(FBS, Flow Laboratories, Irvine, U.K.), penicillin G (100 U/ml), and streptomycin (100 µg/ml) and were plated on 6- or 12-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) at a density of 3×10^5 cells/cm^2. After incubation for 24 h at 37 °C in 5% CO_2 – 95% air, non-adherent cells were washed off with culture medium and cells were cultivated for another 48 h.

3.6. Preparation of pDNA/liposome complexes for in vitro experiments

Man-C4-Chol was mixed with DOPE at a molar ratio of 3:2 in chloroform and the mixture was dried, vacuum desiccated, and resuspend in 1 ml sterile 20 mM HEPES buffer (pH 7.8) in a sterile test-tube. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes. It was passed through a 0.45 µM filter for sterilization. The lipid concentration was determined by phosphorus analysis and was adjusted (1 mg/ml) (Bartlett 1959). Cationic liposomes and pDNA (1.0 µg) in a 12 × 75 mm polystyrene tube were diluted with Opti-MEM at various charge ratios before carrying out the transfection experiment. The theoretical charge ratio of cationic lipid/pDNA was calculated as a molar ratio of Man-C4-Chol (monovalent) to a nucleotide unit (average molecular weight 330). Complex formation was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

3.7. In vitro transfection experiment

Macrophages were seeded in 10.5 cm^2 dishes at a density of 1.1×10^6 cells/cm^2 in RPMI 1640 supplemented with 10% fetal calf serum. After 3 days in culture, the culture medium was replaced with Opti-MEM I containing 0.5 µg/ml pDNA and cationic liposomes. Six hours later, the incubation medium was replaced again with RPMI 1640 supplemented with 10% FBS and incubated for an additional 18 h. Then, the cells were scraped and suspended in 200 µl pH 7.4 phosphate-buffered saline (PBS). One hundred microliters the cell suspension were 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 assay was performed. Ten microliters of supernatant were mixed with 100 µl luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured in a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as the relative light units per mg protein. The protein content of the cell suspension in PBS was determined by the modified Lowry method using BSA as a standard (Wang and Smith 1975).

3.8. Preparation of pDNA/liposome complexes for in vivo experiments

Man-C4-Chol or Gal-C4-Chol was mixed with DOPE at a molar ratio of 3:2 in chloroform and the mixture was dried, vacuum desiccated, and resuspend in 5 ml sterile 5% dextrose in a sterile test-tube. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes. It was passed through a 0.45 µM filter for sterilization. The lipid concentration was determined by phosphorus analysis and was adjusted (3 mg/ml) (Bartlett 1959). Equal volumes of pDNA (50 µg) and stock liposome solution diluted with 5% dextrose solution to produce various ratios of pDNA/liposomes were mixed in 1.5 ml Eppendorf tubes at 4 °C. Then, the DNA solution was added rapidly to the surface of the liposome solution using a Pipetman and the mixture was agitated rapidly by pumping it up and down twice in the pipet tip and was stored at 4 °C for 12 h.

3.9. In vivo transfection experiment

Five-week-old ICR mice were injected intravenenously with 300 µl of pDNA/liposome complexes using a 30-gauge syringe needle. 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, 0.1M 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&G Berthold, Bad Wildbad, Germany). The protein concentration of each tissue extract was determined by the modified Lowry method (Wang and Smith 1975). Luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

3.10. Statistical analysis

Statistical comparisons were performed by analysis of variance. $P < 0.05$ was considered to be indicative of statistical significance.

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Note

Uptake characteristics of mannosylated and fucosylated bovine serum albumin in primary cultured rat sinusoidal endothelial cells and Kupffer cells

Yuriko Higuchi[a], Makiya Nishikawa[b], Shigeru Kawakami[a], Fumiyoshi Yamashita[a], Mitsuru Hashida[a,*]

[a] *Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606 8501, Japan*
[b] *Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606 8501, Japan*

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Abstract

The purpose of this study is to delineate uptake characteristics of mannosylated and fucosylated proteins in primary cultured sinusoidal endothelial cells and Kupffer cells. In cultured sinusoidal endothelial cells, uptake of mannosylated and fucosylated bovine serum albumin (BSA) was significantly inhibited by excess mannosylated and fucosylated BSAs but not by galactosylated BSA, suggesting that both glycosylated proteins might be primarily taken up via mannose receptors. In cultured Kupffer cells, uptake of fucosylated BSA was significantly inhibited by excess galactosylated BSA as well as mannosylated and fucosylated BSAs, although that of mannosylated BSA was inhibited only by mannosylated and fucosylated BSAs. This suggests that uptake of fucosylated BSA by Kupffer cells might be mediated by both Kupffer cell lectin (fucose receptor) and mannose receptor. On the other hand, in vivo hepatic uptake of fucosylated BSA was inhibited to a greater extent by $GdCl_3$ pretreatment than that of mannosylated BSA. Based on in vitro and in vivo experiments, it was concluded that fucosylated BSA is more Kupffer cell-selective because it exhibited a lower sinusoidal endothelial cell uptake than mannosylated BSA.
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Keywords: Sinusoidal endothelial cells; Kupffer cells; Cell targeting; Drug delivery; Mannosylated BSA; Fucosylated BSA

1. Introduction

Receptor-mediated drug targeting is a promising approach to deliver therapeutic agents selectively to target cells and maximize their efficacy (Takakura

* Corresponding author. Tel.: +81 75 753 4525; fax: +81 75 753 4575.
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

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and Hashida, 1996). We have demonstrated the usefulness of carbohydrate receptor-mediated drug delivery systems directed at both liver parenchymal (Nishikawa et al., 1995; Kawakami et al., 1998, 2000; Opanasopit et al., 2001a) and non-parenchymal (Ogawara et al., 1999; Opanasopit et al., 2001b) cells. Glycosylation of macromolecular (Nishikawa et al., 1993) and particulate (Kawakami et al., 1998; Ishida et al., 2004) carriers and proteinous drugs themselves (Fujita et al., 1992) with 2-imino-2-methoxyethyl-1-thioglycoside, which we have used so far, has several advantages including the simplicity of its structure, ease of synthesis, broad applicability, and satisfactory targetability.

In a series of investigations, we have demonstrated that mannosylated (Ogawara et al., 1999) and fucosylated (Opanasopit et al., 2001b) bovine serum albumins (BSAs) are efficiently taken up by liver non-parenchymal cells (NPC), mainly composed of sinusoidal endothelial cells and Kupffer cells, after intravenous injection. Similar results have been obtained with mannosylated liposomes (Opanasopit et al., 2001a), and fucosylated liposomes (Kawakami et al., 2000). Inhibition experiments (Opanasopit et al., 2001b) have shown that efficient NPC uptake of mannosylated and fucosylated delivery systems is primarily due to specialized sugar recognition systems.

However, the uptake by NPC via mannose and fucose receptors appears to be complicated. In vitro binding experiments using isolated receptor proteins have shown that the mannose receptor recognizes mannose and fucose but not galactose (Haltiwanger and Hill, 1986; Otter et al., 1992), and that the fucose receptor recognizes fucose and galactose but not mannose (Lehrman et al., 1986). It is also known that the mannose receptor is present on both Kupffer cells (Kuiper et al., 1994) and sinusoidal endothelial cells (Magnusson and Berg, 1993; Otter et al., 1992), and that the fucose receptor is presented on Kupffer cells (Lehrman and Hill, 1986; Haltiwanger et al., 1986). The multiplicities in substrate specificity and cellular localization of the receptors make it difficult to evaluate the contribution of each pathway to the overall in vivo NPC uptake. Further details of the mechanisms involved in NPC uptake are required to develop efficient drug delivery systems that can target sinusoidal endothelial cells and Kupffer cells. In order to obtain such information, it is necessary to elucidate the contribution of mannose receptor and fucose receptor on the uptake of mannosylated and fucosylated BSA using primary cultured Kupffer cells and sinusoidal endothelial cells.

This dearth of mechanistic information prompted us to investigate the uptake characteristics of mannosylated and fucosylated BSAs in rat Kupffer cells and sinusoidal endothelial cells. After both cell types were isolated from rat liver and cultivated, the cellular uptake characteristics of mannosylated and fucosylated BSAs were evaluated in the presence of inhibitors. In order to clarify the contribution of these cell types in vivo, we examined the hepatic uptake of mannosylated and fucosylated BSAs after intravenous administration of $GdCl_3$, a compound that down-regulates the function of Kupffer cells, to mice (Hardonk et al., 1992).

2. Materials and methods

2.1. Chemicals

BSA, collagenase (type I), dexamethasone, vascular endothelial growth factor (VEGF) and Minimum essential medium eagle Joklik modification for suspension culture were purchased from Sigma Chemicals Inc. (St. Louis, MO, USA). D-Mannose, D-galactose, L-fucose, and chloramine-T were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Indium-111 chloride [^{111}In] was kindly supplied by Nihon Medi-Physics Co. Ltd. (Nishinomiya, Japan). Sodium iodide-125 [^{125}I] was purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). RPMI1640 and Eagle's MEM 'Nissui' were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride was purchased from Dojindo Laboratory (Kumamoto, Japan). Rat tail collagen type I, insulin-transferin-selenious acid (ITS+ Premix), and human recombinant fibronectin were purchased from Becton Dickinson (Franklin Lakes, N.J., USA). All other chemicals were of the highest grade available.

