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

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Received November 11, 2003; accepted March 5, 2004

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-

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0724-8741/04/0600-0932/0 © 2004 Plenum Publishing Corporation

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

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

where $AUC_{(0\sim t)}$ 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 [$AUC_{(0\sim t)}$].

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.

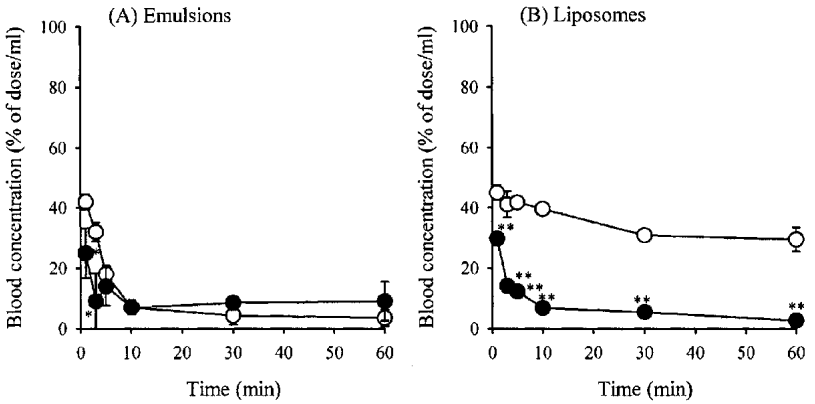

Fig. 1. Blood concentration of [^{3}H]-labeled (A) bare-(○) and Gal-(●) emulsions and (B) bare-(○) and Gal-(●) liposomes after intravenous injection into mice. Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

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-

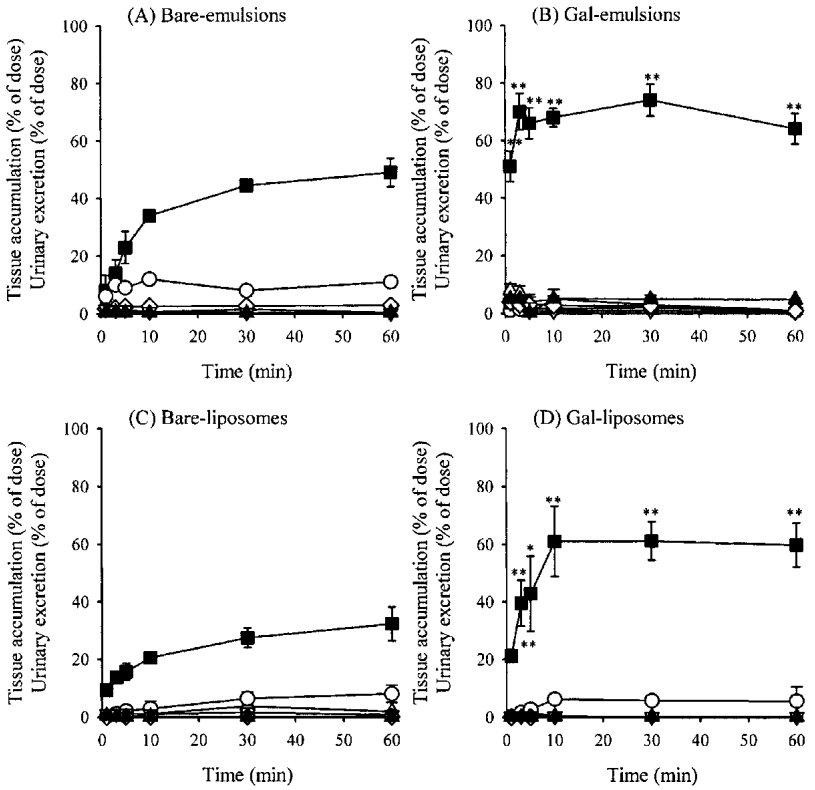

Fig. 2. Tissue accumulation of [^{3}H]-labeled (A) bare-emulsion, (B) Gal-emulsion, (C) bare-liposomes, and (D) Gal-liposomes after intravenous administration into mice. Radioactivity was determined in the liver (■), kidney (△), spleen (○), lung (▽), heart (◇), and urine (▲). Each value represents the 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.

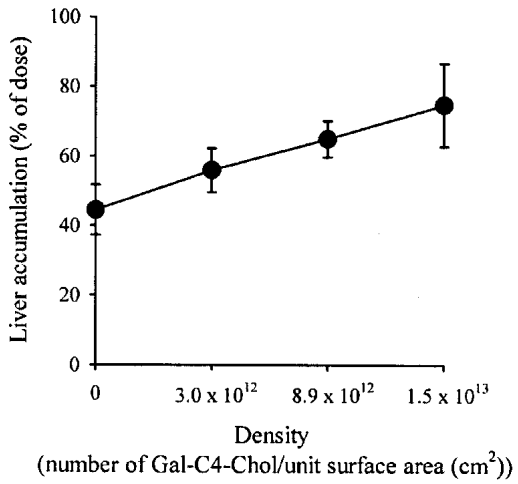

Fig. 3. Liver accumulation of [^{3}H]-labeled Gal-emulsions 30 min after intravenous administration into mice. Gal-emulsions were prepared with various amounts of Gal-C4-Chol. Each value represents the mean ± SD of three experiments.

