Targeting Efficiency of Galactosylated Liposomes to Hepatocytes *in Vivo*: Effect of Lipid Composition

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Purpose. To investigate the effects of the lipid composition of galactosylated liposomes on their targeted delivery to hepatocytes.
Methods. Several types of liposomes with a particle size of about 90 nm were prepared using distearoyl-L-phosphatidylcholine (DSPC), cholesterol (Chol) and cholesten-5-yloxy-N-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl)formamide (Gal-C4-Chol), and labeled with [^{3}H]cholesterol hexadecyl ether. Their tissue disposition was investigated in mice following intravenous injection. The binding and internalization characteristics were also studied in HepG2 cells.
Results. Compared with [^{3}H]DSPC/Chol (60:40) liposomes, [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes exhibit extensive hepatic uptake. Separation of the liver cells showed that galactosylated liposomes are preferentially taken up by hepatocytes, whereas those lacking Gal-C4-Chol distribute equally to hepatocytes and nonparenchymal cells (NPC). Increasing the molar ratio of DSPC to 90% resulted in enhanced NPC uptake of both liposomes, suggesting their uptake via a mechanism other than asialoglycoprotein receptors. DSPC/Chol/Gal-C4-Chol (60:35:5) and DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes exhibited similar binding to the surface of HepG2 cells, but the former were taken up faster by the cells.
Conclusions. The recognition of galactosylated liposomes by the asialoglycoprotein receptors is dependent on the lipid composition. Cholesterol-rich galactosylated liposomes, exhibiting less non-specific interaction and greater receptor-mediated uptake, are better for targeting drugs to hepatocytes *in vivo*.

KEY WORDS: liposome; asialoglycoprotein receptor; hepatocytes; drug targeting; internalization.

INTRODUCTION

Drug carriers with specific ligands for the corresponding receptors on the cell surface are useful for targeted drug delivery. Among various ligands investigated so far, galactose has been shown to be a promising targeting ligand to hepatocytes (liver parenchymal cells) because the cells possess a large number of the asialoglycoprotein receptors that recognize the galactose units on the oligosaccharide chains of glycoproteins or on chemically galactosylated drug carriers (1). The receptor-ligand interaction is known to show a significant "cluster effect" in which a multivalent interaction results in extremely strong binding of ligand to the receptors (2). We have already demonstrated that the *in vivo* recognition of galactosylated macromolecules by asialoglycoprotein receptors correlates with the degree of galactose modification (3–5). A pharmacokinetic analysis of the tissue disposition patterns of galactosylated proteins in mice has clearly shown that the density of galactose units on the protein surface determines the affinity of galactosylated proteins for asialoglycoprotein receptor-mediated hepatic uptake (4). These results indicate that the drug targeting efficiency to hepatocytes using galactosylated macromolecular carriers is dependent on the degree of galactose modification.

Liposomes are another class of drug carriers that have several advantages such as ease of preparation and a large capacity for drug loading (6). In previous papers, we synthesized a galactosylated cholesterol derivative and formulated it into neutral or cationic liposomes to obtain the galactosylated counterparts (7–11). These galactosylated liposomes were able to effectively deliver prostaglandin E_1, probucol and plasmid DNA to hepatocytes *in vivo*, indicating that the galactose units on the liposome surface can increase the affinity of the liposome for asialoglycoprotein receptors on hepatocytes. To ensure hepatocyte-specific targeting of liposomes by galactosylation, however, properties other than galactose density should also be controlled, such as the size and electric charge. The recognition of liposomes by the mononuclear phagocyte system (MPS) is known to be dependent on its lipid composition (12,13), which is an important factor in determining the surface properties of liposomes. Therefore, the clearance of liposomes from the circulation *in vivo* is highly dependent on the lipid composition (14). The surface properties of liposomes, such as the rigidity of the membrane and co-existence of two or more phases, is determined by their composition, which might affect the clustering of galactose units incorporated into a liposome formulation.

To understand the effects of the lipid composition of galactosylated liposomes on their targeted delivery to hepatocytes *in vivo*, various liposomes with or without galactose units were prepared involving different lipid mixing ratios: distearoyl-L-phosphatidylcholine (DSPC), cholesterol (Chol) a and galactosylated cholesterol derivative, and cholesten-5-yloxy-N-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl)formamide (Gal-C4-Chol). The liposomes were adjusted to a size of about 90 nm in diameter and radiolabeled with [^{3}H]cholesteryl hexadecyl ether (CHE). The tissue disposition of each type of liposome was studied in mice after intravenous injection. The distribution of liposomes within the liver (i.e., to hepatocytes or liver nonparenchymal cells (NPC)), was examined after digestion of the liver by collagenase. In addition, the internalization of liposomes was investigated in HepG2 cells *in vitro*. We report here that the lipid composition of the galactosylated liposomes is important for their recognition by asialoglycoprotein receptors.