2.2. Animals

Male ddY mice (5 weeks old) and male Wistar rats (200–250 g) were purchased from Shizuoka Agricul-

tural Co-operate 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 US National Institutes of Health and with the Guidelines of the Kyoto University Animal Experimentation Ethics Committee.

2.3. The synthesis of BSA derivatives with sugar moieties

Coupling of mannose and galactose moieties to BSA was carried out according to the method of Lee et al. (1976). Briefly, cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-D-mannoside was treated with 0.01 M sodium methoxide at room temperature for 24 h, and a syrup of 2-imino-2-methoxyethyl-1-thio-D-mannoside (IME-thiomannoside) was obtained after evaporation of the solvent. A quantity of the resultant syrup was added to BSA (100 mg) in 10 ml 50 mM borate buffer (pH 9.0). Coupling of fucose moieties to BSA was carried out by the method of Lee et al. (1976) with minor modifications (Kawakami et al., 2000). The molecular weight of the glycosylated proteins was determined by SDS-PAGE and the number of sugar residues was determined by the anthrone-sulfuric acid method using galactose, mannose and fucose as standards (Table 1).

2.4. Radiolabeling with indium-111 and iodine-125

^{111}In-labeled BSA was prepared using a bifunctional chelating agent, DTPA anhydride (Hnatowich et al., 1982). ^{125}I-labeling was performed by the chloramine-T method (Hunter and Greenwood, 1962).

2.5. Cell isolation and culture of sinusoidal endothelial cells and Kupffer cells

Liver sinusoidal endothelial cells and Kupffer cells from rat liver were isolated according to the method of Nagelkerke et al. (1983) with some modification. Endothelial cells were plated at 3.0×10^5 cells/cm^2 onto 24-well plates coated with rat tail collagen type I and human recombinant fibronectin, and cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 5 μg/ml vascular endothelial growth factor (VEGF), 1% (v/v) ITS+ Premix, 10 mg/ml amphotericin B, 10 μM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM/ml L-glutamine for 3–4 days. Kupffer cells were cultured on 24-well plates (2.5×10^5 cells/cm^2) in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/ml L-glutamine, and 10 mg/ml amphotericin B for 24 h. Cells were cultured in a 5% CO_2–95% air incubator thermostatically controlled at 37 °C. The purity of the isolated endothelial cells and Kupffer cells was checked by factor VIII immunostaining and uptake by 4.5 μm latex beads, respectively.

2.6. Uptake of ^{125}I-glycosylated BSA in cultured liver sinusoidal endothelial cells and Kupffer cells

Prior to the uptake experiments, cells were equilibrated with serum-free RPMI medium containing 10 mM 2-[4-(2-hydoroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2% BSA at 37 °C for 20–30 min. Then, ^{125}I-glycosylated BSA (100,000 cpm/well) was added to the medium, followed by incubation at 37 °C for 2 h. In some groups, ^{125}I-glycosylated BSA was administered, together with different concentrations of mannosylated, fucosylated or galactosylated BSA. After 2 h incubation, the medium

Table 1
Physicochemical characteristics of synthesized glycosylated BSAs

Compounds	Molecular weight (Da)	Number of sugar residues (mol/mol BSA)
Mannosylated BSA (Man-BSA)	70800	36
Fucosylated BSA (Fuc-BSA)	70800	41
Galactosylated BSA (Gal-BSA)	68000	42

The number of molecular weight of glycosylated BSAs were estimated by SDS-PAGE. The number of sugar residue was determined by the anthron-sulfic method.

was collected, mixed with 45% trichloroacetic acid (TCA), and centrifuged to separate metabolites from intact glycosylated BSA. The radioactivity associated with metabolites in the supernatant was measured using a well-type NaI-scintillation counter (ARC-500; Aloka, Tokyo, Japan). In addition, the cells were washed three times with ice-cold phosphate-buffered saline and lysed with 0.25 ml of 0.3 M NaOH containing 0.1% Triton X-100. An aliquot of the lysate was taken for the determination of ^{125}I radioactivity and protein content. The radioactivity was counted by the scintillation counter while the protein content was measured by the modified Lowry method (Lowry et al., 1951) using BSA as a standard. The result of in vitro uptake experiment was expressed as total uptake amount of count of metabolites in the supernatant and of cell lysate.

2.7. *In vivo hepatic disposition in mice pretreated with $GdCl_3$*

An amount of 30 mg/kg $GdCl_3$ was intravenously injected into mice to down-regulate the functions of Kupffer cells (Hardonk et al., 1992). Twenty-four hours later, ^{111}In-mannosylated, ^{111}In-fucosylated or ^{111}In-galactosylated BSA (0.1 mg/kg) was administered by intravenous injection. At 5 min post injection, mice were killed under anesthesia and the liver was excised and plasma was collected. The radioactivity associated with these samples was determined in a well-type NaI-scintillation counter (ARC-500; Aloka, Tokyo, Japan).

3. Results

3.1. *Uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA by sinusoidal endothelial cells and Kupffer cells*

Fig. 1 shows the uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA in cultured sinusoidal endothelial cells. Without inhibitors, uptake of mannosylated BSA by sinusoidal endothelial cell was 1.4-fold higher than that of fucosylated BSA. In the presence of 50 mg/ml mannosylated or fucosylated BSA, the uptake of ^{125}I-mannosylated BSA was significantly inhibited (Fig. 1A). However, the uptake of ^{125}I-mannosylated BSA was not inhibited by the same concentration of galactosylated BSA (Fig. 1A). Similar inhibition patterns were observed with ^{125}I-fucosylated BSA (Fig. 1B).

Fig. 2 shows the uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA in cultured Kupffer cells. In the absence of inhibitors, ^{125}I-mannosylated and ^{125}I-fucosylated were similar with respect to the degree of uptake by Kupffer cells, and their uptake was significantly greater than that by cultured endothelial cells. The uptake of ^{125}I-mannosylated BSA was inhibited by mannosylated and fucosylated BSAs, but not by galactosylated BSA, similar to that observed in cultured endothelial cells. In contrast, the uptake of ^{125}I-fucosylated BSA was inhibited by galactosylated BSA, as well as mannosylated and fucosylated BSAs. Thus, fucosylated BSA exhibits different uptake characteristics from mannosylated BSA in Kupffer cells.

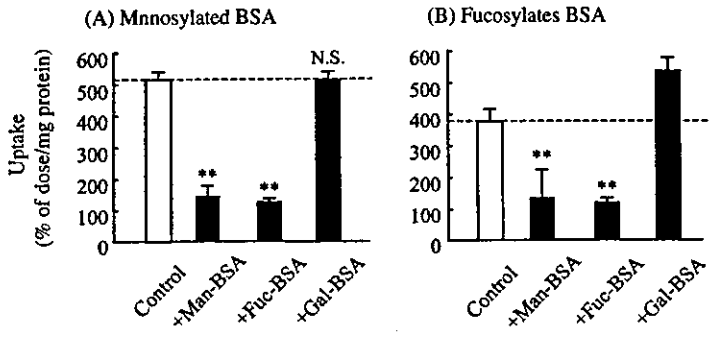

Fig. 1. The uptake of ^{125}I-mannosylated BSA (A), and ^{125}I-fucosylated BSA (B) by cultured endothelial cells. Cells were incubated for 2 h with or without an excess of other forms of glycosylated BSA (50 μg/ml). Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$; N.S., not significant).

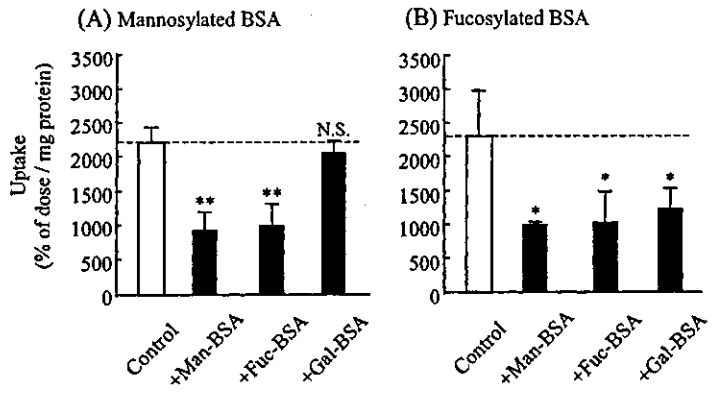

Fig. 2. The uptake of ^{125}I-mannosylated BSA (A), and ^{125}I-fucosylated BSA (B) by cultured Kupffer cells. Cells were incubated for 2 h with or without an excess of other forms of glycosylated BSA (50 μg/ml). Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$; *, $P < 0.05$; N.S., not significant).

