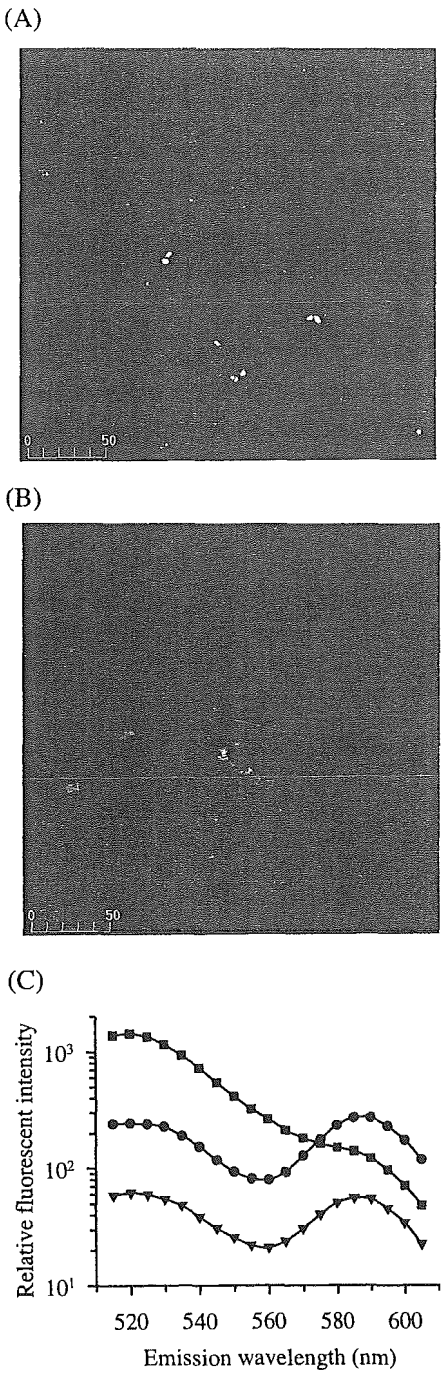

Fig. 4. Interaction between galactosylated lipoplex and blood cells with or without preincubation with serum. pDNA (30 μg) was complexed with galactosylated liposomes at a charge ratio of +2.3. Carboxyfluorescein-pDNA was complexed with Rh-galactosylated liposomes under the same conditions as the in vivo experiments. The galactosylated lipoplex, with or without preincubation with serum, was mixed with erythrocyte suspension or whole blood to adjust the hematocrit. A and B, confocal microscopic images of the galactosylated lipoplex bound to blood cells with (B) or without (A) preincubation with serum. Scale bars indicate 50 μm. C, FRET analysis of supernatant for galactosylated lipoplex integrity. Circles represent control, inverted triangles represent mixing with serum followed by blood cells, and squares represent mixing with whole blood, respectively.

in the liver and other organs were observed with or without incubation with serum and, accordingly, this result did not correlate with the enhanced hepatic transfection activity of the galactosylated lipoplex preincubated with serum.

In Situ Hepatic Disposition of Galactosylated Lipoplex Preincubated with Blood Components. We performed pharmacokinetic analyses using a single-pass rat liver perfusion experiment that allowed us to evaluate the local disposition of the carrier systems. The venous outflow profile of the complexes (Fig. 3) after a bolus input into the isolated perfused liver was analyzed by a two-compartment dispersion model to quantitatively evaluate the difference in each kinetic process. Table 2 shows the results of moment analysis for the venous outflow pattern of galactosylated lipoplex preincubated with rat blood components. Incubation with serum significantly reduced the extraction ratio of galactosylated lipoplex, whereas the effect of incubation with rat whole blood on the extraction ratio was minor. However, the MRT of the galactosylated lipoplex in the liver was significantly reduced after incubation with serum. To clarify each kinetic process in perfused liver, the binding and internalization processes were evaluated by a two-compartment dispersion model. As shown in Fig. 3, the simulation curves using the model were in good agreement with the observed data. The validation of two-compartment dispersion model was discussed in our previous report (Fumoto et al., 2003b). The association rate (k_{12}) of the galactosylated lipoplex was reduced by half after incubation with serum (Table 3). However, the dissociation rate (k_{21}) was reduced by 80% by serum. As a consequence, the tissue binding affinity (k_{12}/k_{21}) was 2.6-fold higher than that without preincubation. On the other hand, incubation with whole blood significantly increased the dissociation rate while maintaining the high

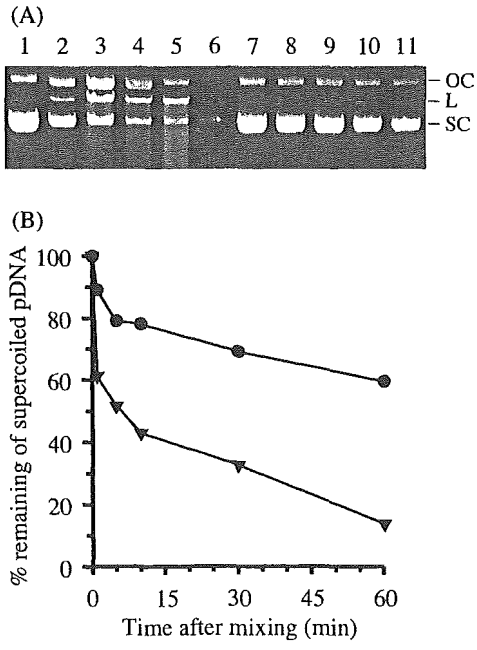

Fig. 5. Effect of preincubation with serum on pDNA stability in galactosylated lipoplex after exposure to blood. A, agarose gel electrophoresis of pDNA extracted from a mixture of the lipoplex and blood components. Lane 1, extract from the lipoplex (control); lanes 2 to 6, incubated with whole blood for 1, 5, 10, 30, and 60 min, respectively; lanes 7 to 11, preincubated with serum for 5 min before mixing with erythrocyte suspension for 1, 5, 10, 30, and 60 min, respectively. OC, L, and SC indicate open circular, linear, and supercoiled forms of pDNA, respectively. B, densitometric analysis of supercoiled pDNA in intact form in the agarose gel electrophoresis image. Circles represent mixing with serum followed by blood cells, and inverted triangles represent mixing with whole blood.

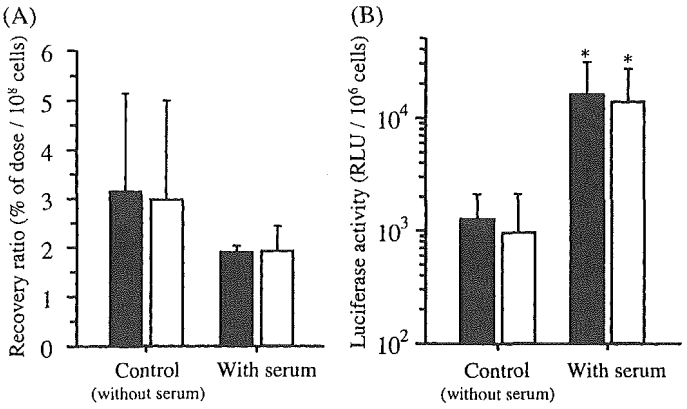

Fig. 6. Hepatic cellular distribution of ^{32}P-galactosylated lipoplex (A) and gene expression (B) after intraportal injection of galactosylated lipoplex, with or without incubation with serum. pDNA (30 μg) was complexed with galactosylated liposomes at a charge ratio of +2.3. Luciferase activity in PC (filled bar) and NPC (open bar) was determined 6 h postinjection. Each value represents the mean ± S.D. of at least four experiments. Statistical comparisons were performed by Steel's test (* indicates comparison with the control group; $P < 0.05$).

association rate. As a result, the tissue binding affinity (k_{12}/k_{21}) was reduced by 60% after incubation with whole blood.

Assessment of Galactosylated Lipoplex Stability in Blood. To evaluate the differences in galactosylated lipoplex stability with or without incubation with serum, we performed FRET analysis. Galactosylated lipoplex was labeled with both fluorescein-labeled pDNA (green fluorescence) and rhodamine-labeled lipid (red fluorescence). A mixture of galactosylated lipoplex and mouse blood components was centrifuged after a 5-min incubation, and subsequently, the erythrocyte compartment and supernatant were examined using confocal laser-scanning microscopy and spectrofluorophotometry, respectively. After incubation with whole blood, the signal of the erythrocyte compartment was found to be red (Fig. 4A), whereas the energy transfer from fluorescein-labeled-pDNA to rhodamine-labeled lipid in the supernatant was reduced (Fig. 4C), suggesting that the pDNA and lipids in the lipoplex had dissociated. On the contrary, preincubation with serum before incubation with erythrocyte suspension did not induce such dissociation of pDNA and lipids in the lipoplex (Fig. 4, B and C). The dissociation of pDNA from cationic liposomes might cause degradation by serum nuclease; therefore, agarose gel electrophoresis was performed to analyze the degradation of pDNA (Fig. 5). Preincubation with serum before mixing with erythrocyte suspension markedly inhibited pDNA degradation, indicating that preincubation with serum stabilized the lipoplex.

Hepatic Cellular Localization of Galactosylated Lipoplex with or without Incubation with Serum. The hepatic cellular localization of ^{32}P-labeled galactosylated lipoplex was investigated after bolus injection into perfused rat liver (Fig. 6A). When the radioactivity associated with PC and NPC per unit cell number was measured, the PC/NPC ratio for the galactosylated lipoplex with pre-exposure to serum was 1.0, which was comparable with the galactosylated lipoplex without pre-exposure to serum (PC/NPC ratio: 1.1). As for the hepatic cellular localization of transfection activity in mice, gene expression in PC and NPC of galactosylated lipoplex with preincubation with serum was one order of magnitude higher than the values for the lipoplex without preincubation (Fig. 6B).