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ABBREVIATIONS: DSPC, distearoyl-L-phosphatidylcholine; Chol, cholesterol; Gal-C4-Chol, cholesten-5-yloxy-N-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl)formamide; NPC, nonparenchymal cells; MPS, mononuclear phagocyte system; CHE, cholesteryl hexadecyl ether; IME-thiogalactoside, 2-imino-2-methoxyethyl-1-thiogalactoside; Gal-BSA, galactosylated bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CL_{liver}, liver uptake clearance; PC, phospholipid.

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MATERIALS AND METHODS

Chemicals

N-(4-aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). DSPC and cholesteryl chloroformate was purchased from Sigma Chemical Co., (St. Louis, MO). Chol and Clear-Sol I were obtained from Nacalai Tesque (Kyoto, Japan), and Soluene 350 was purchased from Packard (Groningen, Netherlands). [^{3}H]CHE was purchased from NEN Life Science Products, Inc. (Boston, MA). 2-imino-2-methoxyethyl-1-thiogalactoside (IME-thiogalactoside) and galactosylated bovine serum albumin (Gal-BSA) were synthesized as reported previously (7). All other chemicals were of the highest purity commercially available.

Synthesis of Gal-C4-Chol

Gal-C4-Chol was prepared as reported previously (7). Briefly, cholesteryl chloroformate and N-(4-aminobutyl)carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. A solution of trifluoroacetic acid and chloroform was added dropwise and the mixture was stirred for 4 h at 4°C. The solvent was evaporated to obtain N-(4-aminobutyl)-(cholesten-5-yloxyl)formamide then combined with IME-thiogalactoside and this mixture was then stirred for 24 h at room temperature. After evaporation, the resultant material was suspended in water, dialyzed against distilled water for 48 h (12 kDa cut-off dialysis tubing), then lyophilized.

Preparation of Liposomes

A mixture of DSPC and Chol, with or without Gal-C4-Chol, was dissolved in chloroform and evaporated to dryness in a round-bottomed flask. Then, the lipid film formed was resuspended in 5 ml sterile phosphate-buffered saline (pH 7.4). After hydration, the dispersion was sonicated for 5–10 min in a bath sonicator to form liposomes. Each suspension was extruded through a 100 nm pore size polycarbonate membrane at 60°C using an extruder (Northern Lipids, Vancouver, Canada). The concentration of Chol in each formulation was measured with a cholesterol E-test Wako kit (Wako Pure Chemicals, Osaka, Japan) and the lipid concentration was adjusted to 5 mg/ml.

The particle size of the liposomes was measured in a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan). Radiolabeling of the liposomes was performed by addition of [^{3}H]CHE to the lipid mixture before formation of a thin film layer.

Tissue Disposition Study

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. Five-week-old male ddY mice (25–27 g body weight) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). [^{3}H]liposomes at a dose of 25 mg/kg and 60 kBq/kg were injected into a tail vein. At predetermined time points, the mice were killed, and blood and urine were collected. The liver, kidney, spleen, heart, and lung were collected, washed with saline, blotted dry, and weighed. Ten microliters blood, 200 μl urine, and a piece of each tissue were digested with 0.7 ml Soluene-350 by incubating overnight at 45°C. Then, isopropanol (200 μl), 30% hydroperoxide (200 μl), 5 M HCl (100 μl) and, finally, Clear-Sol I (5 ml) were added. The samples were stored overnight and the radioactivity was measured using a scintillation counter (LSA-500, Beckman, Tokyo, Japan).

In different sets of experiments, 20 mg/kg Gal-BSA or 250 mg/kg DSPC/Chol liposome was first injected into mice. Then [^{3}H]DSPC/Chol/Gal-C4-Chol liposome at a dose of 25 mg/kg and 60 kBq/kg was injected and the liver was excised after 5 min.

Calculation of Clearance

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

$$\frac{dX_t}{dt} = CL_{uptake} C_b \tag{1}$$

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

$$X_t = CL_{uptake} AUC_{0-t} \tag{2}$$

where $AUC_{0\text{-}t}$ represents the area under the blood concentration-time curve from time *0* to *t*. Eq. (2) divided by C_b gives

$$\frac{X_t}{C_b} = \frac{CL_{uptake} AUC_{0-t}}{C_b} \tag{3}$$

The CL_{uptake} can then be obtained from the initial slope of a plot of X_t/C_b vs. $AUC_{0\text{-}t}/C_b$.