3.2. *Liver accumulation of ^{111}In-mannosylated and ^{111}In-fucosylated BSA with or without $GdCl_3$ pretreatment*

^{111}In-labeled BSA derivatives were used in the in vivo distribution studies, since ^{111}In is trapped in the cell interior after degradation so that the uptake rate can be evaluated precisely (Hnatowich et al., 1982). Fig. 3 shows the plasma concentration and hepatic uptake of ^{111}In-mannosylated and ^{111}In-fucosylated BSAs 5 min after intravenous injection. When mice were pretreated with $GdCl_3$, the plasma clearance and hepatic uptake of these proteins significantly decreased, and the inhibition effect was greater with fucosylated BSA than mannosylated BSA. Since $GdCl_3$ completely inhibits binding of mannosylated and fucosylated BSA to Kupffer cells without adversely affecting either hepatocytes or sinusoidal endothelial cells (Hardonk et al., 1992), it appears that the contribution of Kupffer cells to uptake by whole liver is greater in the case of fucosylated BSA.

4. Discussion

Haltiwanger and Hill (1986) isolated macrophage mannose receptors from alveolar macrophages, and showed that these receptors recognize mannose, fucose, and *N*-acetylglucosamine but not galactose. Otter et al. (1992) found that mannose receptors

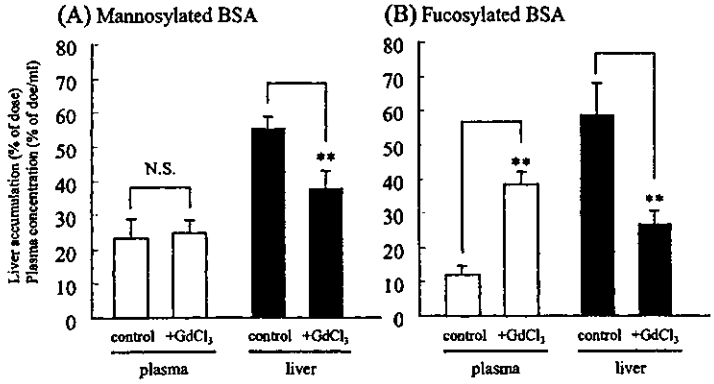

Fig. 3. Liver accumulation (■) and plasma concentration (□) of ^{111}In-mannosylated BSA (A), and ^{111}In-fucosylated BSA (B) (0.1 mg/kg) with or without pretreatment of $GdCl_3$ (30 mg/kg) at 5 min after intravenous injection into mice. $GdCl_3$ was injected 24 h before the experiments. Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$).

exhibiting the same substrate specificity are expressed in liver NPC. Magnusson and Berg (1993) reported that mannose receptors are expressed on isolated sinusoidal endothelial cells in binding experiments using an isolated sinusoidal endothelial cell suspension and ovalbumin, a mannose-terminated glycoprotein. In the present study, mannosylated and fucosylated BSAs were taken up efficiently by liver sinusoidal endothelial cells in primary culture, where the uptake was significantly inhibited by mannosylated and fucosylated BSAs but not by galactosylated BSA (Fig. 1). Therefore, it is likely that uptake of mannosylated and fucosylated BSAs by cultured sinusoidal endothelial cells is mediated primarily by mannose receptors.

Cultured Kupffer cells and sinusoidal endothelial cells exhibited similar inhibition profiles for mannosylated BSA, but not for fucosylated BSA: the uptake of fucosylated BSA was significantly inhibited by galactosylated BSA in Kupffer cells (Fig. 2B). Lehrman and co-workers isolated fucose receptors (Kupffer cell lectin) that are uniquely expressed in Kupffer cells by an immunohistological method (Lehrman and Hill, 1986), and demonstrated that binding of fucosylated BSA to these receptors can be inhibited by excess fucose or galactose, but not by mannose (Lehrman et al., 1986). In the present study, the uptake of fucosylated BSA was inhibited by galactosylated BSA (Fig. 2B), suggesting that fucose receptors might, at least in part, be responsible for the uptake of fucosylated BSA. In addition, mannose receptors also appear to be responsible for the uptake of fucosylated BSA, since the uptake of fucosylated BSA is inhibited by mannosylated BSA (Fig. 2B).

The present result for fucosylated BSA in Kupffer cells differed from that of Sarkar et al. (1996), who demonstrated that the uptake of fucose–human serum albumin (HSA) by isolated Kupffer cells was not inhibited by galactose–HSA. This difference might be due to the method of fucosylation: Sarkar et al. (1996) coupled *p*-aminophenyl-α-L-fucopyranoside and albumin with carbodiimide whereas we coupled albumin with 2-imino-2-methoxyethyl-1-thio-L-fucoside under weak alkaline conditions. Thus, it is likely that the difference in the structure at the 1-position of fucose might affect recognition of fucosylated albumin by Kupffer cell lectin that interacts with galactose. Glycosylation with 2-imino-2-methoxyethyl-1-thioglycoside is also characteristic in that the net charge of the proteins is unchanged since an imine is formed at the amino group modified with sugar. Jansen et al. (1991) demonstrated that para-aminophenyl mannose-terminated HSA was taken up by NPC via scavenger receptors, presumably due to an increase in negativity in accordance with sugar modification of the amino groups. However, they also showed that the liver uptake of mannosylated HSA synthesized using 2-imino-2-methoxyethyl-1-thiomannoside was not inhibited by formaldehyde-treated albumin, a typical scavenger receptor ligand (Jansen et al., 1991). Thus, the chosen method of sugar modification could play an important role in the design of glycosylated proteins.

We previously found that the cellular uptake of fucosylated BSA at 1 h post intravenous injection was 1.1, 6.2, and 21% of dose/10^8 cells for liver parenchymal cells, sinusoidal endothelial cells, and Kupffer cells, respectively (Opanasopit et al., 2001b), while that of mannosylated BSA was 0.841, 13.4, and 10.4% of dose/10^8 cells (Ogawara et al., 1999). To confirm the contribution of each cell type to the total hepatic uptake from a different point of view, we investigated the in vivo distribution of mannosylated and fucosylated BSAs in mice pretreated with $GdCl_3$ (Fig. 3). It is known that $GdCl_3$ completely inhibits the binding of mannosylated and fucosylated BSA to Kupffer cells without adversely affecting either hepatocytes or sinusoidal endothelial cells (Hardonk et al., 1992). The reduction in the hepatic uptake produced by $GdCl_3$ pretreatment was greater for fucosylated BSA than mannosylated BSA (Fig. 3). This finding corresponded to previous results that fucosylated BSA was taken up more selectively by Kupffer cells than mannosylated BSA (Opanasopit et al., 2001b; Ogawara et al., 1999). The in vitro uptake experiment (Figs. 1 and 2) showed that the uptake by cultured sinusoidal endothelial cells was greater for mannosylated BSA than fucosylated BSA whereas the uptake by cultured Kupffer cells was similar for both proteins. Thus, the higher Kupffer cell-selectivity of fucosylated BSA might be due to lower uptake by the sinusoidal endothelium. When the data obtained following $GdCl_3$ pretreatment were compared with the data obtained from cell fractionation studies, account was taken of the fact that the number of sinusoidal endothelial cells was approximately twice that of Kupffer cells (Blomhoff et al., 1982; Pertoft and Smedsrod, 1986).

5. Concluding remarks

Different carbohydrate receptors with relatively broad substrate specificity are present in different hepatic cell types. Mannosylated BSA appears to be taken up via mannose receptors regardless of sinusoidal endothelial cells and Kupffer cells. However, it is likely that the uptake of fucosylated BSA by Kupffer cells is mediated by not only mannose receptors but fucose receptors while its uptake by sinusoidal endothelial cells is mediated primarily by mannose receptors. Moreover, fucosylated BSA is more Kupffer cell-selective because it exhibited a lower endothelial cell uptake than mannosylated BSA. This is potentially useful information for the development of carbohydrate receptor-mediated delivery strategies for biologically active proteins.

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Biodistribution Characteristics of Galactosylated Emulsions and Incorporated Probucol for Hepatocyte-Selective Targeting of Lipophilic Drugs in Mice

Emi Ishida,[1] Chittima Managit,[1] Shigeru Kawakami,[1] Makiya Nishikawa,[2] Fumiyoshi Yamashita,[1] and Mitsuru Hashida[1,3]

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Purpose. Galactosylated emulsions containing cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butly)formamide (Gal-C4-Chol) as a "homing device" were developed for hepatocyte-selective drug targeting. The targeting efficiency of galactosylated emulsions was evaluated by a distribution study in mice.

Methods. Soybean oil/EggPC/cholesterol (Chol) (weight ratio, 70:25:5) (bare) emulsions and soybean oil/EggPC/Gal-C4-Chol (weight ratio, 70:25:5) (Gal) emulsions were prepared and labeled with [^{3}H]cholesteryl hexadecyl ether (CHE). [^{14}C]probucol as a model lipophilic drug was incorporated in the emulsions or EggPC/Chol/Gal-C4-Chol (Gal) liposomes. Their tissue and intrahepatic distribution were evaluated following intravenous injection in mice.