Inhibition Experiment of Hepatic Transfection Activity of Galactosylated Lipoplex. To confirm whether galactosylated lipoplex exposed to serum is recognized by asialoglycoprotein receptors on hepatocytes, we performed an inhibition experiment involving predosing with Gal-BSA (Fig. 7A). The hepatic transfection activity was significantly inhibited by Gal-BSA, suggesting that the serum protein-bound galactosylated lipoplex is recognized by asialoglycoprotein receptors.

Effect of Inactivation of Serum on Transfection Activity and Biodistribution of Galactosylated Lipoplex. Opsonin activity in serum might affect the in vivo distribution and subsequent transfection activity of the lipoplex. To confirm the effect of opsonin activity in serum, serum was inactivated by heating before incubation with the lipoplex. Figure 7B shows the effect of inactivation of mouse serum on the hepatic transfection activity of the lipoplex 6 h after intraportal injection to mice. Approximately 85% of the hepatic transfection activity of the galactosylated lipoplex was attenuated by inactivation of serum, suggesting that some

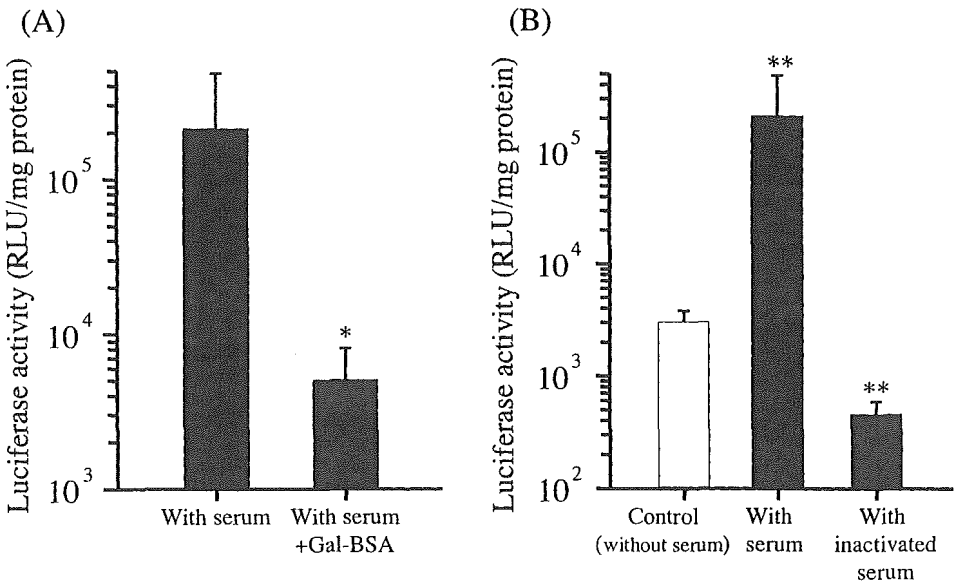

Fig. 7. Inhibitory effect of predosing Gal-BSA (A) and inactivation of serum (B) on hepatic transfection activity of galactosylated lipoplex preincubated with serum after intraportal injection in mice. pDNA (30 μg) was complexed with galactosylated liposomes at a charge ratio of +2.3. Five minutes before injection, the lipoplexes were mixed with 30% serum (A) or inactivated serum (B). Luciferase activity was determined 6 h postinjection of the lipoplex. Each value represents the mean ± S.D. of at least three experiments. A, Gal-BSA was intravenously injected 1 min before the lipoplex injection. Statistical comparisons were performed by the Mann-Whitney test (*, $P < 0.05$). B, mouse serum was inactivated by incubation for 30 min at 56°C before mixing with galactosylated lipoplex. Statistical comparisons with the control group were performed by Steel's test (**, $P < 0.01$). RLU, relative light unit.

factor, such as complement component, is involved in the hepatic transfection activity of the galactosylated lipoplex.

To investigate the inactivation of serum further, we evaluated the in vivo distribution after intraportal injection of ^{32}P-labeled galactosylated lipoplex. Figure 8 shows the blood concentration and tissue accumulation (liver and lung) at the indicated time points. Inactivation with serum significantly reduced the accumulation not only in the liver but also in the lung, whereas an increased blood concentration was observed at the early time points. To estimate the effect of preincubation with inactivated serum on organ uptake of galactosylated lipoplex, we calculated organ uptake clearance (Table 4). The hepatic uptake clearance was the highest of all of the tissues. The hepatic uptake clearance was markedly reduced by incubation with inactivated serum. These differences in distribution caused by inactivation of serum will be involved in the difference seen in the hepatic transfection activity of galactosylated lipoplex preincubated with inactivated serum.

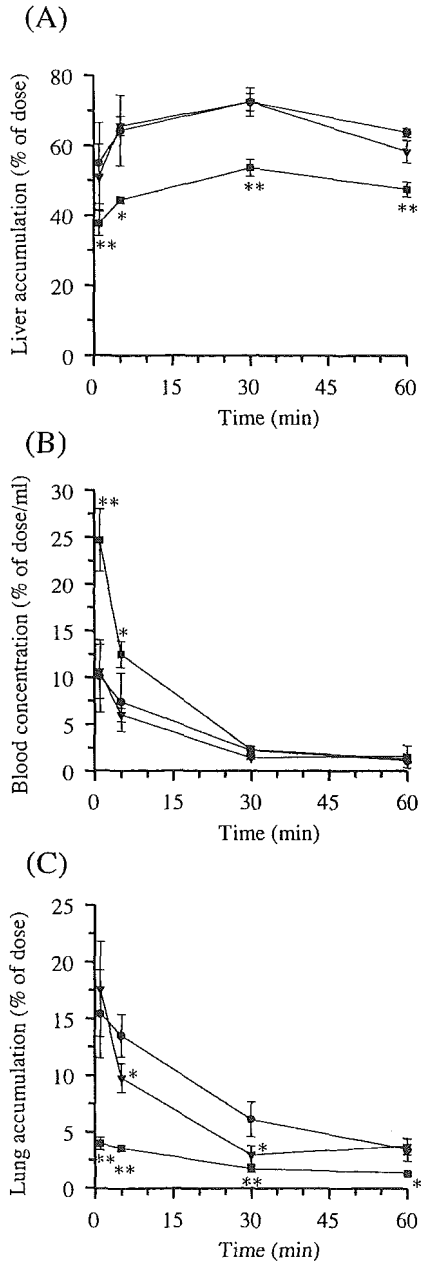

Fig. 8. Effect of preincubation with inactivated serum on the distribution of galactosylated lipoplex after intraportal injection in mice. [^{32}P]pDNA (30 μg) was complexed with galactosylated liposomes at a charge ratio of +2.3. Radioactivities were determined in the liver (A), blood (B), and lung (C). Five minutes before injection, galactosylated lipoplex was mixed with 30% serum (▼), inactivated serum (■), or without incubation (●). Each value represents the mean ± S.D. of at least three experiments. Statistical comparisons with control group were performed by Dunnett's test (*, $P < 0.05$, **, $P < 0.01$).

Effect of Blood Components on Serum Cytokine Level after Intraportal Injection of Galactosylated Lipoplex. It is well known that lipoplex induces production of inflammatory cytokines via the unmethylated CpG motif in pDNA (McLachlan et al., 2000). To study the contribution of the interaction with each blood component to cytokine production, we evaluated the serum IFN-γ level at 6 h after intraportal injection of galactosylated lipoplex preincubated with blood components (Fig. 9). Preincubation with serum did not affect the serum IFN-γ level, whereas preincubation with whole blood significantly reduced it. Preincubation with inactivated serum also significantly reduced the serum IFN-γ level, suggesting that an interaction between galactosylated lipoplex and some serum components is involved in inflammatory cytokine production.

Discussion

A number of possible barriers for targeted gene delivery to hepatocytes are thought to be limiting factors for in vivo transfection, including 1) nonspecific interactions with erythrocytes, serum, and nontarget cells, 2) aggregation of lipoplex, and 3) penetration of endothelial cells. It is necessary to discover the fate of the gene carrier in order to improve and/or develop an effective gene carrier system. In the present study, we evaluated the effect of blood components (serum and whole blood) on asialoglycoprotein receptor-mediated in vivo gene transfer using galactosylated lipoplex. Preincubation with serum greatly enhanced the hepatic transfection activity of the lipoplex, whereas preincubation with whole blood reduced it (Fig. 1); this indicates that interaction with blood components plays a crucial role in in vivo gene transfer.

We initially evaluated the effect of the interaction between galactosylated lipoplex and blood components on the basic physicochemical properties. Because particle size is an important factor that determines the delivery efficiency of various particulates to liver parenchymal cells (Rahman et al., 1982; Ogawara et al., 1999), we measured the change in particle size of the lipoplex produced by exposure to serum. As we expected, exposure of the lipoplex to serum increased the particle size (Table 1). However, the particle size of the galactosylated lipoplex after exposure to serum was comparable with the size of the fenestrae (100–200 nm) in the liver sinusoidal endothelium (Wisse, 1970; Wisse et al., 1985; Gatmaitan et al., 1996). Indeed, the intrahepatic distribution (PC/NPC ratio) of galactosylated lipoplex after pre-exposure to serum was similar to that of galactosylated lipoplex without pre-exposure to serum (Fig. 6A). It was also demonstrated that the transfection activity of galactosylated lipoplex after pre-exposure to serum was equally enhanced in both PC and NPC; as a consequence, the PC/NPC ratio of the transfection activity had a similar value (Fig. 6B).