Intrahepatic Disposition

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and injected intravenously with [^{3}H]liposomes (25 mg/kg and 300 kBq/kg). The body temperature of the mice was kept at around 37°C with a heat lamp during the experiment. At 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 and then with a 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. At the end of the perfusion, the liver was excised and its capsular membranes were removed. The cells were dispersed in ice-cold Hank's-HEPES buffer containing 0.1% BSA by gentle stirring. The dispersed cells were filtered through a cotton mesh sieve, followed by centrifugation at 50 × *g* for 1 min. The pellets containing hepatocytes were washed twice with Hank's-HEPES buffer by centrifuging at 50 × *g* for 1 min. The supernatant containing NPC was similarly centrifuged at least twice. The resulting supernatant was then centrifuged twice at 200 × *g* for 2 min. Hepatocytes and NPC were resuspended separately in ice-cold Hank's-HEPES buffer (4 ml for hepatocytes and 1.8 ml for NPC). The cell number and viability

were determined by the trypan blue exclusion method. The cells were suspended in 0.5 ml saline and the radioactivity assayed as described above.

Uptake by HepG2 Cells

The HepG2 cells were plated on a 12-well cluster dish at a density of 2×10^5 cells/3.8 cm^2 and cultivated in 800 μl DMEM supplemented with 10% FBS. Twenty-four hours later, the culture medium was replaced with an equivalent volume of HBSS containing [^{3}H]liposomes (0.25 mg/ml, 1.8 kBq/ml). For the inhibition study, 20 mM galactose was added to the liposome solution. After incubation for 1 h at 37°C, the solution was removed by aspiration, and the cells were washed five times with ice-cold HBSS buffer. For separation of the internalized and surface bound liposomes, the cells were washed three times with acetate buffer (pH 4) to remove the liposomes bound to the cell surface. The cells were then solubilized in 0.5 ml 1 M NaOH and the radioactivity was assayed as above. The protein content of each sample was determined by a modification of the Lowry method. In another set of experiments, the cells were pre-incubated with HBSS containing 10 mM NaN_3 for 20 min prior to the addition of liposomes.

Statistical Analysis

Differences were statistically evaluated by one-way analysis of variance followed by the Student-Newmann–Keuls multiple comparison test. The level of significance was set at $p < 0.05$.

RESULTS

Liposome Size

Table I summarizes the lipid composition and particle size of the liposomes prepared. All liposomes were similar in size (average diameter approximately 90 nm). The size of the liposomes remained constant over one month at 4°C.

Tissue Disposition of Liposomes after Intravenous Injection

Figure 1 shows the tissue disposition of [^{3}H]DSPC/Chol (60:40), [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5), [^{3}H]DSPC/Chol (90:10), and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes in mice. When intravenously injected, [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) were rapidly taken up by the liver (Fig.1), as well as other galactosylated liposomes (data not shown). On the other hand, [^{3}H]DSPC/Chol (60:40) liposomes exhibited prolonged retention in the blood circulation (Fig. 1A). However, [^{3}H]DSPC/Chol (90:10) liposomes were taken up by the liver almost as fast as their galactosylated counterpart (Fig. 1C and D).

Table I. Lipid Composition and Particle Size of Liposomes

Liposome (molar ratio)	Particle size (mm)[a]
DSPC/Chol (50:50)	88.7 ± 10.5
DSPC/Chol (60:40)	94.6 ± 6.3
DSPC/Chol (70:30)	89.4 ± 6.7
DSPC/Chol (90:10)	91.7 ± 12.8
DSPC/Chol/Gal-C4-Chol (50:45:5)	94.7 ± 7.9
DSPC/Chol/Gal-C4-Chol (60:35:5)	84.0 ± 14.8
DSPC/Chol/Gal-C4-Chol (70:25:5)	93.9 ± 9.7
DSPC/Chol/Gal-C4-Chol (90:5:5)	85.8 ± 10.8

[a] The particle size of the liposomes was measured using a dynamic light scattering spectrophotometer. Results are expressed as the mean ± SD of three determinations.