Results. After intravenous injection, Gal-emulsions were rapidly eliminated from the blood and accumulated in the liver, in contrast to the bare-emulsions. The liver uptake clearance of Gal-emulsions was 3.2- and 1.2-times greater than that of bare-emulsions and Gal-liposomes, respectively. The uptake ratio in liver parenchymal cells (PC) and nonparenchymal cells (NPC) of Gal-emulsions was higher than that of Gal-liposomes, being 7.4 and 3.0, suggesting that Gal-emulsions are an effective PC-selective carrier. The hepatic uptake of Gal-emulsions, but not that of bare-emulsions, was significantly inhibited by the pre-dosing of not only lactoferrin but also Gal-liposomes, suggesting asialoglycoprotein receptor–mediated endocytosis. Furthermore, [^{14}C]probucol incorporated in Gal-emulsions was efficiently delivered to the liver compared with Gal-liposomes.

Conclusion. Gal-emulsions have been proven to be an alternative carrier for hepatocyte-selective drug targeting.

KEY WORDS: galactosylated emulsions; hepatocytes; lipophilic drug; targeting.

INTRODUCTION

Receptor-mediated drug targeting is a promising approach to cell-selective drug delivery (1). One particular method exploits the sugar recognition mechanisms that specific cell types possess. Receptors for carbohydrates, such as the asialoglycoprotein receptor on hepatocytes and the mannose receptor on several macrophages and liver endothelial cells, recognize the corresponding sugars on the nonreducing terminal of sugar chains. Recently, we synthesized a novel galactosylated cholesterol derivative, that is, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol), to modify liposomes with galactose moieties for hepatocytes drug targeting (2). However, for certain lipophilic drugs, liposomal carrier systems are of limited use for delivery because of their restricted solubilizing capacity. Even though a drug carrier exhibits a favorable *in vivo* disposition profile, limited solubility of the incorporated drugs may lead to failure in achieving sufficient therapeutic efficacy.

Lipid emulsions are considered to be superior to liposomes due to the fact that they can be produced on an industrial scale, are stable during storage, are highly biocompatible, and have a high solubilizing capacity as far as lipophilic drugs are concerned (3,4) because lipid emulsions possess an oil phase in particulate form, so that it can dissolve large amounts of highly lipophilic drugs. In this context, lipid emulsions have widely been used as drug carriers, especially as long-circulating drug carriers for passive targeting (5–7). Cell-specific drug targeting is sometimes urgently required for a variety of clinical purposes; however, there are few reports of cell-specific drug targeting using lipid emulsions. Recently, Rensen *et al.* developed novel apo E–associated emulsions for hepatocytes targeting (8,9). These apo E–associated emulsions are reported to be selectively taken up by liver parenchymal cells and are useful for delivery of antiviral drugs, such as iododeoxyuridine, to hepatocytes. However, introduction of apo E to the carrier is rather complicated, and so there can be problems as far as the reproducibility and stability of apo E emulsions are concerned. The lipid emulsion (oil-in-water) surface exhibits aqueous properties; thus, a galactose moiety could be covered on the emulsion surface when Gal-C4-Chol was added because galactose is a hydrophilic molecule, and so the galactose moiety would be fixed on the emulsions surface.

The purpose of this study was to elucidate the biodistribution characteristics of galactosylated (Gal-) emulsions after intravenous administration as a novel drug carrier to hepatocytes. Then, we examined the applicability of probucol, which is a model lipophilic drug, to investigate the usefulness of the drug carrier. The targeting efficiency of probucol incorporated in emulsions was compared with that in EggPC/Chol/Gal-C4-Chol liposomes, which is the optimized lipid composition for the targeted delivery of probucol by Gal-liposomes (10). [^{3}H]Cholesteryl hexadecyl ether (CHE) was used as an emulsion marker (11).

MATERIALS AND METHODS

Chemicals

N-(4-aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate was obtained from Sigma Chemicals (St. Louis, MO, USA). Egg phosphatidylcholine (EggPC) and soybean oil were purchased from Wako Pure Chemicals Industry Ltd. (Osaka, Japan). Cholesterol (Chol) and Clear-Sol I were purchased from Nacalai Tesque (Kyoto, Japan). Soluene 350 was obtained from Packard (Groningen, The Netherlands). [^{3}H]Cholesteryl hexadecyl ether (CHE) was pur-

[1] Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.

[2] Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.

[3] To whom correspondence should be addressed. (e-mail: hashidam@pharm.kyoto-u.ac.jp)

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chased from NEN Life Science Products Inc. (Boston, MA, USA). [^{14}C]Probucol was purchased from Daiichi Radioisotopes (Tokyo, Japan). All other chemicals were of the highest purity available.

Synthesis of Gal-C4-Chol

Gal-C4-Chol was synthesized by the method described previously (2). Briefly, cholesteryl chloroformate was reacted with *N*-(4-aminobutyl) carbamic acid *tert*-butyl ester in chloroform for 24 h at room temperature and then incubated with trifluoroacetic acid for 4 h at 4°C. *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide was obtained after evaporation of the solvent. A quantity of the resultant material was added to an excess of 2-imino-2-methoxyethyl-1-thiogalactoside (12) in pyridine containing triethylamine. After 24 h incubation at room temperature, the reaction mixture was evaporated, resuspended in water, and dialyzed against distilled water for 48 h using a semipermeable membrane (12 kDa cutoff). Finally, the dialyzate was lyophilized.

Preparation of Emulsions and Liposomes

Bare-emulsions consisted of soybean oil, EggPC, and Chol at a weight ratio of 70:25:5. Gal-emulsions contained soybean oil, EggPC, and Gal-C4-Chol at a weight ratio of 70:25:5. Bare-liposomes consisted of EggPC and Chol at a molar ratio of 60:40. Gal-liposomes consisted of EggPC, Chol, and Gal-C4-Chol at a molar ratio of 60:35:5. The lipid mixture was dissolved in chloroform, vacuum-desiccated, and resuspended in 5 ml sterile phosphate-buffered saline (pH 7.4). The suspension was sonicated for 20 min (200 W) under a current of nitrogen. As for liposomes, after hydration, the suspension was sonicated for 3 min (200 W), and the resulting liposomes were passed through 200-nm (5 times) and 100-nm (5 times) polycarbonate membrane filters using an extruder device. The concentration of the emulsions and liposomes was adjusted to 0.5% based on radioactivity measurement so that the total EggPC, Chol, and Gal-C4-Chol content was equivalent to 0.5 g per 100 ml. Radiolabeling of the emulsions and liposomes was performed by addition of [^{3}H]CHE (500 μCi) and/or [^{14}C]probucol (50 μCi) with probucol (13.8 μg) to the lipid mixture before formation of a thin film layer. [^{14}C]Probucol dissolved serum was prepared by addition of mouse serum into a thin film of [^{14}C]probucol (50 μCi) and probucol (13.8 μg). [^{14}C]Probucol dissolved serum was then filtrated through a Mullex HV sterile filter (Millipore, Bedford, USA) before the animal experiments. The particle sizes of the emulsions and liposomes without radioisotope were measured in a dynamic light-scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan). The zeta potential of emulsions and liposomes without radioisotope was measured electrophoretically using a zeta-potential analyzer (LEZA-500T, Otsuka Electronics). The density of Gal-C4-Chol on emulsions and liposomes was determined by calculating the galactose content of Gal-emulsions and liposomes using the anthrone sulfuric acid method (13).