The reduced ζ-potential after incubation with serum (Table 1) suggests that the surface of the galactosylated lipoplex is

TABLE 4
Effect of incubation with serum or inactivated serum on the pharmacokinetic parameters of galactosylated lipoplex after intraportal injection in mice

Incubation	AUC_p	Organ Uptake Clearance				
		Liver	Lung	Spleen	Kidney	Heart
	% of dose · h/ml			*μl/h*		
None (control)	3.26	62,800	17,600	1170	790	950
With serum	2.37	62,000	21,400	580	980	1000
With inactivated serum	4.45	20,700	2170	490	750	430

extensively covered by serum protein, because most serum proteins have negative charges. However, a reduced surface charge is expected to reduce the liver accumulation. In fact, the in situ liver perfusion experiments showed that incubation with serum reduced the extraction ratio (Table 2); however, the extraction ratio (65% of the dose) was high in absolute terms despite a single passage through the perfused liver. Furthermore, two-compartment dispersion model analysis demonstrated that incubation with serum reduced not only the association rate but also the dissociation rate. As a consequence, the tissue binding affinity of galactosylated lipoplex preincubated with serum was increased (Table 3). These results indicate that preincubation with serum reduces the initial amount of lipoplex bound to tissue, although the binding of retained lipoplex to tissue is stronger than that in the case of no incubation. As shown in Fig. 7A, the hepatic transfection activity of galactosylated lipoplex preincubated with serum was significantly inhibited by predosing with Gal-BSA, suggesting that asialoglycoprotein receptor-mediated endocytosis markedly contributes to the high tissue binding affinity. In contrast, incubation with whole blood did not reduce the association rate but increased the dissociation rate in the liver perfusion experiments (Table 3). Under in vivo conditions, dissociation by blood cell would be more marked than in perfused liver because of the abundance of blood cells in the continuous blood flow. This analysis might explain why the liver accumulation of lipoplex without incubation after intraportal injection (Fig. 2) was relatively lower than the extraction ratio in the liver perfusion experiment (Table 2). As a result, the liver accumulation of the lipoplex, with or without incubation with serum, exhibited a similar profile.

We next assessed the stability of the galactosylated lipoplex in blood. It is known that mixing lipoplex with anionic liposomes induces the dissociation of pDNA from the lipoplex (Xu and Szoka, 1996; Sakurai et al., 2000). Blood cells have a negative surface charge; thus, they might induce dissociation of pDNA from the galactosylated lipoplex. After incubation with whole blood, dissociation of pDNA from the galactosylated lipoplex was observed (Fig. 4, A and C). On the contrary, incubation with serum inhibited such dissociation of pDNA from the galactosylated lipoplex (Fig. 4, B and C). In addition, the stability of pDNA in the galactosylated lipoplex was improved by preincubation with serum (Fig. 5). It has been reported that prolonged incubation with serum also induces pDNA dissociation and degradation (Li et al., 1999). Furthermore, we have demonstrated that the presence of blood cells together with galactosylated lipoplex enhances pDNA dissociation and degradation. The injected lipoplex is immediately mixed with blood before contact with the liver; therefore, this result partially supports the enhanced transfection activity of the galactosylated lipoplex. Taking these results into consideration, not only ligand modification (i.e., galactosylation) of the lipoplex but also controlling the stability of the galactosylated lipoplex is important for achieving cell-selective gene transfection under in vivo conditions.

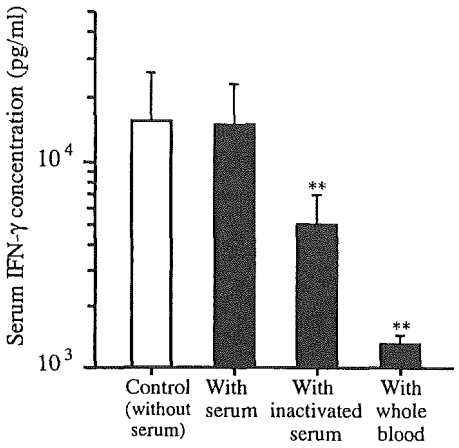

Fig. 9. Serum IFN-γ concentration after intraportal injection of galactosylated lipoplex preincubated with blood components in mice. pDNA (30 μg) was complexed with galactosylated liposomes at a charge ratio of +2.3. Five minutes before injection, galactosylated lipoplex was mixed with 30% serum, inactivated serum, or whole blood. Serum IFN-γ was determined 6 h postinjection of the lipoplex. Each value represents the mean ± S.D. of at least three experiments. Statistical comparisons with the control group were performed by Steel's test (**, $P < 0.01$).

Inactivation of serum before incubation with galactosylated lipoplex significantly reduced both liver accumulation (Fig. 8) and hepatic transfection activity (Fig. 7). It is known that the opsonin activity in serum affects the disposition of liposomes (Chonn et al., 1992; Patel, 1992), suggesting that the disposition of galactosylated lipoplex would also be affected by opsonization. The complement component C1q, factor B, and fibronectin are heat-labile (Hamuro et al., 1978; McManus and Nakane, 1980; Rivedal, 1982); thus, both classical and alternative pathways of complement are no longer able to work after inactivation of serum. As a result, the uptake via recognition of these factors bound to the galactosylated lipoplex was expected to fall. In fact, the liver and lung accumulation of galactosylated lipoplex with inactivated serum was significantly reduced (Fig. 8; Table 4). Barron et al. (1998) reported that complement depletion by intraperitoneal injection of cobra venom factor and anti-C3 antibodies did not affect the distribution and gene expression of lipoplex after intravenous injection. Cobra venom factor depletes factor B and component C3; therefore, other factors apart from factor B and component C3 might be involved in the reduced accumulation of galactosylated lipoplex preincubated with inactivated serum. Further studies are needed to clarify how

complement component(s) affect the disposition and transfection activity of the lipoplex.

It is known that lipoplex initiates inflammatory cytokine production via pDNA containing the unmethylated CpG motif (McLachlan et al., 2000). When the galactosylated lipoplex was preincubated with serum, there was no significant change in the serum IFN-γ level; therefore, incubation with serum may be a useful method for enhancing transfection activity without increasing the inflammatory response. On the contrary, incubation with both whole blood and inactivated serum significantly reduced the serum IFN-γ level. Although the mechanism is unclear, this information may useful for development of safe gene carrier with less inflammatory response.

In summary, we have shown that the interaction with blood components plays a crucial role in in vivo gene transfer. Although incubation of galactosylated lipoplex with whole blood reduced the hepatic transfection activity, incubation of the lipoplex with serum enhanced it. The stability of pDNA in blood rather than its in vivo distribution partially explains this difference. In contrast, inactivation with serum reduced the hepatic transfection activity, suggesting that other factor(s) in serum are involved in the hepatic transfection activity of galactosylated lipoplex. Hence, the information in this study will be valuable for the future use, design, and development of galactosylated lipoplex for in vivo asialoglycoprotein receptor-mediated gene transfer.

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Biodistribution characteristics of mannosylated and fucosylated O/W emulsions in mice

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Abstract

Cell-specific drug delivery is one of the most promising strategies for improving therapeutic efficiency and minimizing systemic toxicity. Carrier systems devoted to receptor-mediated targeting need to be developed. In the case of liver-non-parenchymal cell-specific targeting systems, glycosylated emulsions have been developed as carriers for lipophilic drugs and/or peptides. This present study demonstrates the *in vivo* disposition behaviour and pharmacokinetic characteristics of mannosylated (Man-) and fucosylated (Fuc-) emulsions incorporated with cholesten-5-yloxy-N-(4-((1-imino-2-D-thiomannosylethyl)amino)alkyl)formamide (Man-C4-Chol) and its fucosylated derivatives (Fuc-C4-Chol), respectively. Man- (or Fuc-) emulsions are composed of soybean oil, EggPC and Man-C4-Chol (or Fuc-C4-Chol) in a weight ratio of 70:25:5. After intravenous administration to mice, these two types of [^{3}H]cholesteryl hexadecyl ether (CHE)-labelled glycosylated emulsions were rapidly eliminated from the blood circulation and preferentially recovered in the liver. In contrast, bare (Bare-) emulsions composed of soybean oil:EggPC:cholesterol (Chol) in a weight ratio of 70:25:5 were more retained in the blood circulation. The hepatic uptake clearances of Man- and Fuc-emulsions were 3.3- and 4.0-times greater than that of Bare-emulsions. Interestingly, the hepatic uptake clearance of Fuc-emulsions was significantly higher that that of Man-emulsions. The uptake ratios by non-parenchymal cells (NPC) and parenchymal cells (PC) (NPC/PC ratio) for Bare-, Man- and Fuc-emulsions were found to be 0.4, 2.0 and 2.9, respectively. The hepatic uptakes of [^{3}H]CHE-labelled Man- and Fuc-emulsions were reduced by pre-dosing with glycosylated proteins and liposomes. These results clearly support the conclusion that Man- and Fuc-emulsions are promising carrier systems for liver NPC-specific targeting via receptor-mediated mechanism.

Keywords: *Mannosylated emulsion, fucosylated emulsion, non-parenchymal cells, mannose receptor, fucose receptor, targeting*

Introduction

To date, there have been numerous investigations into the effect of particle size in lipid emulsions on their biological fate. After intravenous administration of lipid emulsions, conventional lipid emulsions with a particle size of 200–300 nm are rapidly eliminated from the blood circulation and easily trapped in the reticuloendothelial system (RES), especially in the liver. On the other hand, conventional lipid emulsions with a small particle size (less than 100 nm) are more retained in the bloodstream (Takino et al. 1994, Sakaeda and Hirano 1998, Seki et al. 2004). Furthermore, the particle sizes of lipid emulsions also affect their hepatic uptake mechanism. We have demonstrated that large-size lipid emulsions are predominantly localized in liver-NPC after intravenous administration to mice (Takino et al. 1995). These results suggest predominant uptake of large-size emulsions by liver-NPC. However, it has been suggested that large-size emulsions might affect the immune system after intravenous administration (Hamawy et al. 1985, Keipert et al. 1994, Noveck et al. 2000).

Receptor-mediated drug targeting is a promising approach to cell-specific drug delivery to improve therapeutic efficiency and minimize systemic toxicity (Takakura and Hashida 1996). One particular strategy involves the sugar recognition mechanism whereby

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receptors can recognize the corresponding sugars on the non-reducing terminal of sugar chains and then mediate cellular uptake. For instance, asialoglycoprotein receptors localized on PC recognize galactose residues, while mannose and fucose receptors on liver non-parenchymal cells (NPC) recognize mannose and fucose residues (Ashwell and Harford 1982).