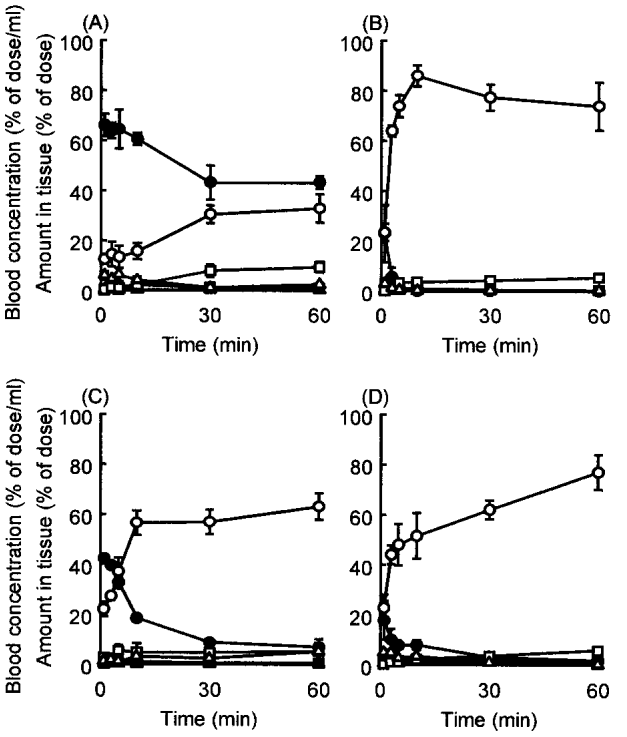

Fig. 1. Time-courses of the concentration in blood and amounts in tissues of ^{3}H-radioactivity after intravenous injection of (A) [^{3}H]DSPC/Chol (60:40), (B) [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5), (C) [^{3}H]DSPC/Chol (90:10) and (D) [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes into mice at a dose of 25 mg/kg. Results are expressed as the mean ± SD of three mice. Keys: (●) blood; (○) liver; (■) kidney; (□) spleen; (▲) heart; (△) lung.

Pharmacokinetic Analysis of Liposomes

Figure 2 summarizes the liver uptake clearance (CL_{liver}) of all the liposomes investigated after intravenous injection into mice. The CL_{liver} of liposomes lacking galactose units was much lower than that of galactosylated liposomes. Among the control liposomes ([^{3}H]DSPC/Chol liposomes), the DSPC/Chol (90:10) liposomes with the smallest Chol ratio had the highest CL_{liver}.

The CL_{liver} of [^{3}H]DSPC/Chol/Gal-C4-Chol (50:45:5), [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5), and [^{3}H]DSPC/Chol/Gal-C4-Chol (70:25:5) liposomes was, respectively, 51.4, 52.2, and 48.8-times greater than that of the counterparts without Gal-C4-Chol, whereas that of [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes was only 2.9-times greater than that of [^{3}H]DSPC/Chol (90:10) liposomes. Although all the galactosylated liposomes tested were equivalent in terms of the number of galactose units per liposome, [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes had the highest CL_{liver}.

Cellular Localization of Liposomes in the Liver

Figure 3 shows the distribution of [^{3}H]DSPC/Chol (60:40), [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5), [^{3}H]DSPC/Chol

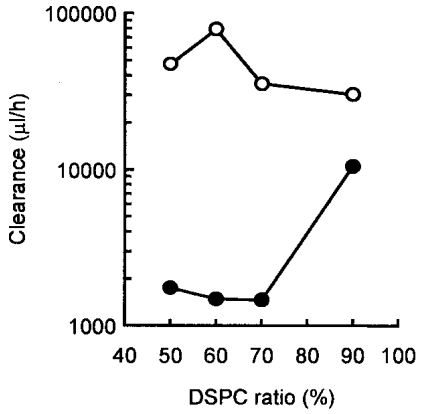

Fig. 2. Hepatic clearance (CL_{liver}) of liposomes after intravenous injection in mice at a dose of 25 mg/kg. The CL_{liver} was calculated by dividing the amount in the liver at 10 min by the area under the blood concentration-time curve up to the same time as described in Materials and Methods. The CL_{liver} values were plotted against the percentage of DSPC in liposome total lipids. Keys: (●) liposomes without Gal-C4-Chol; (○) liposomes with Gal-C4-Chol.

(90:10), and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes between hepatocytes and NPC at 30 min after intravenous injection. [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes were taken up preferentially by hepatocytes compared with NPC (hepatocytes/NPC ratio of 15), whereas [^{3}H]DSPC/Chol (60:40) liposomes were taken up to an equal extent by both hepatocytes and NPC. On the other hand, NPC uptake was marked in the case of [^{3}H]DSPC/Chol (90:10) liposomes. [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes exhibited greater distribution to hepatocytes compared with [^{3}H]DSPC/Chol (90:10) liposomes, but the uptake by hepatocytes did not exceed that in NPC. The hepatocyte/NPC ratio was calculated to be 0.78, which was much smaller than that for [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes.