In vivo Distribution

Five-week-old male ddY mice (25.0–30.0 g) were obtained from 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 U.S. National Institutes of Health and the Guideline for Animal Experiments of Kyoto University. [^{3}H]CHE (1.0 μCi/100 μl) and/or [^{14}C]probucol (0.1 μCi/100 μl)-labeled emulsions or liposomes were injected into the tail vein of mice at a dose of 5 mg/kg. In the hepatic uptake inhibition experiments, various compounds were intravenously injected 1 min prior to the intravenous injection of emulsions or liposomes. At given times, blood was collected from the vena cava under anesthesia and mice were then sacrificed. The liver, kidney, spleen, heart, and lung were removed, washed with saline, blotted dry, and weighed. A complete urine collection was obtained by combining the excreted urine and that remaining in the bladder. Ten microliters of blood, 200 μl of urine, and a small amount of each tissue were digested with 0.7 ml Soluene-350 by incubating the samples 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 the radioactivity was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

Hepatic Cellular Localization

The separation of liver parenchymal cells and nonparenchymal cells was performed according to collagenase perfusion method (14). Briefly, mice were anesthetized with pentobarbital sodium (40–60 mg/kg) and given an intravenous injection of [^{3}H]CHE (0.5–1.0 μCi/100 μl)-labeled emulsions or liposomes. The body temperatures were kept at 37°C with a heat lamp during the experiment. Then, 30 min after administration, the liver was perfused first with Ca^{2+}, Mg^{2+}-free perfusion buffer [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 137 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, and 0.4 mM Na_2HPO_4, pH 7.2] for 10 min followed by perfusion buffer supplemented with 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I; pH 7.5) for 10 min. As soon as the perfusion started, the vena cava and aorta were cut, and the perfusion rate was maintained at 3–4 ml/min. Following the discontinuation of perfusion, the liver was excised, and its capsular membranes were removed. The cells were dispersed by gentle stirring in ice-cold Hank's-HEPES buffer containing 0.1% BSA. The dispersed cells were filtered through cotton mesh sieves, followed by centrifugation at 50 × *g* for 1 min. The pellets containing parenchymal cells (PC) were washed twice with Hank's-HEPES buffer by centrifuging at 50 × *g* for 1 min. The supernatant containing nonparenchymal cells (NPC) was similarly centrifuged twice. The resulting supernatant was then centrifuged twice at 200 × *g* for 2 min. PC and NPC were resuspended separately in ice-cold Hank's-HEPES buffer (4 ml for PC and 1.8 ml for NPC). The cell numbers and viability were determined by the trypan blue exclusion method. Then, the radioactivity in the cells (0.5 ml) was determined as for the other tissue samples.

Calculation of Organ Clearance

Tissue distribution data were evaluated using the organ distribution clearances as reported previously (15). Briefly, the tissue uptake rate can be described by the following equation:

$$\frac{dX_t}{dt} = CL_{uptake} \cdot C_b \quad (1)$$

where X_t is the amount of [^{3}H]-labeled emulsions or liposomes in the tissue at time t, CL_{uptake} is the tissue uptake clearance, and C_b is the blood concentration of [^{3}H]-labeled emulsions or liposomes. Integration of Eq. (1) gives

$$X_t = CL_{uptake} \cdot AUC_{(0-t)} \quad (2)$$

where $AUC_{(0-t)}$ represents the area under the blood concentration-time curve from time 0 to t. The CL_{uptake} value can be obtained from the initial slope of a plot of the amount of [^{3}H]-labeled emulsions or liposomes in the tissue at time t (X_t) vs. the area under the blood concentration-time curve from time 0 to t [$AUC_{(0-t)}$].

Statistical Analysis

Statistical comparisons were performed using Student's unpaired t test. $p < 0.05$ was considered to be indicative of statistical significance.

RESULTS

Physicochemical Properties of Emulsions

Sinusoids in the liver lobules have a unique type of endothelial lining consisting of endothelial cells with flattened processes perforated by small fenestrae about 100 nm in size (16). Therefore, emulsions and liposomes with a diameter less than this can readily pass through the fenestration into the Disse space. Accordingly, we prepared emulsions and liposomes less than 100 nm in diameter in order to allow free access to hepatocytes.

Table I summarizes the lipid composition, particle sizes, and zeta potential of the emulsions and liposomes prepared. These emulsions and liposomes were very similar in size with a mean diameter of approximately 100 nm. As shown by the zeta potential, the surface charge of each particle was almost neutral. In addition, the particle size and zeta potential of the emulsions and liposomes were kept constant for a period of at least 2 months at 4°C (data not shown).

Biodistribution of [^{3}H]-Labeled Emulsions

[^{3}H]CHE was selected as a tracer of emulsion (11,17) and liposomes (10,11). Figures 1 and 2 show the blood concentration-, tissue accumulation-, and urine excretion-time course of [^{3}H]-labeled soybean oil/EggPC/Chol (70:25:5) (bare) emulsions, soybean oil/EggPC/Gal-C4-Chol (70:25:5) (Gal) emulsions, EggPC/Chol (60:40) (bare) liposomes, and EggPC/Chol/Gal-C4-Chol (60:35:5) (Gal) liposomes after intravenous injection. In contrast to the bare-emulsions, Gal-emulsions were rapidly eliminated from the blood circulation and mostly recovered in the liver, accounting for 75% of the dose, within 30 min. Gal-liposomes, which have the same Gal-C4-Chol density as that of Gal-emulsions, were also rapidly eliminated from the blood circulation and mostly recovered in the liver, accounting for 60% of the dose, within 30 min.

Pharmacokinetic Analysis of Biodistribution of [^{3}H]-Labeled Emulsions

To compare the disposition profiles of emulsions and liposomes, initial distribution in the early phase up to 10 min, in which the contribution of metabolites can be ignored, was quantified using tissue uptake clearances parameter.

Table II summarizes the area under blood concentration-time curve (AUC) and tissue uptake clearances calculated for the initial 10 min for liver (CL_{liver}), kidney (CL_{kidney}), spleen (CL_{spleen}), lung (CL_{lung}), heart (CL_{heart}), and urine (CL_{urine}) of the [^{3}H]-labeled emulsions and liposomes. The AUC of Gal-emulsions was much lower than that of bare-emulsions. The liver uptake clearance of Gal-emulsions was 3.2-times greater than that of bare-emulsions. Also, the liver uptake clearance of Gal-emulsions was 1.2-times higher than that of Gal-liposomes.

Effect of Gal-C4-Chol Content of Emulsions on Hepatic Accumulation

The amounts of [^{3}H]-labeled emulsions recovered in the liver at 30 min after intravenous injection of Gal-emulsions containing various amounts of Gal-C4-Chol was evaluated. The density of Gal-C4-Chol in the emulsions was calculated to be 3.0×10^{12}, 8.9×10^{12}, and 1.5×10^{13}/unit surface area (cm^2) for Gal-emulsions containing, respectively, a 1%, 3%, and 5% weight ratio of Gal-C4-Chol. All emulsions prepared have almost similar particle sizes (data not shown). As shown in Fig. 3, the liver accumulation of Gal-emulsions increased with the amount of Gal-C4-Chol in the emulsions. The relationship between the liver accumulation and the galactose density of the emulsions on the emulsions surface correlates well, suggesting that the galactose density on the surface of the emulsions is important as far as recognition by the asialoglycoprotein receptors on hepatocytes is concerned.

Table I. Lipid Composition, Mean Diameter, and Zeta Potential of the Tested Emulsions and Liposomes

Formulations	Lipid composition	Mean diameter[a] (nm)	Zeta potential[b] (mV)
Bare-emulsions	Soybean oil:EggPC:Chol (70:25:5) (weight ratio)	100.0 ± 2.3	4.0 ± 1.4
Gal-emulsions	Soybean oil:EggPC:Gal-C4-Chol (70:25:5) (weight ratio)	104.5 ± 3.8	4.7 ± 0.5
Bare-liposomes	EggPC:Chol (60:40) (molar ratio)	93.3 ± 11.2	4.4 ± 0.2
Gal-liposomes	EggPC:Chol:Gal-C4-Chol (60:35:5) (molar ratio)	96.2 ± 5.8	9.0 ± 1.7

EggPC, egg phosphatidylcholine; Chol, cholesterol.

[a] Diameter of emulsions and liposomes was measured by dynamic light-scattering spectrophotometry. Results are expressed as the mean ± SD of three experiments.

[b] Zeta potential of emulsions and liposomes was measured by electrophoretic light-scattering spectrophotometry. Results are expressed as the mean ± SD of three experiments.

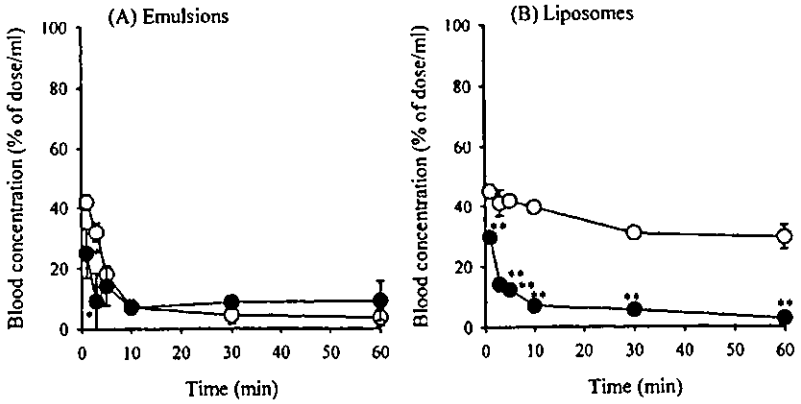

Fig. 1. Blood concentration of [^{3}H]-labeled (A) bare-(○) and Gal-(●) emulsions and (B) bare-(○) and Gal-(●) liposomes after intravenous injection into mice. Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

Hepatic Cellular Localization of [^{3}H]-Labeled Emulsions

Figure 4 shows the hepatic cellular localization of [^{3}H]-labeled emulsions and liposomes 30 min after intravenous injection. Compared with bare-emulsions, Gal-emulsions accumulated selectively in PC with a PC/NPC ratio of 7.4. Moreover, Gal-liposomes also accumulated selectively in PC with a PC/NPC ratio of 3.0. Thus, the PC selectivity of the Gal-emulsions is higher than that of Gal-liposomes.