We previously synthesized novel mannosylated and fucosylated cholesterol derivatives, namely cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)butyl)formamide (Man-C4-Chol) and cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiofucosyl ethyl)amino)butyl)formamide (Fuc-C4-Chol) (Kawakami et al. 2000a). Our previous studies demonstrated that liposomes modified with Man-C4-Chol and Fuc-C4-Chol were specifically delivered to liver-NPC (Kawakami et al. 2000a, Opanasopit et al. 2002) and the plasmid DNA/Man-liposome complex led to high gene expression in liver-NPC (Kawakami et al. 2000b, 2004, Yamada et al. 2004). Nevertheless, for lipophilic drugs, lipid emulsions are more efficient than other carrier systems (Ishida et al. 2004). Besides their physical stability and biocompatibility, lipid emulsions possess a high solubilizing capacity for highly lipophilic drugs resulting in their stable incorporation in the formulations (Prankerd and Stella 1990, Takino et al. 1993, Yamaguchi and Mizushima 1994, Kawakami et al. 2000c). Although cell-specific drug targeting is sometimes urgently required for a variety of clinical purposes, there are few reports of cell-specific drug targeting using O/W (oil-in-water) lipid emulsions. These findings prompted us to study the feasibility of producing modified emulsions such as mannosylated (Man-) and fucosylated (Fuc-) emulsions with a small particle size for use as a liver-NPC-specific targeting system.

The purpose of this present study was to characterize the biodistribution behaviour of Man- and Fuc-emulsions as novel drug carriers targeting liver-NPC after intravenous injection in mice. These emulsions were radiolabelled with [^{3}H]CHE (Takino et al. 1995, Ishida et al. 2004). We have successfully demonstrated that Man- and Fuc-emulsions predominately accumulate in the liver-NPC; in addition, these emulsions are taken up by mannose and fucose receptors. These results suggest that Man- and Fuc-emulsions are promising carriers for liver-NPC specific drug delivery systems.

Materials and methods

Materials

Soybean oil was supplied from Wako Pure Chemicals Industry, Ltd. (Osaka, Japan). EggPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesteryl chloroformate and pyridine were purchased from Sigma-Aldrich, Co. (St. Louis, MO). [^{3}H]CHE was supplied by Perkin Elmer, Inc. (Boston, MA) and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cholesterol was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was obtained from Packard Co., Inc. (Groningen, The Netherlands). Other chemicals used were of the highest purity available.

Synthesis of Man- and Fuc-C4-Chol

Man- and Fuc-C4-Chol were synthesized by the method described previously (Kawakami et al. 2000a). 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. A quantity of the resulting material was added to an excess of 2-imino-2-methoxyethyl-1-thio-mannoside and -fucoside (Lee et al. 1976), respectively, in pyridine containing triethylamine. After 24 h incubation at room temperature, the reaction mixture was evaporated, resuspended in water and dialysed against distilled water for 48 h using a dialysis membrane (12 kDa cut-off) and finally lyophilized.

Preparation of O/W emulsions

Emulsions were prepared by the method described previously (Takino et al. 1995, Ishida et al. 2004). Bare-, Man- or Fuc-emulsions consisted of soybean oil, EggPC and Chol or Man-C4-Chol or Fuc-C4-Chol at a weight ratio of 70:25:5, respectively. The lipid mixtures were dissolved in chloroform, vacuum-desiccated and re-suspended in pH 7.4 phosphate-buffer saline (PBS). The suspensions were sonicated for 1 h (200 W) under a current of nitrogen. The particle sizes were determined by dynamic light scattering spectrophotometry (Photal, Otsuka Electronic Co., Ltd., Osaka, Japan). The concentration of the emulsions was adjusted to 0.5% based on the radioactivity measurement of [^{3}H]CHE-labelled emulsions. In the case of emulsions with a large particle size, the concentration of soybean oil was fixed, while that of EggPC was 0.1 mg ml^{-1}. After hydration with PBS, this suspension was sonicated for 10 min (200 W) under a current of nitrogen.

Stability of emulsions

Emulsion stability was determined by monitoring the particle size change as a function of time using a Zetasizer Nano ZS (Malvern Instruments, Ltd.) and by monitoring the phase separation using the turbidity measurement method described by Pearce and Kinsella (1978). Briefly, the 0.05% emulsions were

transferred to a 1 ml-syringe immediately after preparation and stored vertically at room temperature or 4°C. The turbidity of the samples at a given time from the under side of the syringe at ~ 30% of the emulsion height was measured at 600 nm using a Biorad Model 550 Microplate Reader (Scientific Support, Inc., CA).

In vivo *distribution of emulsions*

Four-to-five-week-old male ddY mice (20–30 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 propagated by the US National Institutes of Health and the Guideline for Animal Experiments of Kyoto University. [^{3}H]CHE (0.1 μCi per 100 μl)-labelled emulsions were injected into the tail vein of mice at a dose of 25 mg kg^{-1}. Various compounds were administered intravenously 1 min prior to the [^{3}H]CHE-labelled emulsions in the hepatic uptake inhibition experiments. At given times, mice were sacrificed and then blood from the vena cava, liver, spleen, heart, lung, kidney, muscle and urine were collected and dissolved in Soluene-350 for radioactivity measurement using a Beckman Model LS2500 Liquid Scintillation Counter (Beckman Coulter, Inc., Tokyo, Japan).

Hepatic cellular localization of emulsions

The separation of liver-PC and NPC was performed by the collagenase perfusion method (Seglen 1973, Kawakami et al. 2001, Ishida et al. 2004). Briefly, mice were anaesthetized with pentobarbital (40–60 mg kg^{-1}) and then intravenously injected with [^{3}H]CHE (0.5 μCi per 100 μl)-labelled emulsions. Thirty minutes after injection, the liver was perfused via the portal vein with perfusion medium (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) of collagenase (type I) at 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^{-1}. Following the discontinuation of perfusion, the liver was excised and its capsular membranes were removed. The cells were suspended by gentle stirring in ice-cold Hank's-HEPES buffer containing 0.1% BSA. The dispersed cells were filtered through gauze, followed by centrifugation at 50 × *g* for 1 min. The pellets containing the liver-PC were washed twice with Hank's-HEPES solution and then centrifuged at 50 × *g* for 1 min. The supernatant containing liver-NPC was similarly centrifuged a further two times. The resulting material was then centrifuged twice at 200 × *g* for 2 min. PC and NPC were re-suspended separately in ice-cold Hank's-HEPES solution (4 ml for PC and 2 ml for NPC). The number of viable cells was determined by the trypan blue exclusion method. Then, the radioactivity in the cells (0.5 ml) was measured using a Beckman Model LS2500 Liquid Scintillation Counter (Beckman Coulter, Inc.).

Determination of tissue uptake clearances of emulsions

Tissue distribution data were evaluated using the organ distribution clearances as reported previously (Takakura et al. 1987). 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]CHE-labelled emulsions in the tissue at time t, CL_{uptake} is the tissue uptake clearance and C_b is the blood concentration of [^{3}H]CHE-labelled emulsions. Integration of equation (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]CHE-labelled emulsions 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

Statistic analysis was performed using Student's paired t-test for two groups and one-way ANOVA for multiple groups. $P < 0.05$ was considered to be indicative of statistical significance.

Results

Particle size of emulsions

As shown in Table I, the mean particle sizes of Bare- and glycosylated emulsions were similar, ~110–130 nm in diameter. Therefore, there was no significant difference in physicochemical properties among these emulsions.

Stability of emulsions

Figure 1a shows the stability of emulsions in term of particle size change during storage at 4°C and room temperature. The mean particle size of the emulsions remained stable for at least 1 month at 4°C while the particle size increased at room temperature over a storage period of 1 month. Simultaneously, the emulsions separated slowly into two phases, the

Table I. The lipid composition and mean particle sizes of emulsions.

Emulsions (lipid composition, weight ratio)	Mean particle size (nm)
Bare-emulsions (soybean oil:EggPC:Chol, 70:25:5)	110 ± 44
Man-emulsions (soybean oil:EggPC:Man-C4-Chol, 70:25:5)	126 ± 51
Fuc-emulsions (soybean oil:EggPC:Fuc-C4-Chol, 70:25:5)	115 ± 48

Each value represents the mean ± SD values ($n = 3$).

cream layer and water phase without coalescence of oil droplets, as shown in Figure 1b.

Biodistribution of [^{3}H]CHE-labelled emulsions

Figure 2 shows the blood concentration and tissue accumulation profiles of 0.5% Bare- and glycosylated emulsions after intravenous injection into mice. Bare-, Man- and Fuc-emulsions were rapidly eliminated from the blood circulation.

The tissue distribution profiles as well as the urinary excretion of Bare- and glycosylated emulsions were compared. Man- and Fuc-emulsions were predominantly recovered in the liver within the first 10 min, accounting for 70–83% of dose, respectively (Figure 2b and c). Although Bare-emulsions also mainly accumulated in the liver, the amount restricted to the liver, ~ 50% of the dose, was significantly ($p < 0.01$) lower than for glycosylated emulsions (Figure 2a). The amount of Fuc-emulsions accumulating in the liver was significantly higher than that of Man-emulsions at all time points ($p < 0.05$).

Pharmacokinetic analysis of [^{3}H]CHE-labelled emulsions

The tissue uptake clearances were analysed in the early phase up to 10 min to avoid the tissue uptake efflux of [^{3}H]CHE. Table II summarizes the pharmacokinetic parameters including the area under the blood concentration-time curve (AUC) and the uptake clearances of Bare- and glycosylated emulsions. The uptake clearances were determined for liver (CL_{liver}), spleen (CL_{spleen}), heart (CL_{heart}), lung (CL_{lung}), kidney (CL_{kidney}), muscle (CL_{muscle}) and urine (CL_{urine}). This pharmacokinetic analysis described the uptake clearance characteristics of these emulsions with a small AUC and a large hepatic uptake clearance. Although their hepatic uptake clearance was the highest among the tissues examined, the hepatic uptake clearances of glycosylated emulsions were similar and 3.5–4.0 times greater than that of Bare-emulsions. The hepatic uptake clearances of Man- and Fuc-emulsions were ~ 60 500 and 73 000 μl h^{-1}, respectively.