Effect of Gal-BSA or DSPC/Chol (90:10) Liposomes on Hepatic Uptake of Galactosylated Liposomes

Figure 4 shows the effect of preinjection of a large amount of Gal-BSA or DSPC/Chol (90:10) liposomes on the hepatic uptake of [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and

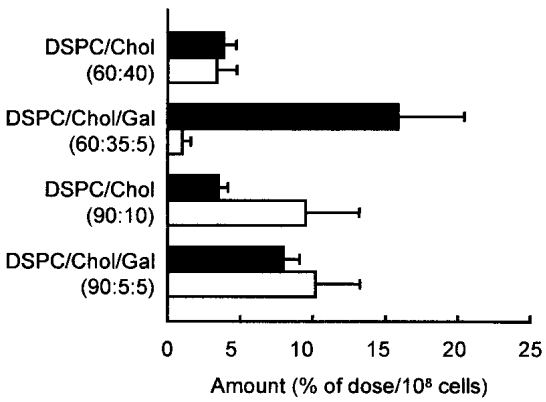

Fig. 3. Distribution of ^{3}H-radioactivity between hepatocytes and liver NPC at 30 min after intravenous injection of [^{3}H]liposomes into mice at a dose of 25 mg/kg. Results are expressed as the mean ± SD of three mice. Key: (filled) hepatocytes; (open) NPC.

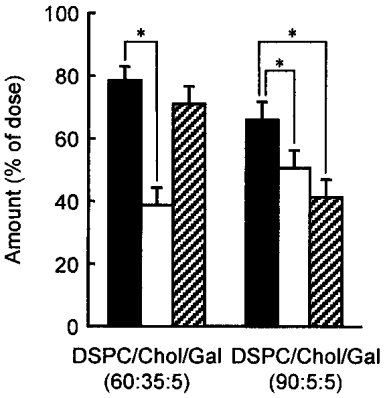

Fig. 4. Amount of ^{3}H-radioactivity in the liver after intravenous injection of [^{3}H]liposomes with or without preinjection of 20mg/kg Gal-BSA or 250mg/kg DSPC/Chol (90:10) liposomes. The distribution was examined at 5 min after intravenous injection of [^{3}H]liposomes. Results are expressed as the mean ± SD of three mice. Keys: (filled) no preinjection; (open) preinjection of Gal-BSA; (hatched) preinjection of DSPC/Chol liposomes. * Statistically significant differences ($p < 0.05$) from each no preinjection group.

[^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes. Gal-BSA significantly inhibited the hepatic uptake of both [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes. The extent of the inhibition by Gal-BSA, however, was greater for [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes than for [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes.

Preinjection of DSPC/Chol (90:10) liposomes had no significant effects on the hepatic uptake of [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes whereas the injection of DSPC/Chol (90:10) liposome prior to [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes significantly inhibited the hepatic uptake of the latter.

Uptake by HepG2 Cells

Figure 5 shows the *in vitro* uptake of [^{3}H]liposomes in HepG2 cells. The uptake of [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) lipo-

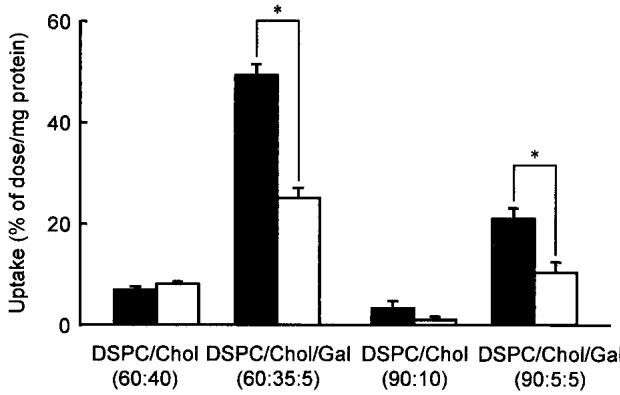

Fig. 5. Uptake of [^{3}H]liposomes by HepG2 cells. Cells were incubated with each type of [^{3}H]liposome with or without 20 mM galactose. The amount of ^{3}H-radioactivity associated with the cells was measured following 1 h of incubation. Results are expressed as the mean ± SD of three wells. Keys: (filled) control; (open) +20 mM galactose. * Statistically significant differences ($p < 0.05$).

somes was greater than that of [^{3}H]DSPC/Chol (60:40) and [^{3}H]DSPC/Chol (90:10) liposomes, respectively. The uptake of the galactosylated liposomes was significantly inhibited by the addition of 20mM galactose, whereas no change was observed with control liposomes. [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes showed significantly higher uptake than [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes.

The amount of surface binding and internalization of the galactosylated liposomes was evaluated according to an acid-wash procedure (Fig. 6). The surface binding of both [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes was similar although the amount of [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes internalized was greater than that of [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes. When the cells were treated with a metabolic inhibitor, NaN_3, the amount associated with the cells was similar in both galactosylated liposomes, and comparable with the acid-washable amounts under normal conditions.

DISCUSSION

Asialoglycoprotein receptor-mediated targeting of pharmaceuticals to hepatocytes is a promising approach to achieve cell (hepatocyte)-specific delivery after systemic administration because (i) the asialoglycoprotein receptors are specifically expressed in hepatocytes, (ii) molecules entering the systemic circulation easily get access to the cells through the discontinuous endothelium of the liver, and (iii) the liver has a high blood flow. These physiologic and biologic features of the liver and hepatocytes give galactosylated carriers the opportunity to deliver drugs to hepatocytes via asialoglycoprotein receptor-mediated endocytosis.