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

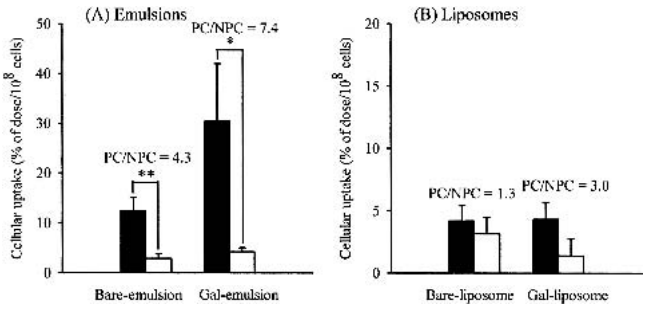

Fig. 4. Hepatic cellular localization of (A) [^{3}H]-labeled emulsions and (B) [^{3}H]-labeled liposomes after intravenous administration into mice. Radioactivity was determined 30 min postinjection in PC (■), and NPC (□). Each value represents the mean + SD of three experiments. Statistically significant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

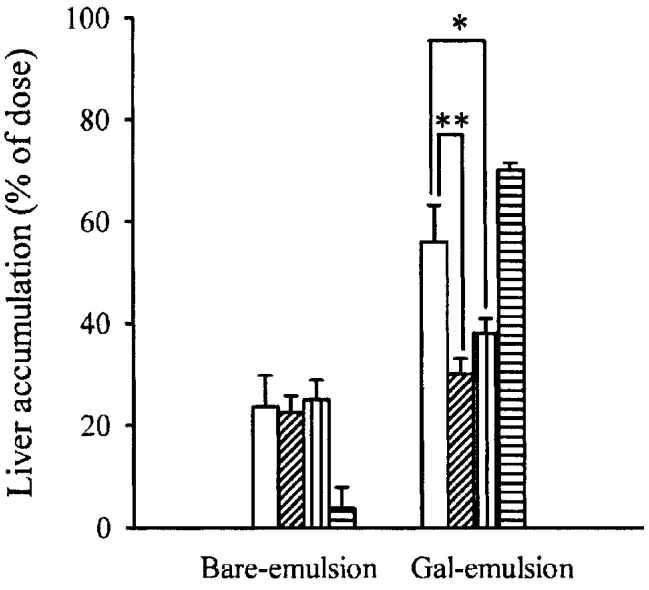

Fig. 5. Inhibition of liver uptake of [^{3}H]-labeled emulsions after intravenous preinjection of various compounds. Liver accumulation was determined at 5 min. Emulsions were injected without (□) or with preinjection of lactoferrin (▨), Gal-liposomes (▥), or an excess of bare-emulsions (▤). Each value 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 chylomicron 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

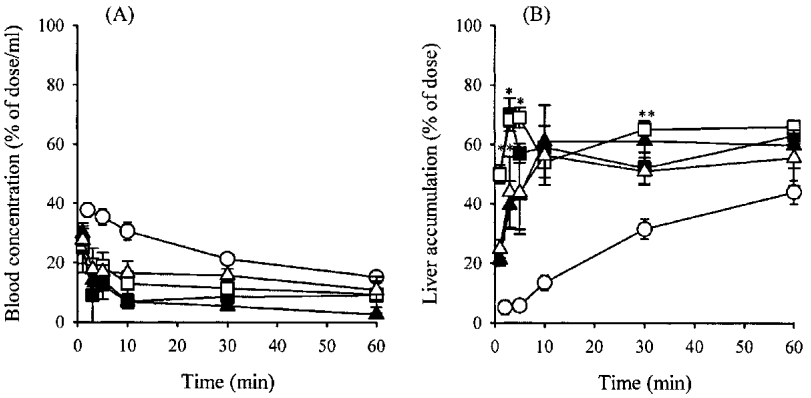

Fig. 6. (A) Blood concentration and (B) liver accumulation of [^{14}C]-labeled probucol dissolved in serum (○) or [^{3}H]CHE-labeled Gal-emulsions (■) and [^{3}H]CHE-labeled Gal-liposomes (▲) or [^{14}C]-labeled probucol incorporated Gal-emulsions (□) and [^{14}C]-labeled probucol incorporated Gal-liposomes (△) 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 [^{4}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.

ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by Health and Labor Sciences Research Grants for Research on Hepatitis and BSE from the Ministry of Health, Labor and Welfare of Japan.

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