Inhibition of Hepatic Uptake of Emulsions by Pre-dosing Various Agents

Figure 5 shows the effect of pre-dosing with various agents on the hepatic accumulation of [^{3}H]-labeled bare- and Gal-emulsions. The liver uptake of Gal-emulsions was significantly inhibited by pre-dosing lactoferrin, which is a ligand of chylomicron remnant receptors on liver parenchymal cells, and Gal-liposomes, which contain a ligand for asialoglycopro-

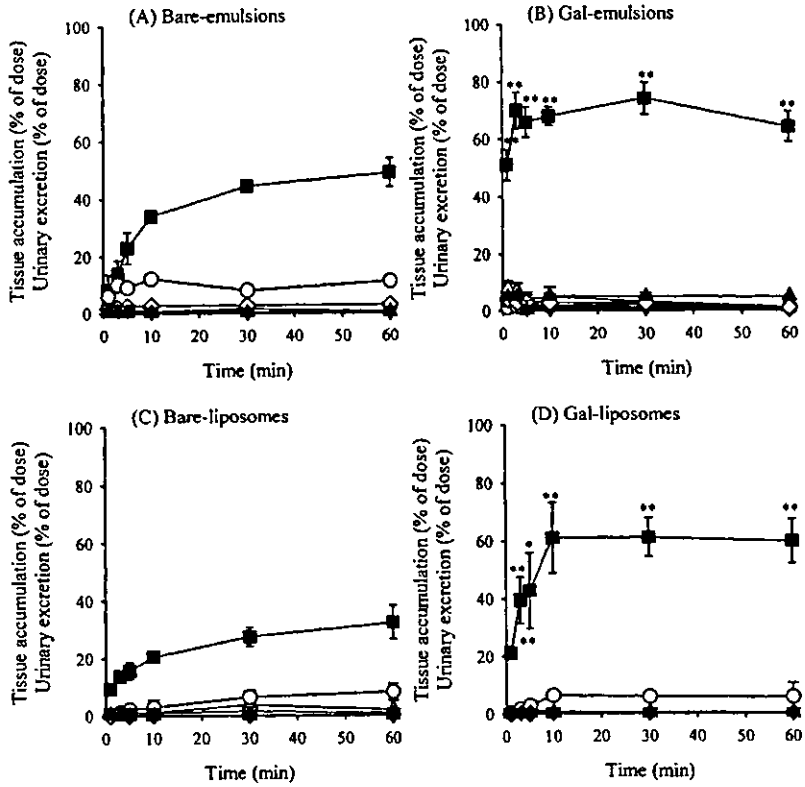

Fig. 2. Tissue accumulation of [^{3}H]-labeled (A) bare-emulsion, (B) Gal-emulsion, (C) bare-liposomes, and (D) Gal-liposomes after intravenous administration into mice. Radioactivity was determined in the liver (■), kidney (△), spleen (○), lung (▽), heart (◇), and urine (▲). Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from each bare emulsion (A vs. B) or bare liposomes (C vs. D).

Table II. Area Under the Blood Concentration-Time Curve (AUC) and Tissue Uptake Clearance of [^{3}H]-Labeled Emulsions and Liposomes After Intravenous Injection Into Mice[a]

Formulations	AUC (% of dose × h/ml)	Clearance[a] (μl/h)					
		CL_{liver}	CL_{kidney}	CL_{spleen}	CL_{lung}	CL_{heart}	CL_{urine}
Bare-emulsions	3.7	9480	201	570	179	31	3
Gal-emulsions	1.9	31,200	1350	1040	239	705	1360
Bare-liposomes	7.0	2960	121	407	152	102	6
Gal-liposomes	2.4	25,300	313	2610	129	76	67

[a] AUC and clearance (CL) were calculated for the period until 10 min after injection. An average of three experiments is shown.

tein receptors on liver parenchymal cells, whereas no inhibition was observed in the case of bare-emulsions.

Blood Elimination and Hepatic Accumulation of [^{14}C]Probucol Incorporated into [^{3}H]-Labeled Gal-Emulsions and Gal-Liposomes

Figure 6 shows the blood concentration and liver accumulation of [^{14}C]probucol dissolved in serum, which represents the original distribution of probucol, [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions and Gal-liposomes after intravenous injection. [^{14}C]Probucol was dissolved in serum to analyze the inherent distribution of probucol to investigate the controlled distribution of probucol by Gal emulsions and liposomes. Rapid blood elimination of [^{14}C]probucol was observed in Gal-emulsions, followed by Gal-liposomes. Similarly, the fastest blood elimination of [^{3}H]CHE was observed in Gal-emulsions, followed by Gal-liposomes. As for the liver accumulation, rapid liver uptake of [^{14}C]probucol was observed in Gal-emulsions followed by Gal-liposomes. Similarly, the highest hepatic uptake of [^{3}H]CHE was observed in Gal-emulsions, followed by Gal-liposomes.

The Uptake Clearance of [^{14}C]Probucol Incorporated into [^{3}H]-Labeled Gal-Emulsions and Gal-Liposomes

Table III summarizes the AUC and tissue uptake clearances of [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions and Gal-liposomes. The lowest AUC was observed in Gal-emulsions followed by Gal-liposomes. The liver uptake clearance of [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions was 1.6-times higher than that into Gal-liposomes.

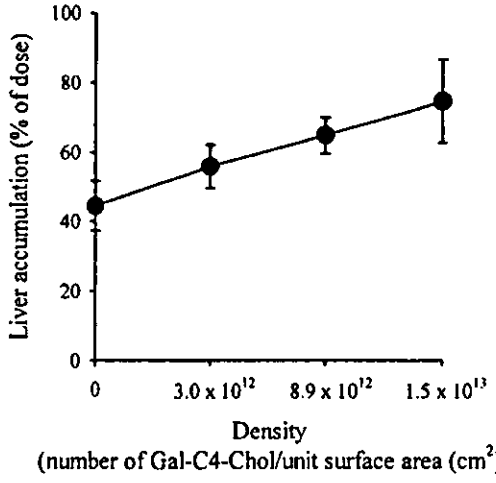

Fig. 3. Liver accumulation of [^{3}H]-labeled Gal-emulsions 30 min after intravenous administration into mice. Gal-emulsions were prepared with various amounts of Gal-C4-Chol. Each value represents the mean ± SD of three experiments.

DISCUSSION

This manuscript summarizes our initial efforts to investigate whether Gal-emulsions would offer a significant advantage as an alternative carrier for drug targeting to hepatocytes. Several investigators have demonstrated that the liposomes that were modified with native glycoproteins (18,19) or synthetic glycolipids possessing tris-galactosides (20) were efficiently recognized by asialoglycoprotein receptors. However, there are several potential problems in using these compounds due to their complicated structures, difficulty in achieving industrial-scale production, and possible antigenicity. Recently, we synthesized Gal-C4-Chol having bifunctional properties of a lipophilic anchor moiety for stable incorporation into liposomes and a galactose moiety for recognition by the asialoglycoprotein receptors (21). Our strategy for efficient targeting of liposomes by glycosylation is to achieve stable fixation of the sugar moiety on the surface of the liposomes under *in vivo* conditions. Therefore, cholesterol was chosen as a hydrophobic anchor, which is stably associated with the liposomal membrane (22,23), and only monogalactoside was introduced to the lipid as a ligand because introduction of many hydrophilic galactose moieties to a lipid anchor would result in their removal from liposomes by interaction with lipoproteins and/or other lipid compartments under *in vivo* conditions (20). In fact, we found that eggPC or

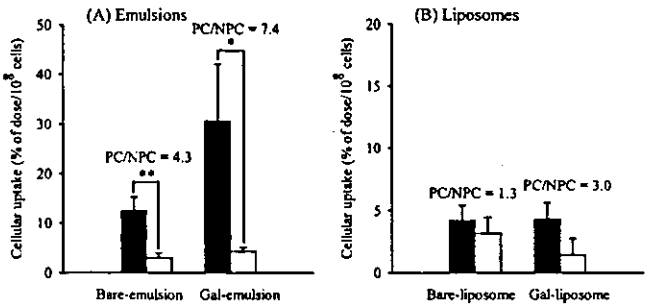

Fig. 4. Hepatic cellular localization of (A) [^{3}H]-labeled emulsions and (B) [^{3}H]-labeled liposomes after intravenous administration into mice. Radioactivity was determined 30 min postinjection in PC (■), and NPC (□). Each value represents the mean + SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