As far as the interaction of a galactosylated ligand with the receptors is concerned, the affinity of the ligand seems to be governed by the valency of the galactose residues and their

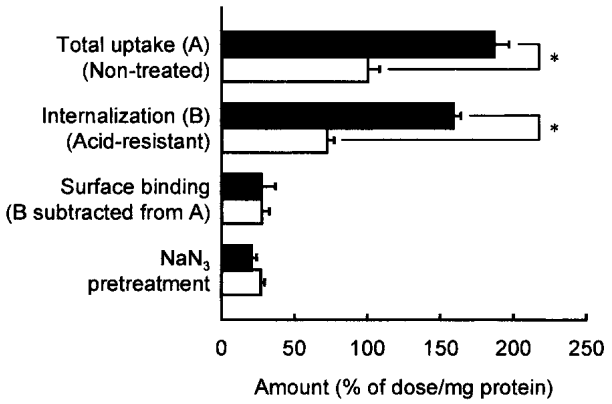

Fig. 6. Amount of [^{3}H]galactosylated liposomes associated with HepG2 cells. Cells were incubated with [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) or [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes. At 1 h, the cells were washed with an acid buffer to separate the surface bound liposomes. The difference in cellular association between acid-treatment and no treatment was regarded as the amount associated with the cell surface. In another group, the cells were pre-incubated with HBSS containing 10 mM NaN_3 for 20 min prior to the addition of liposomes. Results are expressed as the mean ± SD of three wells. Keys: (filled) [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposome; (open) [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposome. * Statistically significant differences ($p < 0.05$).

appropriate spacing. Clustering of galactosides greatly enhances the affinity for the receptor in the following order: tetra- > tri- >> bi- >> mono-antennary galactosides (17). Such an effect can be explained by the simultaneous occupation of the carbohydrate recognition domains of the receptor and optimal structures of cluster galactosides for binding have been proposed (18). Based on these findings, various oligosaccharides containing multiple galactose terminals have been developed as "homing" devices with a high affinity for hepatocytes (19–21). These synthetic oligosaccharide-containing molecules as well as naturally occurring asialoglycoproteins are believed to have a high affinity for asialoglycoprotein receptors.

In previous papers, we have described the modification of various macromolecules with IME-thiogalactoside to obtain galactosylated derivatives. The pharmacokinetic study of galactosylated macromolecules in mice revealed that a derivative having a sufficient number of galactose units is very efficiently recognized by the receptors *in vivo* and is taken up by the liver at a rate equal to the hepatic plasma flow (3–5). These results indicate that, although monosaccharides such as IME-thiogalactoside have a much weaker receptor affinity than oligosaccharides, the clustering of monosaccharides on a carrier can compensate for this weak affinity. The estimated density of galactose units on the protein surface was found to determine the affinity of galactosylated proteins for the asialoglycoprotein receptors *in vivo* (4). Based on these findings, we have designed a series of mono-glycosylated derivatives of cholesterol to obtain glycosylated lipophilic carriers such as liposomes and lipid emulsions (7,22,23). Galactosylated liposomes consisting of Gal-C4-Chol and other lipids have been shown to be promising carriers for the delivery of drugs or genes to asialoglycoprotein receptor-positive cells (7–11,22,23).

As clearly shown with galactosylated proteins (4), extensive modification of liposomes with Gal-C4-Chol, for example, 5 mol % of total lipid, can give them the ability to target hepatocytes (9,10). In the present study, both galactosylated liposomes, i.e., [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes, exhibited marked accumulation in the liver following intravenous injection in mice. However, the uptake of [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes was not selective for hepatocytes, whereas that of [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes was highly selective for these cells. The introduction of a galactose moiety onto a liposome might not necessarily be a universal approach to endow the liposome with the ability to target hepatocytes, because the tissue disposition of galactosylated liposomes is determined not only by the recognition by asialoglycoprotein receptors, but by the other interactions within the body after systemic administration. Low-Chol liposomes are known to have a very short half-life in the circulation, largely due to their extensive uptake by MPS. On the other hand, high-Chol liposomes (30–50 mol % of total lipid) are relatively stable in the circulation (14). Semple *et al.* (13) found that cholesterol-free DSPC liposomes bind significant amounts of serum proteins, whereas cholesterol-rich DSPC liposomes are much less active. These characteristics of low-Chol DSPC liposomes would explain the uptake of [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes by liver NPC (Fig. 3). Inhibition studies clearly showed that the hepatic uptake of [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes takes place by at least two different pathways:

galactose-specific hepatocyte uptake and DSPC/Chol (90:10) liposome-sensitive NPC uptake.