Hepatic cellular localization of [^{3}H]CHE-labelled emulsions

Figure 3 shows the hepatic cellular distribution of 0.5% emulsions after 30 min after intravenous administration. The distribution in NPC and PC was determined by the NPC/PC ratio after normalization of cell numbers. Man- and Fuc-emulsions were clearly preferentially localized in NPC with a high

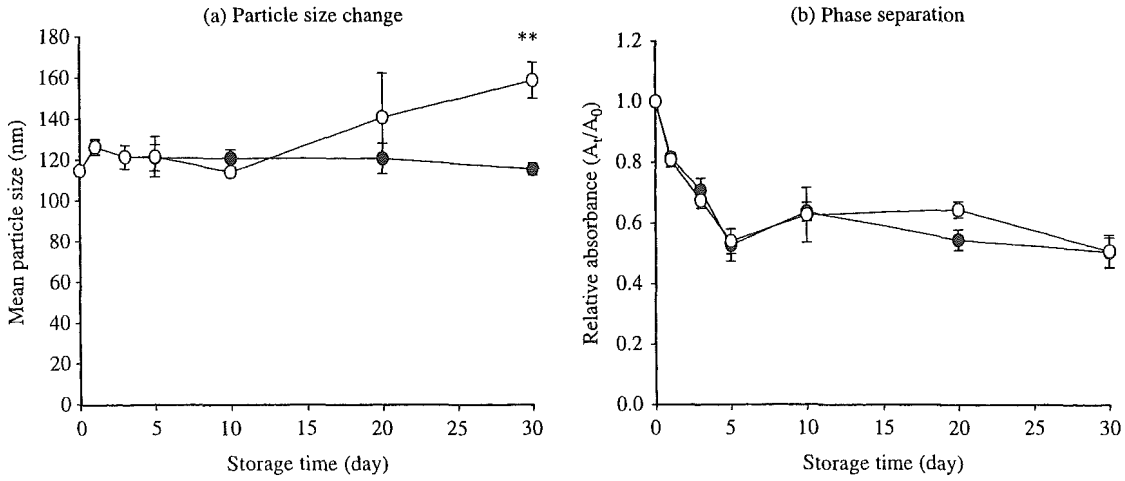

Figure 1. Stability of Fuc-emulsions (soybean oil:EggPC:Fuc-C4-Chol at weight ratio 70:25:5) on storage at 4°C (●) and room temperature (○). (a) Particle size change; (b) Phase separation. Relative absorbance was determined by the ratio of absorbance at a given time (A_t) to that at time zero (A_0). Each value represents the mean ± SD of three experiments. Statistically significant differences (**$p < 0.01$) for stability at room temperature compared with stability at 4°C.

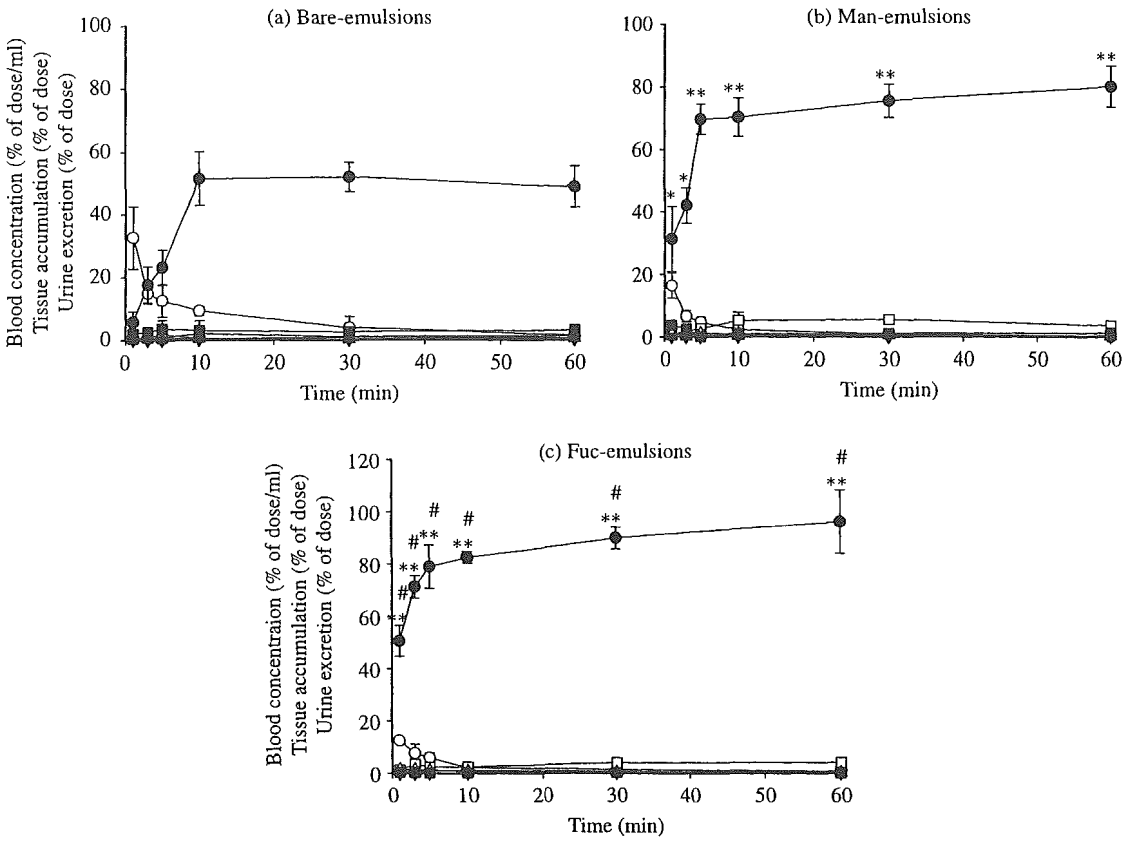

Figure 2. Blood concentration and tissue accumulation of [^{3}H]CHE-labelled emulsions following intravenous injection in mice. Radioactivity was determined in the blood (○), liver (●), spleen (□), heart (■), lung (△), kidney (▲), muscle (▽) and urine (▼). Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) for Man- or Fuc-emulsions and (#$p < 0.05$) for Fuc-emulsions compared with Bare- and Man-emulsions, respectively.

NPC/PC ratio, 2.0 and 2.9, respectively ($p < 0.01$) while Bare-emulsions were predominately localized in PC with a low NPC/PC ratio, 0.4 ($p < 0.05$).

Inhibition of hepatic uptake of [^{3}H]CHE-labelled emulsions

Five minutes after intravenous injection of emulsions, the inhibition of hepatic uptake was performed with or without pre-dosing of an excess amount (5%) of glycosylated liposomes (Gal-, Man- and Fuc-liposomes) and (20 mg kg^{-1}) glycosylated bovine serum albumin (Man- and Fuc-BSA), as shown in Figure 4. The hepatic uptakes of Man- and Fuc-emulsions were significantly inhibited by pre-dosing with all forms of glycosylated liposomes and BSA, but not Bare-emulsions ($p < 0.05$ or $p < 0.01$) (Figure 4). However, the hepatic uptake of Bare-emulsions was significantly suppressed by pre-dosing with only Bare-emulsions ($p < 0.05$).

Stability of large-size emulsions

Figure 5 shows the stability of large-size emulsions compared with Fuc-emulsions in terms of particle size change (Figure 5a) and phase separation (Figure 5b) on storage at 4°C and room temperature. The mean particle size of large-size emulsions significantly increased and tended to coalesce over time ($p < 0.05$). Concomitantly, large-size emulsions also

Table II. Area under the blood concentration-time curve (AUC) and tissue uptake clearance of [^{3}H]CHE-labelled emulsions after intravenous injection into mice.*

Emulsions	AUC* (% of dose h ml^{-1})	Tissue uptake clearance (μl h^{-1})						
		CL_{liver}	CL_{spleen}	CL_{heart}	CL_{lung}	CL_{kidney}	CL_{muscle}	CL_{urine}
Bare-emulsions	2.8	18 300	724	1067	161	129	26	12
Man-emulsions	1.2	60 500	4493	699	621	138	13	12
Fuc-emulsions	1.1	73 000	2150	211	764	968	45	61

*The AUC and tissue uptake clearances were calculated for the periods up to 10 min after intravenous injection. An average of three experiments is shown.

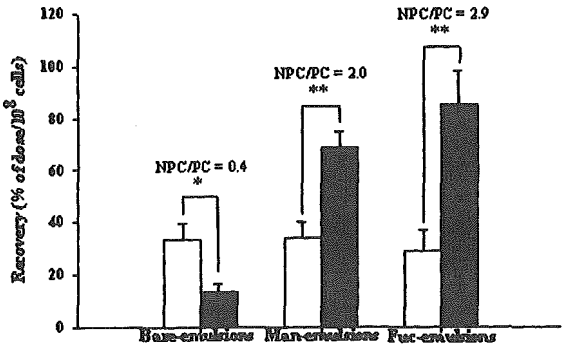

Figure 3. Hepatic cellular localization of [^{3}H]CHE-labelled emulsions after intravenous injection in mice. Radioactivity was determined 30 min post-injection in PC (□) and NPC (■). Each value represents the mean + SD of three experiments. Statistically significant differences (*$p < 0.05$, ** $p < 0.01$) between NPC and PC in each group.

separated instantly into two phases with low relative absorbance (A_t/A_0) compared with Fuc-emulsions ($p < 0.01$) and separated small oil droplets were observed on the first day of storage.