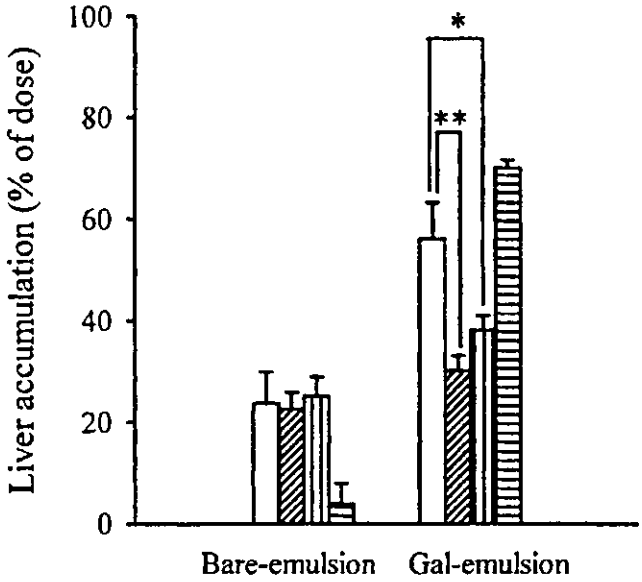

Fig. 5. Inhibition of liver uptake of [^{3}H]-labeled emulsions after intravenous preinjection of various compounds. Liver accumulation was determined at 5 min. Emulsions were injected without (□) or with preinjection of lactoferrin (▨), Gal-liposomes (▥), or an excess of bare-emulsions (▤). Each value represents the mean + SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

DSPC/Chol/Gal-C4-Chol (Gal-) liposomes were rapidly taken up by hepatocytes via asialoglycoprotein receptor–mediated endocytosis (10,21). Thus, the Gal-C4-Chol combination with soybean oil, EggPC, and Chol gave emulsion formulations with suitable pharmaceutical characteristics for targeting under *in vivo* conditions.

As shown in Figs. 2 and 4, [^{3}H]-labeled Gal-emulsions exhibited marked hepatic uptake and rather high PC/NPC ratios, suggesting that Gal-emulsions were more efficiently taken up into hepatocytes compared with bare-emulsions. To investigate the uptake mechanism of Gal-emulsions, we performed a competitive inhibition experiment involving predosing lactoferrin, Gal-liposomes, and bare-emulsions (Fig. 5). Pre-dosing lactoferrin, which is a ligand of chylomicron remnant receptors on liver parenchymal cells (24), significantly inhibited the liver uptake of Gal-emulsions. It has also been reported that lactoferrin can bind to asialoglycoprotein receptors on liver parenchymal cells (24,25); therefore, this result does not make it clear whether the uptake of Gal-emulsions involves chylomycron remnant receptor– or asialoglycoprotein receptor–mediated endocytosis. However, the liver uptake of Gal-emulsions was also markedly inhibited by Gal-liposomes, which contain a ligand of asialoglycoprotein receptors (10), but was not inhibited by bare-emulsions (Fig. 5). Taking these findings into consideration, this suggests that Gal-emulsions were taken up by asialoglycoprotein-mediated endocytosis after intravenous administration.

We have established methods for introducing galactose moieties directly into various molecular species and developed various macromolecular drug carrier systems (26,27), protein derivatives (28,29), and liposomes (30–33) that show superior liver targeting via asialoglycoprotein receptor–mediated endocytosis. As far as the molecular design of the galactosylated protein is concerned, we have demonstrated that the recognition of galactosylated protein by the liver cells is based on the estimated surface density of the galactose residues (34). In this study, we showed that the galactose density on the surface of the Gal-emulsions is an important factor for recognition by the asialoglycoprotein receptors on hepatocytes, suggesting that enhancing the recognition by asialoglycoprotein receptors can be controlled by the amount of added Gal-C4-Chol. This observation correlates well with the effect of the galactose density of galactosylated liposomes, which possess a mono-galactoside, that have been studied by Murahashi *et al.* (35).

In order to achieve drug targeting by a cell-selective approach, the drug release properties from emulsions are important for drug targeting, and so we investigated the relationship between the movement of Gal-emulsions and incorporated probucol. We previously investigated the *in vivo* disposition of drugs with a variety of lipophilicities, incorporated into liposomes, lipid emulsions, and micelles, based on

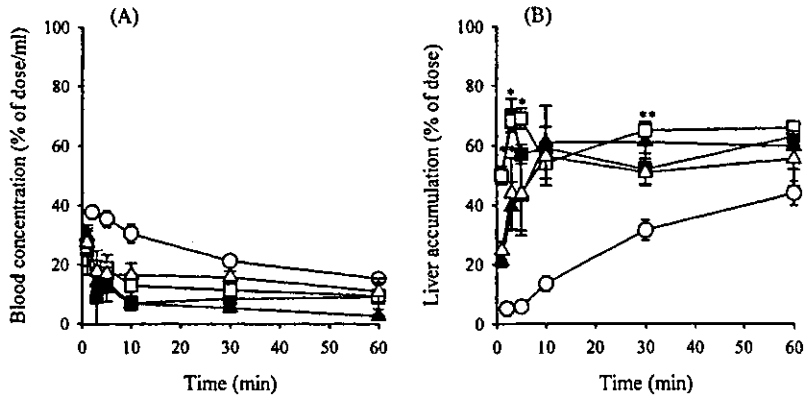

Fig. 6. (A) Blood concentration and (B) liver accumulation of [^{14}C]-labeled probucol dissolved in serum (○) or [^{3}H]CHE-labeled Gal-emulsions (■) and [^{3}H]CHE-labeled Gal-liposomes (▲) or [^{14}C]-labeled probucol incorporated Gal-emulsions (□) and [^{14}C]-labeled probucol incorporated Gal-liposomes (△) after intravenous administration into mice. Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) between Gal-emulsions and Gal-liposomes.

Table III. Area Under the Blood Concentration-Time Curve (AUC) and Tissue Uptake Clearance of [^{3}C]probucol Dissolved in Serum, [^{14}C]probucol Incorporated Emulsions and Liposomes After Intravenous Injection into Mice[a]

Formulations	AUC (% of dose × h/ml)	Clearance[a] (μl/h)					
		CL_{liver}	CL_{kidney}	CL_{spleen}	CL_{lung}	CL_{heart}	CL_{urine}
Serum	5.9	2300	60.5	1250	131	55.9	0
Gal-emulsions	2.3	28,700	540	1800	150	403	170
Gal-liposomes	3.2	17,596	680	1620	430	160	145

[a] AUC and clearance (CL) were calculated for the period until 10 min after injection. An average of three experiments is shown.

physicochemical considerations (11,15). We found that the required lipophilicity of drugs for stable incorporation into lipid carries was 10^6 for liposomes and 10^9 for emulsions based on values for the partitition coefficient between *n*-octanol and water (PC_{oct}). Also, we investigated the relationship between the movement of galactosylated liposomes and incorporated drug after intravenous injection in relation to the lipid composition of the liposomes. Thus, probucol, with a PC_{oct} of $10^{10.8}$, was selected as a model lipophilic drug having a suitable lipophilicity for incorporation of both of emulsions and liposomes. In this study, we examined the applicability of probucol to examine the usefulness of Gal-emulsions. As shown in Fig. 6, [^{14}C]probucol incorporated Gal-emulsion exhibited blood concentration and liver accumulation profile similar to that of [^{3}H]CHE emulsions and different from free [^{14}C]probucol, suggesting that probucol was stably incorporated in Gal-emulsions. In contrast, a slight difference was observed between the blood concentration and liver accumulation profiles of [^{3}H]CHE and [^{14}C]probucol in Gal-liposomes, suggesting that probucol was released from liposomes to some extent. Pharmacokinetic analysis demonstrated that the liver uptake clearance of [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions was 1.6-times higher than that into Gal-liposomes. Thus, these results suggest that Gal-emulsions are more efficient carriers of probucol, which is a model lipophilic drug, for hepatocyte-selective targeting than Gal-liposomes.

In the current study, we showed that introduction of galactosylated cholesterol derivatives into emulsions can be prepared Gal-emulsions. Our current results provide evidence that introduction of ligand-grafted lipids such as mannose (36,37), fucose (38), folate (39,40), and transferrin (41), for cell-selective targeting, to emulsions also allows cell-selective targeting.

In conclusion, we have demonstrated that Gal-emulsions, which incorporate Gal-C4-Chol in emulsions, are alternative hepatocyte-selective carriers for highly lipophilic drugs. We have also demonstrated that the recognition mechanism of Gal-emulsions is mediated by asialoglycoprotein receptors, and the recognition efficiency depends on the galactose density on the emulsion surface. These observations provide information to help in the design of Gal-emulsions for hepatocyte-selective carrier systems for lipophilic drugs.