The uptake experiments using HepG2 cells *in vitro* were performed to gain further insights into the receptor-mediated uptake of the two different galactosylated liposomes. Compared with galactose-free control liposomes, both [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) and [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes showed greater and galactose-inhibitable cellular uptake. However, the uptake by HepG2 cells was greater for [^{3}H]DSPC/Chol/Gal-C4-Chol (60:35:5) liposomes than for [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes. The separation of surface-bound liposomes from their internalized counterparts by acid washing shows that the difference in their uptake can be fully explained by the amount internalized. In a previous paper (25), we analyzed the hepatic uptake of Gal-BSAs with varying numbers of galactose units in isolated, perfused rat liver, and found that the internalization rate of Gal-BSA was greater for derivatives with many galactose residues (i.e., 17 or 36 galactose units/BSA) than for one with only 10 residues. In this case, however, it should be noted that the two galactosylated liposomes investigated contain the same concentration of galactose units. A possible explanation for this is that distribution of Gal-C4-Chol in bilayers may be heterogeneous so that it can affect the rate of internalization via asialoglycoprotein receptor-mediated endocytosis. At lower Chol concentrations, a lateral-phase separation occurs in DSPC/Chol liposomes, (i.e., a pure phospholipid (PC) phase and a PC/Chol mixture phase), whereas high-Chol liposomes possesses a homogeneous, liquid ordered structure (26,27). Such a structural difference depending on the Chol concentration would affect the distribution and mobility of Gal-C4-Chol in bilayers. It has also been reported that membrane lipids are rearranged in bilayers following an interaction with plasma proteins (28), ganglioside-containing liposomes (29), and a polycation (30). This would depend on the rate of lateral diffusion in liposomal bilayers. Overall, slower accumulation of [^{3}H]DSPC/Chol/Gal-C4-Chol (90:5:5) liposomes in the liver compared with Chol-rich galactosylated liposomes (Fig. 1) could also be explained by inappropriate distribution of galactose units on the liposomal surface, although a partial shielding of galactose units by serum protein binding cannot be ruled out.

In conclusion, the lipid composition of galactosylated liposomes has been shown to be important for their targeted delivery to hepatocytes. Besides non-specific serum protein binding, the rate of internalization via a specialized uptake process is affected. The Chol-rich galactosylated liposomes appear to be promising carriers that can expand the specificity of their delivery to the target.