Discussion

To date, there have been few published reports about cell-selective delivery of lipid emulsions. The surface of (oil-in-water) lipid emulsions exhibits aqueous properties; thus, sugar moieties could be covered on the emulsion surface when Man- and Fuc-C4-Chol are added because sugar is a hydrophilic molecule and, therefore, the sugar moieties would be fixed on the emulsion surface. It was expected that Man- and Fuc-emulsions would be taken up by the mannose and fucose receptors on NPC. In fact, our previous study demonstrated that intravenously administered Gal-emulsions rapidly disappeared from the blood circulation and rapidly accumulated the liver up to ~ 80% of the dose within 10 min post-dosing. In addition, it was preferentially taken up by PC compared with NPC (Ishida et al. 2004). More recently, we have demonstrated that Gal-emulsions are taken up by asialoglycoprotein receptor-mediated endocytosis in a series of *in vitro* experiments (Managit et al. 2005). In this present study, we have described Man- and Fuc-emulsions with Man-C4-Chol and Fuc-C4-Chol as novel drug carriers for liver-NPC selective delivery systems.

The mean particle sizes of Bare-, Man- and Fuc-emulsions were ~ 110, 126 and 115 nm, respectively. Since the physicochemical properties of these three emulsions are almost identical, the effect of the emulsion ligand types could be analysed. First, we analyse the *in vivo* disposition characteristics of Man- and Fuc-emulsions after intravenous administration. As shown in Figure 2, we have demonstrated that ~50, 70–83% of the injected dose accumulated in the liver after intravenous administration of Bare-, Man- and Fuc-emulsions, respectively, up to 10 min. Pharmacokinetic

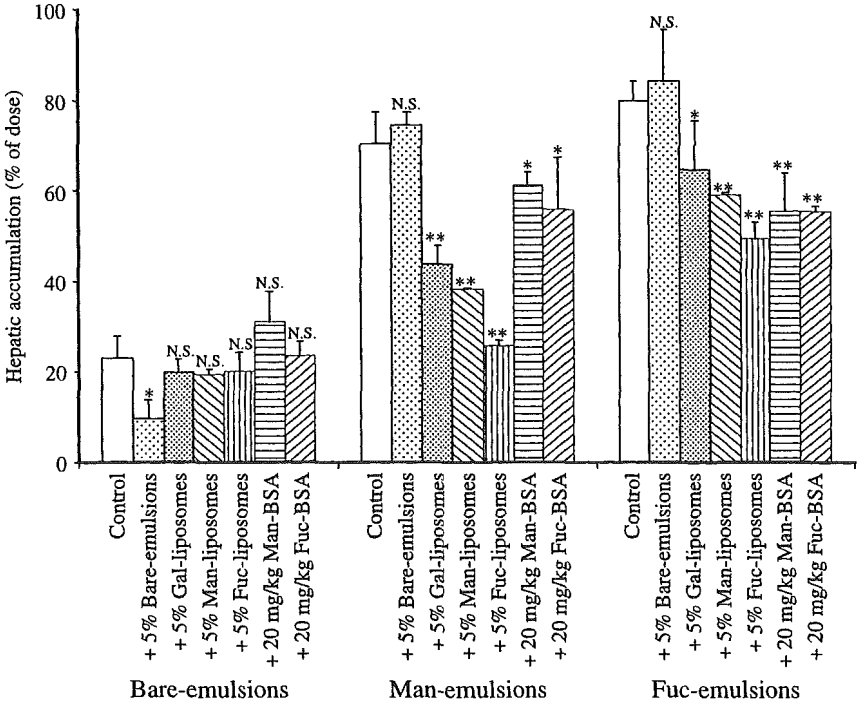

Figure 4. Inhibition of hepatic accumulation of [^{3}H]CHE-labelled emulsions following intravenous injection following pre-dosing with excess Bare-emulsions, glycosylated-BSA or liposomes in mice. Hepatic accumulation was determined at 5 min following injection. Each value represents the mean + SD of three experiments. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$; N.S., not significant) compared with the control group.

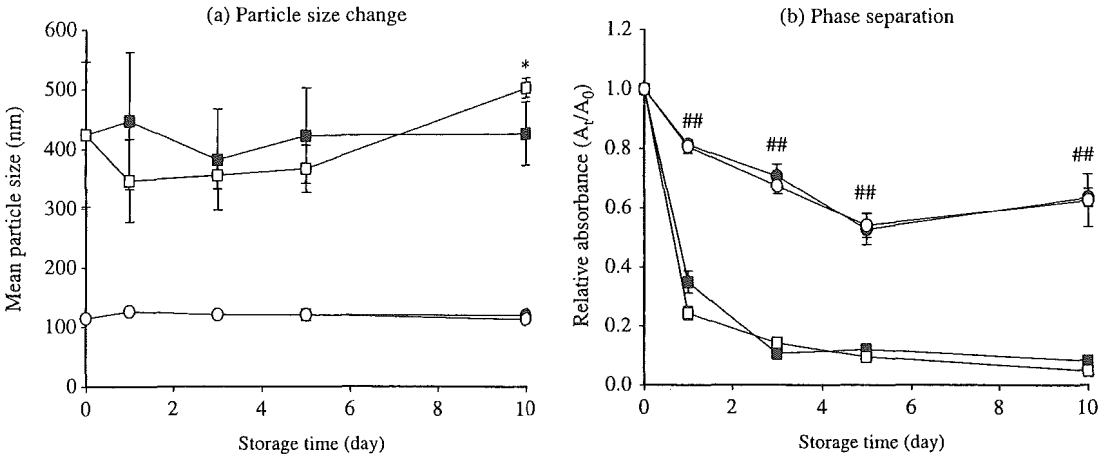

Figure 5. Stability of Fuc- (●, ○) and large-size (■, □) emulsions on storage time at 4°C (●, ■) and room temperature (○, □). (a) Particle size change; (b) Phase separation. Relative absorbance was determined by the ratio of absorbance at given times (A_t) to that at time zero (A_0). Each value represents the mean ± SD of three experiments. Statistically significant differences (* $p < 0.05$) for the particle size change at room temperature compared with that at 4°C and (##$p < 0.01$) for relative absorbance both at 4°C and room temperature of Fuc-emulsions compared with those of large-size emulsions.

analysis demonstrated that the hepatic uptake clearances of Man- (60 500 μl h^{-1}) and Fuc-emulsions (73 000 μl h^{-1}) were nearly equal to the hepatic plasma flow rate (66 000 μl h^{-1}) (Gerlowski and Jain 1983), indicating highly efficient hepatic cellular uptake of Man- and Fuc-emulsions by the liver.

In order to confirm whether the increased hepatic uptakes of Man- or Fuc-emulsions resulted from recognition by the mannose or fucose receptors on NPC, intra-hepatic distribution and competitive inhibition studies were performed. Man- and Fuc-emulsions were predominately recovered in the liver-NPC compared with Bare-emulsions (Figure 3). Furthermore, competitive inhibition experiments demonstrated that the hepatic uptakes of Man- and Fuc-emulsions were significantly inhibited by pre-dosing with excess glycosylated BSAs (Man- and Fuc-BSA) and liposomes (Man- and Fuc-liposomes) except for pre-dosing with Bare-emulsions (Figure 4). However, the uptake inhibition of Bare-emulsions was observed only following pre-dosing with an excess of Bare-emulsions, suggesting the uptake of Bare-emulsions via other receptors including low density lipoprotein (LDL) receptors and LDL related protein/α_2-macroglobulin receptors (LRP) (Rensen et al. 1997). These results lead us to propose that Man- and Fuc-emulsions are taken up by mannose and fucose receptors.

However, the uptake by NPC via mannose and fucose receptors appears to be a complex process. *In vitro* binding experiments using isolated receptor proteins have shown that the mannose receptors recognize mannose and fucose but not galactose (Haltiwanger and Hill 1986, Otter et al. 1992) and that the fucose receptors recognize fucose and galactose but not mannose (Lehrman et al. 1986). It is also known that the mannose receptors are present on both Kupffer cells (Kuiper et al. 1994) and sinusoidal endothelial cells (Otter et al. 1992, Magnusson and Berg 1993) and fucose receptors are present on Kupffer cells (Haltiwanger et al. 1986, Lehrman and Hill 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* uptake by NPC. As shown in Figure 4, the uptake of Fuc-emulsions was inhibited by Man-liposomes and Man-BSA. Similarly, the uptake of Man-emulsions was inhibited by Fuc-liposomes and Fuc-BSA. These phenomena correspond to our previous reports about Man- and Fuc-BSA (Ogawara et al. 1999, Opanasopit et al. 2001, Higuchi et al. 2004) and Man- and Fuc-liposomes (Kawakami et al. 2000a). Therefore, these observations lead us to believe that Man- and Fuc-emulsions are taken up by both mannose and fucose receptors to some extent. Since the uptake characteristics by NPC via mannose and fucose receptors are complicated, further studies will be needed to clarify their uptake mechanisms using primary cultured sinusoidal endothelial cells and Kupffer cells.

In this present study, the hepatic uptakes of Man- and Fuc-emulsions were significantly inhibited by 5% Gal-liposomes (Figure 4). These results may be explained by the observation in our previous report about the glycosylated liposomes (Kawakami et al. 2000a). That is to say, it was suggested by our previous study that Gal-liposomes with a low content of Gal-C4-Chol (0.5%) are recognized by asialoglycoprotein receptors on PC having a high affinity for these liposomes but, as the dose increases, saturation of the asialoglycoprotein receptors occurs and the contribution of the galactose particle receptors on NPC with a lower affinity becomes predominant (Kawakami et al. 2000a). Kuiper et al. (1994) have

suggested that the galactose particle receptors on Kupffer cells may be fucose receptors, since an antibody capable of inhibiting the uptake of galactose particles can react with a protein having the same molecular mass as the fucose receptors. Our data on the uptake inhibition of Fuc-emulsions by 5% Gal-liposomes strongly support their hypothesis.

