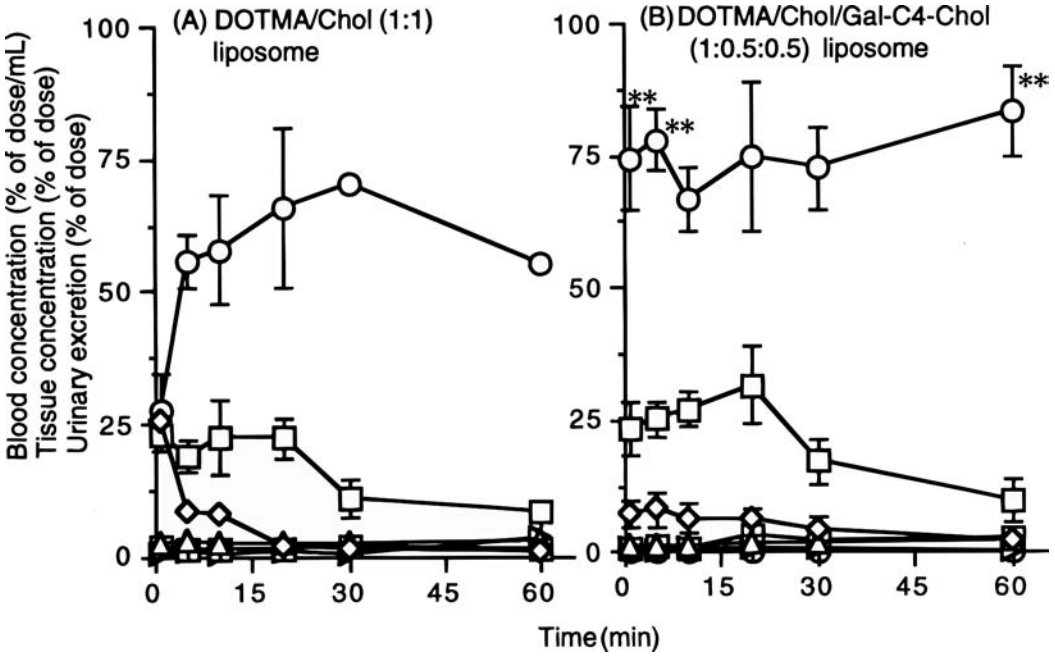

Fig. 7. *In vivo* distribution of [^{32}P] radioactivities after intraportal injection of DOTMA/Chol(1:1) (A) and DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) (B) liposomes complexed with [^{32}P] DNA in mice. Raioactivities were determined in the blood (◇), lung (□), liver (○), kidney (△), spleen (⊞), heart (▷), and urine (⊕). Plasmid DNA (30 μg) was complexed with cationic liposomes at a charge ratio of 2.3. Statistical analysis was performed by analysis of variance (** indicated $P < 0.01$; N.S., not significant). Each value represents the mean ± S.D. values ($n = 3$).

excess cationic charge ratio in the complex required for efficient gene transfer in *in vivo* experiments (17–19,22). However, these sale of gene transfection is non-specific adsorption mediated endocytosis. In this study, we demonstrated that DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes exhibited optimal activity at a charge ratio of 2.3–3.1 for receptor-mediated gene transfer in *in vivo* and an excess in cationic liposomes resulted in reduced gene expression of the liver (Figs. 5, 6). Under these conditions with a higher charge ratio, the complexes may be prevented from binding to receptors due to being coated with negatively charged serum proteins. Gene expression in the lung with DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes increased on increasing their charge ratios, in a similar manner to DOTMA/Chol(1:1) liposomes. On the other hand, gene expression in the liver with DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes decreased at the charge ratio of 7.0. Thus, it should be noted that non-specific gene expression in particular increases on increasing the charge ratio.

In vitro transfection studies demonstrated that Gal-C4-Chol/DC-Chol/DOPE-based liposomes are much more effective than DOTMA/Chol/Gal-C4-Chol-based liposomes (Fig. 1), while the *in vivo* gene transfection studies gave the opposite result at the charge ratio of 2.3 (Fig. 3(A) (B)). Several groups have shown that liposomes containing Chol as a neutral lipid, such as DOTMA/Chol (17–18), dimethyldioctadecylammonium bromide (DDAB)/Chol (21), and DOTAP/Chol (22) liposomes, exhibit greater *in vivo* transfection activity than those containing DOPE. Hong *et al.* (21) found that DOPE-containing liposome/DNA complexes form very large multilamellar and hexagonal lipid structures after incubation with 50% serum, while cholesterol-containing complexes maintain small liposomal structures. The enhancement of *in vivo* transfection by cholesterol may be due to the liposomal stability in *in vivo* conditions. Stable liposomal formulation seems to be better not only non-specific gene expression but also cell-selective gene expression.

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 (23). Referring to this report, the complexes were prepared with 5% dextrose solution in this study. In a preliminary study, DOTMA/Chol/Gal-C4-Chol(1:0.5:0.5) liposome/DNA complexes prepared in pH 7.8 phosphate-buffered saline had a mean diameter of about 16 μm. When these complexes were intraportally injected, gene expression in the liver was significantly reduced, whereas it was increased in the lung. Recently, Kwoh *et al.* also reported that aggregated asialooromucoid poly-l-lysine/DNA complexes did not result in efficient delivery to hepatocytes (24). Thus, selection of a suitable injection vehicle is also an important issue in *in vivo* receptor-mediated gene transfer.

In conclusion, superior *in vivo* delivery and expression of genes in the liver via asialoglycoprotein receptor-mediated endocytosis was achieved using Gal-C4-Chol containing liposomes. Optimization of the colipid type of liposomes, the charge ratio of the liposome/DNA complexes, solvent of the liposome/DNA complexes and other factors based on physicochemical considerations will lead to further improvements in hepatic gene delivery using galactosylated liposomes. We also developed mannosylated liposomes and their superior *in vivo* gene transfection potential in the liver will be reported in a future paper.

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