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In vivo gene transfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits

Shigeru Kawakami [a,1], Ayaka Harada [b], Koji Sakanaka [b], Koyo Nishida [a], Junzo Nakamura [a], Toshiyuki Sakaeda [c], Nobuhiro Ichikawa [b], Mikiro Nakashima [b], Hitoshi Sasaki [b,*]

[a] *Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[b] *Department of Hospital Pharmacy, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan*
[c] *Department of Hospital Pharmacy, School of Medicine, Kobe University, 7-5-2, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*

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Abstract

To optimize the in vivo ocular transfection efficiency of plasmid DNA (pDNA)/cationic liposome complexes, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)/dioleoylphosphatidylethanolamine (DOPE) (1:1 molar ratio) liposomes and DOTMA/cholesterol (Chol) (1:1 molar ratio) liposomes were prepared with varying amounts of pDNA. pDNA/cationic liposome complexes were intravitreally injected (100 μL) in rabbits, and luciferase activity in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina was measured. Transfection efficiency of pDNA alone did not change with pDNA ranging from 40 to 85 mg. In contrast, transfection efficiency of pDNA complexed with DOTMA/Chol liposomes significantly increased with the amount of pDNA ranging from 40 to 85 μg ($P < 0.05$). pDNA complexed with DOTMA/DOPE liposomes could not be prepared with pDNA greater than 60 μg. Among these experiments, pDNA (85 μg) complexed with DOTMA/Chol liposomes (pDNA:cationic liposome charge ratio (−:+) = 1.0:2.0) showed the highest transfection efficiency in the ocular tissue and its transfection-mediated luciferase activity peaked at 3 days. Among the ocular tissues, the highest gene expression was observed in the aqueous humor.
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Keywords: Transfection; Vitreous body; Eye; Cationic liposomes; Plasmid DNA

1. Introduction

Although the ocular transfection efficiency of viral vectors is high, safety concerns have been raised in clinical trials because of their immunogenicity (Reichel et al., 1998). Thus, the use of non-viral vectors has attracted great interest for clinical applications of in vivo gene transfection because such vectors lack some of the risks inherent to viral vector systems (Mahato et al., 1997), and consequently gene transfer to the eye utilizing the non-viral vectors has been widely studied (Masuda et al., 1996; Matsuo et al., 1996; Chaum et al., 1999; Hudde et al., 1999; Abul-Hassan et al., 2000; Urtti et al., 2000; Pleyer et al., 2001). One of the most promising classes of

* Corresponding author. Tel.: +81-95-849-7245; fax: +81-95-849-7251.
E-mail address: sasaki@net.nagasaki-u.ac.jp (H. Sasaki).
[1] Present address: Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.

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non-viral vectors developed so far is cationic liposomes. However, the transfection efficiency of cationic liposomes is lower than that of viral vectors, and thus it is essential to try to enhance the transfection efficiency to the eye.

Recent studies have demonstrated that the optimization of the lipid composition of cationic liposomes and/or the charge ratio (−:+) of plasmid DNA (pDNA) complexed with cationic liposomes greatly enhanced the transfection efficiency after intravenous administration (Huang and Li, 1997; Liu et al., 1997; Song et al., 1997). As for the lipid composition of cationic liposomes, the appropriate selection of neutral lipid types dramatically enhances the transfection efficiency after intravenous administration (Huang and Li, 1997; Liu et al., 1997; Kawakami et al., 2000). However, there is little information about the effects of the physicochemical properties of cationic liposomes or complexes on transfection efficiency to the eye. Once the relationship between the in vivo gene expression and these physicochemical properties is known, it will be possible to design liposomes or pDNA/liposome complexes to enable efficient in vivo gene transfection.

In the present study, we investigated the effect of the neutral lipids of cationic liposomes and the charge ratios of pDNA/cationic liposome complexes on the in vivo ocular transfection efficiency in rabbits. *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was selected as the cationic lipid of cationic liposomes because it is often used as a cationic lipid for gene transfection (Felgner et al., 1987; Zou et al., 1993; Sakurai et al., 2001).

2. Materials and methods

2.1. Materials

DOTMA was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Chol) were obtained from Avanti Polar-Lipids (Alabaster, AL, USA) and Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were obtained commercially as reagent grade products.

2.2. Animals

Male Nippon albino rabbits, 1.5–2.5 kg, were used in the study. The rabbits were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan) with free access to water. The rabbits were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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

pCMV-Luc was constructed by subcloning the Hind III/X ba 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 *E. coli* strain DH5 α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purified pDNA was diluted in sterile 5% (w/v) dextrose. The concentration of pDNA was measured by UV absorption at 260 nm and adjusted to 2 mg/mL.

2.4. Preparation of cationic liposomes

DOTMA/DOPE (1:1 molar ratio) and DOTMA/Chol (1:1 molar ratio) liposomes were prepared according to a previous report (Kawakami et al., 2001). Briefly, DOTMA (9.5 mg), DOPE (7.9 mg), and Chol (5.8 mg) were dissolved in chloroform. Mixtures of DOTMA/DOPE or DOTMA/Chol were dried as a thin film in a test tube using an evaporator at 25 °C, and then were vacuum-desiccated for approximately 4 h. The film was resuspended in sterile 5% dextrose (5 mL) by vortexing and the dispersions were sonicated at 100 W for 3 min on ice. The resulting liposomes were extruded through a 450 mm polycarbonate membrane filter.

2.5. Preparation of pDNA/cationic liposome complexes

Both pDNA and cationic liposomes were diluted with suitable volumes of sterile 5% dextrose to adjust the charge of pDNA/cationic liposome complexes and

were incubated in sterile 5% dextrose for 30 min at 50 °C. The mixing volume of pDNA and cationic liposomes was 60 mL per experiment (total 120 μL) and 100 μL of pDNA/cationic liposome complexes was used for each intravitreal injection.

2.6. Calculation of theoretical charge ratio

The theoretical charge ratio of pDNA/cationic lipid was calculated as the molar ratio of DOTMA (monovalent) per nucleotide unit (average molecular weight 330) (Li and Huang, 1997).

2.7. In vivo transfection experiments

Rabbits were placed in a restraint box and anesthetized with sodium pentobarbital injected into a marginal ear vein. Naked pDNA (100 μL) or pDNA/cationic liposome complexes (100 μL) were injected directly into the vitreous using a 30-gauge needle attached to a syringe. The rabbits were sacrificed by intravenous administration of an overdose of sodium pentobarbital at 1, 3 or 7 days. After the eyes were enucleated, the aqueous humor (200–300 μL) and vitreous fluid (800–1000 μL) were collected using a syringe inserted and then, the six ocular tissues were dissected so that they could be assayed for gene expression. The cornea, iris–ciliary body, lens, and retina were washed twice with ice-cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris–HCl, pH 7.8) (Li and Huang, 1997). The lysis buffer was added at a weight ratio of 4 mL/mg of the sample. After three cycles of freezing with liquid nitrogen for 3 min and thawing at 37 °C for 3 min, the tissue homogenates, aqueous humor, and vitreous body were centrifuged at 15,000 × *g* for 3 min at room temperature. Twenty microliters of supernatant were mixed with 100 μL of luciferase assay buffer (Picagene®, TOYO B-Net Co, Tokyo, Japan) and the light produced was immediately measured using a luminometer (MiniLumat LB9506, EG&G Berthold, Bad Wildbad, Germany). Luciferase activity in the tissue was normalized by relative light units (RLU) per g or mL of the tissue. The gene expression levels of 2×10^3 RLU/g of tissue were considered positive because each tissue mixed with the substrates without the injection of pDNA showed approximately 2×10^3 RLU/g or mL (blank level). The results of each experiment were expressed after the raw results minus the blank level.

2.8. Statistical analysis

Statistical comparisons were performed by analysis of variance and post-hoc test was performed by Turkey–Kramer test. $P < 0.05$ was considered to be indicative of statistical significance.

3. Results

3.1. Effect of neutral lipid of pDNA/cationic liposome complexes on gene expression

Fig. 1 shows the luciferase activity as a measure of transfection efficiency 3 days after intravitreal injection of naked pDNA or pDNA complexed with DOTMA/DOPE or DOTMA/Chol at pDNA doses of 40, 60, and 85 μg in rabbits. The levels of the luciferase activity induced by pDNA (40 μg) complexed with DOTMA/DOPE liposomes in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina were approximately 3.3, 2.4, 2.5, 2.0, 7.0, and 1.1-fold higher than those obtained with naked pDNA, respectively. At pDNA doses of more than 60 μg, however, it was difficult to prepare the pDNA complexed with DOTMA/DOPE liposomes due to aggregation. On the other hand, the transfection efficiency of pDNA complexed with DOTMA/Chol liposomes was enhanced by increasing the dose of pDNA (Fig. 1(c)). Especially, pDNA (85 mg)/DOTMA/Chol liposomes showed the highest transfection efficiency, producing luciferase activities in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina that were about 2, 280, 28, 31, 120, and 5-fold higher than those obtained with naked pDNA, respectively. Transfection efficacy by DOTMA/Chol liposomes in the vitreous body was significantly higher than that by naked pDNA ($P < 0.05$). Therefore, pDNA (85 μg)/DOTMA/Chol liposomes was used in the following experiments.

Fig. 2 shows the time dependency of luciferase expression at 1, 3, and 7 days after intravitreal injection of pDNA (85 μg)/DOTMA/Chol in various ocular tissues. Luciferase gene expression was not observed