As far as liver-NPC targeting is concerned, emulsions with a large particle size can be candidate carriers since they are rapidly cleared from the blood circulation and predominantly taken up by the RES particularly in the liver, spleen and lung (Seki et al. 2004). Moreover, our previous study (Takino et al. 1995) and a study by others (Fukui et al. 2003) suggested that large-size emulsions (200–300 nm) mainly accumulate in liver-NPC compared with smaller ones (less than 100 nm) after intravenous injection. We have described their pharmacokinetic parameters showing that the hepatic uptake clearance of large-size emulsions was 12900 $\mu l\, h^{-1}$ (Takino et al. 1998). In addition, the hepatic uptake clearances of Man- and Fuc-emulsions were very similar to the mouse hepatic plasma flow rate. These results lead us to conclude that Man- and Fuc-emulsions with Man-C4-Chol and Fuc-C4-Chol possess a higher affinity for liver-NPC than large-size emulsions.

To achieve effective carriers for efficient delivery systems, not only do powerful carriers need to be developed, but also the physical and chemical stability of the carriers need to be improved. In general, large-size emulsion formulations (without secondary emulsifiers) are prone to separate into two phases and coalesce into large oil droplets during storage (Tamilvanan 2004). To confirm the effect of particle size on their physical stability, the particle size change and phase separation of large-size emulsions were investigated. As shown in Figure 5, large-size emulsions rapidly separated into two phases during storage both at room temperature and 4°C compared with smaller ones, Fuc-emulsions. Furthermore, there was a wide size distribution of large-size emulsions, whereas there was no significant particle size change in smaller emulsions during storage.

As far as therapeutic applications are concerned, the particle sizes of the lipid emulsions affect the immune system after intravenous administration in mice and humans (Waitzberg et al. 2002). For example, perfulbron emulsions with a large particle size (more than 200 nm) were associated with a significant febrile response in rats (Keipert et al. 1994) and in humans (Noveck et al. 2000) after intravenous administration. On the other hand, Hamawy et al. (1985) have demonstrated that administration of lipid emulsions as total parenteral nutrition with a particle size of ~ 200–300 nm affected the host defense system against bacterial infection in rats. The high accumulation of lipid emulsions in macrophages after intravenous administration was suggested to result in a blockage of the RES. Therefore, the pathogens were only cleared slowly exposing the animals to infection. In this present study, we developed Man- and Fuc-emulsions with a small particle size (~ 110–130 nm) for liver-NPC selective delivery. All of these observations lead us to surmise that Man- and Fuc-emulsions are superior carriers than large-size emulsions as far as liver-NPC selective targeting is concerned.

In conclusion, we have demonstrated that Man- and Fuc-emulsions preferentially accumulate in the liver-NPC after intravenous injection in mice. The hepatic uptakes of Man- and Fuc-emulsions are mediated by mannose and fucose receptors. These observations demonstrate the feasibility of Man- and Fuc-emulsions for lipophilic drugs as liver-NPC selective targeting systems offering maximum therapeutic efficiency and minimum systemic toxicity.

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The role of dioleoylphosphatidylethanolamine (DOPE) in targeted gene delivery with mannosylated cationic liposomes via intravenous route

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Abstract

We have previously reported that mannosylated cationic liposome consisting with the mannosylated cationic cholesterol derivative Man-C4-Chol (Man) and dioleoylphosphatidylethanolamine (DOPE) (Man/DOPE) could deliver DNA to the liver by intravenous administration via mannose receptor-mediated endocytosis, however, rapid degradation in lysosomes might be a rate-limiting step in its gene transfection. In this study, we tried to evaluate the role of DOPE in in vivo gene transfer by comparing its transfection efficacy with mannosylated liposomes composed of Man and dioleoylphosphatidylcholine (DOPC) (Man/DOPC). In vitro studies showed that the cellular association of both liposome/pCMV-Luc complexes was almost the same, although Man/DOPE complex showed about 10-fold higher transfection activity than Man/DOPC complex. After intraportal administration into mice, Man/DOPE complex showed higher gene expression than Man/DOPC complex, suggesting that DOPE improves intracellular trafficking in target cells under in vivo conditions. An intravenous administration study demonstrated that Man/DOPE complex was accumulated in the liver more efficiently and achieved a higher gene expression in the liver than Man/DOPC complex. Thus, we conclude that the property of DOPE in mannosylated liposomes contributes to the efficient gene expression in the target site through enhanced distribution to the target site and intracellular sorting in the target cells under in vivo conditions.

Keywords: Gene delivery; Mannose receptor; Mannosylated liposomes; Cationic liposomes; DOPE

1. Introduction

Gene delivery to macrophages has great therapeutic potential in a variety of circumstances including DNA vaccination, treatment of genetic metabolic diseases like Gaucher's disease [1], and inhibition

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of HIV replication [2] in cells. Despite the high transfection efficiency of viral vectors, safety concerns have been raised in clinical trials because of their highly toxic nature [3]. Non-viral vectors, such as cationic liposomes and polymers, are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, more attractive vectors for clinical applications [4–6]. In spite of their usefulness, non-viral vectors lack cell specificity in delivery and transfection after systemic administration and it is necessary to attach a ligand that can be recognized by a specific mechanism on the target cells.

Mannose receptor-mediated targeting is a promising approach to achieve cell-specific delivery after systemic administration because (i) the expression of mannose receptors is restricted to the liver NPC and other macrophages, (ii) a complex entering the systemic circulation has easy access to the liver NPC, and (iii) the liver has a high blood flow. These physical and biological features give a mannosylated vector the opportunity to deliver plasmid DNA (pDNA) to the liver NPC via mannose receptor-mediated endocytosis.

To date, application of polylysine linked to mannose with naked pDNA has been reported to enhance gene expression in macrophages [7]. Nevertheless, the transfection efficiency of these vectors is limited due to endosomal or lysosomal degradation. One of the most promising forms of non-viral vectors developed so far is cationic liposome because they have several advantages including a high transfection efficiency, flexibility in introducing various functional components, and reproducibility.

Recently, we have developed a novel mannosylated cholesterol derivative, Man-C4-Chol, consisting of modified cationic liposomes with mannose moieties for NPC-selective gene delivery via mannose receptors on NPC [8]. Also, we have reported that the mannosylated cationic liposomes (Man liposome) we developed can deliver pDNA to liver non-parenchymal cells [8] and splenic DCs [9] and improve immune activation in DNA vaccines [9]. Although there are few vectors available, ours has been able to achieve targeted gene delivery to macrophages under in vivo conditions. However, the transfection activity of Man liposome still needs to be improved for more efficient gene therapy.

The physicochemical properties of the cationic carrier/pDNA complex have an impact on the distribution processes for pDNA delivery to the target cells (tissue distribution), internalization, intracellular sorting and resultant transfection activity in the target site [10–12]. Since our pharmacokinetic study revealed that more than 80% of the Man liposome/pDNA complex accumulated in the liver after intravenous administration, the rate-limiting step in transfection with Man liposomes was considered to be intracellular trafficking. In fact, our previous study investigating intracellular trafficking of the Man liposome/pDNA complex demonstrated that gene delivery with Man liposome facilitates the release of pDNA into the cytosolic space in the liver NPC after intravenous administration and a rapid sorting of the Man liposome/pDNA complex from endosomes to lysosomes was also observed [13]. These results suggest that modulation of its intracellular sorting needs to be improved in order to obtain efficient transfection with Man liposomes.

To modulate the intracellular distribution of pDNA, Man liposomes were prepared by combination of Man-C4-Chol and neutral phospholipid DOPE that has been considered to have a strong destabilizing effect on lipid bilayers due to its small head group and bulky acyl chains [14,15]. This results in the facilitation of fusion with the endosomal membrane of transfected cells and subsequent cytosolic release and enhanced gene expression [14–16]. Although it is essential for the further development of cationic liposome-mediated targeted gene delivery to evaluate the role of DOPE in targeted gene delivery systems, there is no information about the activity of DOPE following in vivo targeted gene delivery since few vectors have succeeded in in vivo targeted gene delivery.

Therefore, in this study, we tried to investigate the role of DOPE on targeted gene delivery using Man liposome via intravenous route mice. To achieve this, we prepared DOPE-containing Man liposomes (Man/DOPE liposome) and others containing dioleoylphosphatidylcholine (DOPC) with a choline head group instead of the ethanolamine head group on DOPE and changes in many properties including the pH sensitivity that DOPE possesses [15–17] (Man/DOPC liposome) and compared their transfection activity and biodistribution after in vivo application.

2. Materials and methods

2.1. Chemicals

N-(4-aminobutyl) carbamic acid *tert*-butyl ester was obtained from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate, DOPE, and DOPC were obtained from Sigma Chemicals (St. Louis, MO, USA) and Avanti Polar-Lipids (Alabaster, AL, USA), respectively. Fetal bovine serum (FBS) was obtained from Biowhittaker (Walkersville, MD, USA). Opti-MEM® I and other culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). Collagenase type I was purchased from Sigma (St. Louis, MO, USA). [α-^{32}P] dCTP was obtained from Amersham (Tokyo, Japan). Man-BSA was synthesized as previously reported [26,27]. All other chemicals were of the highest purity available.

2.2. Mice

Female ICR mice (4–5 week-old, 18–22 g) were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

2.3. Construction and preparation of plasmid DNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the *Hin*dIII/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the *E. coli* strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purity was confirmed by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

2.4. Synthesis of Man-C4-Chol

As reported previously [29], *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide was obtained from cholesteryl chloroformate and *N*-(4-aminobuthyl) carbamic acid *tert*-butyl ester. The product was reacted with 5 equivalents of 2-imino-2-methoxyethyl-1-thiomannoside [28] in pyridine containing 1.1 equivalents of triethylamine for 24 h. After evaporation of the reaction mixture in vacuo, the resultant material was suspended in water and dialyzed against water for 48 h. Then, the materials were lyophilized.

2.5. Particle size measurements

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

2.6. Harvesting and culture of macrophages

Female ICR mice weighing 18–22 g were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. Elicited macrophages were harvested from mice 4 days after intraperitoneal injection of 1 ml 2.9% thioglycolate medium (Nissui pharmaceutical Co. Ltd., Tokyo, Japan). The washed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Flow laboratories, Irvine, UK), 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, USA) 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.