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REFERENCES

1. G. Ashwell and J. Harford. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* **51**:531–554 (1982).
2. Y. C. Lee, R. R. Townsend, M. R. Hardy, J. Lönngren, J. Arnarp, M. Haraldsson, and H. Lönn. Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin. Dependence on fine structural features. *J. Biol. Chem.* **258**:199–202 (1983).
3. M. Nishikawa, H. Hirabayashi, Y. Takakura, and M. Hashida. Design for cell-specific targeting of proteins utilizing sugar-recognition mechanism: effect of molecular weight of proteins on targeting efficiency. *Pharm. Res.* **12**:209–214 (1995).
4. M. Nishikawa, C. Miyazaki, F. Yamashita, Y. Takakura, and M. Hashida. Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. *Am. J. Physiol.* **268**:G849–G856 (1995).
5. H. Hirabayashi, M. Nishikawa, Y. Takakura, and M. Hashida. Development and pharmacokinetics of galactosylated poly-L-glutamic acid as a biodegradable carrier for liver-specific drug delivery. *Pharm. Res.* **13**:880–884 (1996).
6. T. M. Allen. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol. Sci.* **15**:215–220 (1994).
7. S. Kawakami, F. Yamashita, M. Nishikawa, Y. Takakura, and M. Hashida. Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. *Biochem. Biophys. Res. Commun.* **252**:78–83 (1998).
8. S. Kawakami, S. Fumoto, M. Nishikawa, F. Yamashita, and M. Hashida. In vivo gene delivery to the liver using novel galactosylated cationic liposomes. *Pharm. Res.* **17**:306–313 (2000).
9. S. Kawakami, C. Munakata, S. Fumoto, F. Yamashita, and M. Hashida. Targeted delivery of prostaglandin E_1 to hepatocytes using galactosylated liposomes. *J. Drug Target.* **8**:137–142 (2000).
10. Y. Hattori, S. Kawakami, F. Yamashita, and M. Hashida. Controlled biodistribution of galactosylated liposomes and incorporated probucol in hepatocyte-selective drug targeting. *J. Control. Release* **69**:369–377 (2000).
11. S. Kawakami, C. Munakata, S. Fumoto, F. Yamashita, and M. Hashida. Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs. *J. Pharm. Sci.* **90**:105–113 (2001).
12. A. Chonn, S. C. Semple, and P. R. Cullis. Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. *J. Biol. Chem.* **267**:18759–18765 (1992).
13. S. C. Semple, A. Chonn, and P. R. Cullis. Influence of cholesterol on the association of plasma proteins with liposomes. *Biochemistry* **35**:2521–2525 (1996).
14. J. H. Senior. Fate and behavior of liposomes in vivo: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* **3**:123–193 (1987).
15. T. Takino, C. Nakajima, Y. Takakura, H. Sezaki, and M. Hashida. Controlled biodistribution of highly lipophilic drugs with various parenteral formulations. *J. Drug Target.* **1**:117–124 (1993).
16. A. G. Morell, R. A. Irvine, I. Sternlieb, I. H. Scheinberg, and G. Ashwell. Physical and chemical studies on ceruloplasmin. V. Metabolic studies on sialic acid-free ceruloplasmin in vivo. *J. Biol. Chem.* **243**:155–159 (1968).
17. D. T. Connolly, R. R. Townsend, K. Kawaguchi, W. R. Bell, and Y. C. Lee. Binding and endocytosis of cluster glycosides by rabbit hepatocytes. Evidence for a short-circuit pathway that does not lead to degradation. *J. Biol. Chem.* **257**:939–945 (1982).
18. Y. C. Lee, R. R. Townsend, M. R. Hardy, J. Lonngren, J. Arnarp, M. Haraldsson, and H. Lonn. Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin. Dependence on fine structural features. *J. Biol. Chem.* **258**:199–202 (1983).
19. R. T. Lee, P. Lin, and Y. C. Lee. New synthetic cluster ligands for galactose/N-acetylgalactosamine-specific lectin of mammalian liver. *Biochemistry* **23**:4255–4261 (1984).
20. H. H. Spanjer, T. J. C. van Berkel, G. L. Scherphof, and H. J. M. Kempen. The effect of a water-soluble tris-galactoside terminated cholesterol derivative on the in vivo fate of small unilamellar vesicles in rats. *Biochim. Biophys. Acta* **816**:396–402 (1985).
21. E. A. Biessen, D. M. Beuting, H. C. Roelen, G. A. van de Marel, J. H. van Boom, and T. J. C. van Berkel. Synthesis of cluster galactosides with high affinity for the hepatic asialoglycoprotein receptor. *J. Med. Chem.* **38**:1538–1546 (1995).
22. S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, and M. Hashida. Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. *Gene Ther.* **7**:292–299 (2000).
23. S. Kawakami, J. Wong, A. Sato, Y. Hattori, F. Yamashita, and M. Hashida. Biodistribution characteristics of mannosylated, fuco-

sylated, and galactosylated liposomes in mice. *Biochim. Biophys. Acta* **1524**:258–265 (2000).
24. S. C. Semple, A. Chonn, and P. R. Cullis. Interactions of liposomes and lipid-based carrier systems with blood proteins: relation to clearance behaviour in vivo. *Adv. Drug Deliv. Rev.* **32**:3–17 (1998).
25. K. Ogawara, M. Nishikawa, Y. Takakura, and M. Hashida. Pharmacokinetic analysis of hepatic uptake of galactosylated bovine serum albumin in a perfused rat liver. *J. Control. Release* **50**:309–317 (1998).
26. M. R. Vist and J. H. Davis. Phase equilibria of cholesterol/ dipalmitoylphosphatidylcholine mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry* **29**: 451–464 (1990).
27. T.-H. Huang, C. W. B. Lee, S. K. Das Gupta, A. Blume, and R. G. Griffin. A ^{13}C and ^{2}H nuclear magnetic resonance study of phosphatidylcholine/cholesterol interactions: characterization of liquid-gel phases. *Biochemistry* **32**:13277–13287 (1993).
28. C. A. Hunt. Related Articles Liposomes disposition in vivo. V. Liposome stability in plasma and implications for drug carrier function. *Biochim. Biophys. Acta* **719**:450–463 (1982).
29. V. A. Slepushkin, A. I. Starov, A. G. Bukrinskaya, A. B. Imbs, M. A. Martynova, L. S. Kogtev, E. L. Vodovozova, N. G. Timofeeva, J. G. Molotkovsky, and L. D. Bergelson. Interaction of influenza virus with gangliosides and liposomes containing gangliosides. *Eur. J. Biochem.* **173**:599–605 (1988).
30. A. A. Yaroslavov, A. A. Efimova, V. I. Lobyshev, Y. A. Ermakov, and V. A. Kabanov. Reversibility of structural rearrangements in lipid membranes induced by adsorption-desorption of a polycation. *Membr. Cell Biol.* **10**:683–688 (1997).