2.7. Preparation of pLiposome/pDNA complexes for in vitro experiments

Man-C4-Chol was mixed with DOPE or DOPC at a molar ratio of 3:2 in chloroform and the mixture was dried, vacuum desiccated, and resuspended 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 produce liposomes. These were passed through a 0.45 μm filter to sterilize them. The lipid concentration was determined by phosphorus analysis and then adjusted (1 mg/ml) [30]. Cationic liposomes and pDNA (0.5 μg) in a 12 × 75 mm polystyrene tube were diluted with Opti-MEM I at various charge ratios before carrying out the transfection experiment.

2.8. In vitro uptake experiment

The macrophages were plated on a 12-well cluster dish at a density of 3×10^5 cells/cm^2. The culture medium was replaced with an equivalent volume of HBSS containing 1 kBq/ml [^{32}P] pDNA, 0.5 mg/ml cold DNA and cationic liposomes. After incubation for given time periods, the solution was immediately removed by aspiration, the cells were washed five times with ice-cold HBSS buffer and solubilized in 1 ml 0.3 N NaOH solution with 10% Triton X-100. The radioactivity was measured by liquid scintillation counting (LSC-500, Beckman, Tokyo, Japan) and the protein content was determined by DOJINDO protein quantification kit (Dojindo, Tokyo, Japan). The effect of co-presence of mannose was determined in the same system.

2.9. 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 bovine 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 of 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 assay was performed. Ten microliters of supernatant was 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 is 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 [31].

2.10. Preparation of Liposome/pDNA complexes for in vivo experiments

Man-C4-Chol was mixed with DOPE or DOPC at a molar ratio of 3:2 in chloroform and the mixture was dried, vacuum desiccated, and resuspended 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 produce liposomes. These were passed through a 0.45 μm filter to sterilize them. The lipid concentration was determined by phosphorus analysis and then adjusted (3.5 mg/ml) [30]. Then, liposome/pDNA complexes were prepared at the optimal charge ratio as described in our previous report [8,11,32]. Briefly, equal volumes of pDNA (50 μg) and stock liposome solution diluted with 5% dextrose solution to produce liposomes/pDNA complexes at a charge ratio of 1.0:2.3 (−:+) 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 then stored at 4 °C for 12 h.

2.11. Tissue distribution of the liposome/[^{32}P]pDNA complexes

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

2.12. In vivo delivery and gene expression

Five-week-old ICR mice were injected intravenously or intraportally with 300 μl of liposome/pDNA complexes using a 26-gauge or 30-gauge syringe needle, respectively. Six hours after injection, mice were killed and the lung, liver, kidney, spleen and heart were removed and assayed for gene expression. The organs were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). The lysis buffer was added in a weight ratio of 5 μl/mg for the liver samples or 4 μl/mg for the other organ samples. After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000×*g* for 10 min at 4 °C and 20 μl supernatant was analyzed to determine the luciferase activity using a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany). The protein concentration of each tissue extract was determined by the modified Lowry method. The luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

2.13. Cellular localization of luciferase activity in the liver

As described previously [8], 6 h after intravenous injection of liposome/pDNA complexes, each mouse was anesthetized with pentobarbital sodium (40–60 mg/kg) and the liver was perfused with 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 0.5% (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 ml/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 by gentle stirring. The dispersed cells were filtered through cotton mesh sieves, followed by centrifuging at 50×*g* for 1 min. The supernatant containing non-parenchymal cell (NPC) was similarly centrifuged twice more. The resulting supernatant was then centrifuged twice at 200×*g* for 2 min. Parenchymal cell (PC) and NPC were resuspended separately in ice-cold Hank's–HEPES buffer (2 ml for both PC and NPC). The cell numbers and viability were determined by the trypan blue exclusion method. After three cycles of freezing and thawing and following centrifugation at 10,000×*g* for 10 min at 4 °C, 20 μl supernatant was analyzed for luciferase activity in a luminometer A value lower than the blank+S.D. was treated as undetectable gene expression.

2.14. Interaction with erythrocytes

Fresh blood from ICR mice was collected in a heparinized syringe. Erythrocytes were washed three times on ice in order to remove serum proteins and resuspended in 150 mM NaCl, 10 mM HEPES buffer. Six hundred microliters of 0.1% erythrocyte suspension (erythrocyte volume/erythrocyte suspension volume %) was mixed with 50 ml of the complexes at a charge ratio of 1.0:2.3 (−:+) and incubated for 10 min at 37 °C.

2.15. Statistical analysis

Statistical comparisons were performed using a one-way ANOVA for multiple groups. $P<0.05$ was considered to be indicative of statistical significance.

3. Results

3.1. Sizes of liposomes and those complexed with pDNA

The particle size of each liposome/pCMV-Luc complex was measured with laser scattering particle size analyzer. The mean particle sizes of Man/DOPC and Man/DOPE liposome/pDNA complex were 110.4 ± 4.3 and 107.5 ± 10.2 nm, respectively. This result shows that DOPE does not have any effect on the particle size of the liposome/pDNA complex.

3.2. Cellular association of liposome/pDNA complexes in macrophages

To investigate the effect of function of DOPE in cellular association, the cellular association time-courses of Man/DOPE and Man/DOPC liposome/^{32}P-labeled pCMV-Luc complexes in elicited macrophages were evaluated. As shown in Fig. 1, Man/DOPE liposome complexes show a similar asso-

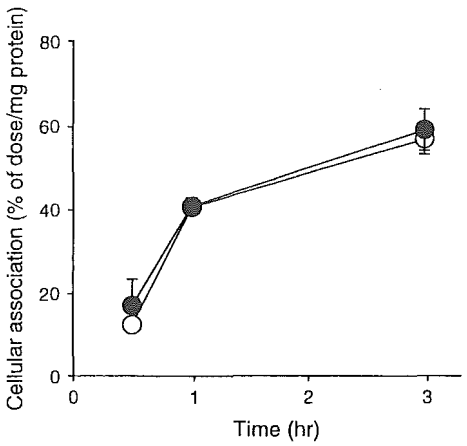

Fig. 1. Cellular association time-course of ^{32}P-labeled pDNA complexed with Man/DOPE (●) and Man/DOPC (○) liposomes in macrophage at 37 °C. pDNA (0.5 μg/ml) was complexed with cationic liposomes at the charge ratio of 1.0:1.6 (−:+). Each value represents the mean ± S.D. (n = 3).

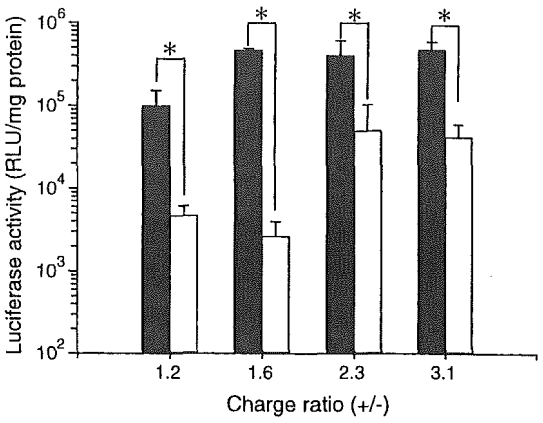

Fig. 2. Transfection activity of Man/DOPE (■) and Man/DOPC (□) liposomes/pDNA complexes at various charge ratios (+/−) in macrophages. pDNA (0.5 μg/ml) was complexed with Man/DOPE and Man/DOPC liposomes at various charge ratios. Each value represents the mean + S.D. (n = 3). Statistical analysis was performed by Student's t-test (*P < 0.05).

ciation profile to that of Man/DOPC liposome complexes. This result shows that the function of DOPE does not affect cellular association of mannosylated cationic liposome/pDNA complex to macrophages.

3.3. Transfection experiment and inhibition experiment in macrophages

We next investigated the effect of function of DOPE in transfection activity of mannosylated cationic liposome; the transfection activities of Man/DOPC and Man/DOPE liposome/pDNA complexes to elicited macrophages were evaluated. The pDNA and mannosylated-liposomes were complexed at charge ratios of 1.2, 1.6, 2.3 and 3.1 (+/−).The transfection activities of Man/DOPE liposome complexes were significantly higher than those of Man/DOPC liposome complexes at every charge ratio (Fig. 2). To evaluate the contribution of mannose receptor-related mechanism in transfection activities of both liposome/pDNA complexes, transfection activities of both liposome complexes were evaluated in the presence/absence of 1 mg/ml mannan (Fig. 3). The transfection efficiency of both complexes was significantly reduced in the presence of 1 mg/ml mannan in the medium. These results show that function of DOPE enhances transfection activities of mannosylated cationic liposome through improvement of intracellular trafficking in macrophages.

3.4. Biodistribution experiment in mice

We next investigate the role of DOPE in biodistribution of mannosylated cationic liposome/pDNA complex after intravenous administration. Fig. 4 shows the lung and liver accumulation after intrave-

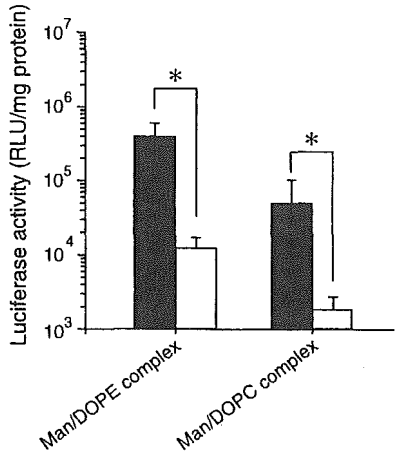

Fig. 3. Inhibitory effect of the co-presence of 1 mg/ml mannan on the transfection activity of liposome/pDNA complexes in macrophages. DNA (0.5 μg/ml) was complexed with Man/DOPE and Man/DOPC liposomes at a charge ratio of 1.0:2.3 (−:+). Cells were transfected with liposome/pDNA complexes in the presence (□) and absence (■) of 1 mg/ml mannan. Each value represents the mean + S.D. (n = 3). Statistical analysis was performed by Student's t-test (*P < 0.05).