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Note

Uptake characteristics of galactosylated emulsion by HepG2 hepatoma cells

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Abstract

Galactosylated (Gal) emulsions containing various molar ratios of cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl)formamide (Gal-C4-Chol) as a ligand for asialoglycoprotein receptors were prepared to study the effect of the galactose content of Gal-emulsions labeled with [^{3}H]cholesteryl hexadecyl ether on their targeted delivery to hepatocytes. The uptake characteristics of Gal-emulsions having Gal-C4-Chol of 1, 3, 4, 6, and 9 mol% were evaluated in HepG2 cells which posses asialoglycoprotein receptors and NIH3T3 cells which are lack of asialoglycoprotein receptors. The uptake and internalization by HepG2 cells was enhanced by the addition of Gal-C4-Chol to the Gal-emulsions whereas the uptake of Gal-emulsions by NIH3T3 cells was not much and was comparable with that of bare-emulsions. In the presence of excess Gal-BSA, the uptake of Gal-emulsions having Gal-C4-Chol of 4, 6, and 9% was inhibited suggesting asialoglycoprotein receptor mediated uptake. Moreover, Gal-emulsions having Gal-C4-Chol of 4, 6, and 9% showed a slight increase in surface binding and exhibited extensive uptake and internalization into HepG2 cells. The present study strongly suggested that the Gal-emulsions are taken up by the asialoglycoprotein receptor-mediated endocytosis and galactose density of Gal-emulsions is important for effective recognition and cell internalization.
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Keywords: Galactosylated emulsion; Drug delivery system; Targeting; Hepatocytes

1. Introduction

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 (Hansrani et al., 1983; Yamaguchi and Muzushima, 1994) 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 been widely used as drug carriers, especially as long-circulating

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drug carriers for passive targeting (Wheeler et al., 1994; Liu and Liu, 1995; Kawakami et al., 2000a). 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.

Rensen et al. (1995, 1997) developed apo E associated emulsions for hepatocytes targeting. These apo E associated emulsions are reported that they were selectively taken up by liver parenchymal cells (PC). 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. Recently, we synthesized a novel galactosylated cholesterol derivative, i.e., cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl) amino)butyl) formamide (Gal-C4-Chol), to modify liposomes with galactose moieties for PC-selective targeting via asialoglycoprotein receptor mediated endocytosis (Kawakami et al., 1998). 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. It was expected that Gal-emulsions were taken up by the asialoglycoprotein receptor on PC. In fact, our previous study demonstrated that intravenously administered galactosylated (Gal-) emulsions rapidly disappeared from the blood and exhibited rapid liver accumulation with up to about 80% of the dose within 10 min and were preferentially taken up by PC compared with non-parenchymal cells (NPC) in the liver (Ishida et al., 2004).

Although the uptake by asialoglycoprotein receptor were suggested by the inhibition study by lactoferrin, which is a ligand of chylomicron remnant and/or asialoglycoprotein receptors under in vivo conditions, but their uptake characteristics was not clear still because of complication by in vivo study, i.e. interaction with endogenous components, etc. Further details of mechanisms involved in PC uptake are required to develop efficient drug delivery systems that can target PC. Since in vitro experiments are simpler than in vivo experiments, detailed information about Gal-emulsions uptake characteristics could be obtained. In the present study, we evaluated the in vitro uptake characteristics of Gal-emulsions having various amount of Gal-C4-Chol by HepG2 cells, which are expressed asialoglycoprotein receptors, and NIH 3T3 cells, which are not expressed asialoglycoprotein receptors (Kawakami et al., 1998). These Gal-emulsions were radiolabeled with [^{3}H]cholesteryl hexadecyl ether (CHE) (Takino et al., 1998). The present study strongly suggested that the Gal-emulsions are taken up by the asialoglycoprotein receptor-mediated endocytosis and galactose density of Gal-emulsions is important for effective recognition and cell internalization.

2. Materials and methods

2.1. Materials

N-(4-Aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). BSA and cholesteryl chloroformate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cholesterol (Chol) and Clear-Sol I were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Egg phosphatidylcholine (Egg PC), soybean oil and galactose were obtained from Wako Pure Chemical Co. (Kyoto, Japan). Soluene 350 was purchased from Packard Bioscience Co. (Groningen, Netherlands). [^{3}H]Cholesteryl hexadecyl ether (CHE) was purchased from NEN Life Science Products Inc. (Boston, MA). Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). 2-Imino-2-methoxyethyl-1-thiogalactoside (IME-thiogalactoside) was synthesized as reported previously (Lee et al., 1976). All other chemicals were of the highest purity commercially available.

2.2. Methods

2.2.1. Synthesis of Gal-C4-Chol

Gal-C4-Chol was synthesized by the method described previously (Kawakami et al., 1998). 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 in pyridine containing triethylamine (Lee et al., 1976). 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 semi-permeable membrane (12 kDa cut-off). Finally, the dialyzate was lyophilized.

2.2.2. *Preparation of emulsions*

A mixture of soybean oil and Egg PC with Chol, or 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 20 min (200 W) under a current of nitrogen. The concentration of emulsions was adjusted to 5 mg/ml total lipids based on radioactivity measurement. Radiolabeling of emulsions was performed by addition of [^{3}H]CHE (500 μCi) to the lipid mixture before formation of a thin film layer. The particle sizes of emulsions without radioisotope were measured in a dynamic light-scattering spectrophotometer (LS-900, Otsuka Electronics Co. Ltd., Osaka, Japan).

2.2.3. *Lectin-induced aggregation of emulsions*

Emulsions (0.1 mg/ml total lipid) were incubated with 100 μl *Ricinus communis agglutinin*, RCA120 (1.0 mg/ml) in a cuvette. After rapid mixing, aggregation of the emulsions was estimated at room temperature by the time dependent increase in turbidity, as measured by the absorbance at 350 nm with a UV-3100 spectrometer (Shimadzu Co., Kyoto, Japan). The reversibility of the aggregation was assessed by the addition of 100 μl (10 mg/ml) free galactose.

2.2.4. *In vitro uptake study*

The HepG2 or NIH3T3 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% fetal bovine serum (Invitrogen Co., Carlsbad, CA, USA). Twenty-four hours later, the culture medium was replaced with an equivalent volume of HBSS containing [^{3}H]emulsions (0.25 mg/ml, 1.8 kBq/ml). For the inhibition study, 20 mM Gal-BSA, Man-BSA or Fuc-BSA was added to the emulsion 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 emulsions, the cells were washed three times with acetate buffer (pH 4.0) to remove the emulsions bound to the cell surface (Murao et al., 2002). The cells were then solubilized in 0.5 ml 1N NaOH and the radioactivity was assayed using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan). The protein content of each sample was determined by a modification of the Lowry method (Lowry et al., 1951). 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 emulsions.

2.2.5. *Statistical analysis*

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

3. Results

3.1. *The particle size of emulsions*

Table 1 summarizes the lipid composition and particle sizes of emulsions prepared. The mean diameters

Table 1
Lipid composition and mean diameter of emulsions

Formulations	Lipid composition (molar ratio)	Mean diameter (nm)[a]
Bare-emulsion	Soybean oil/Egg PC/Chol (70:25:5)	100.0 ± 2.3
Gal 1-emulsion	Soybean oil/Egg PC/Gal-C4-Chol (70:29:1)	108.1 ± 2.8
Gal 3-emulsion	Soybean oil/Egg PC/Gal-C4-Chol (70:27:3)	109.0 ± 1.9
Gal 4-emulsion	Soybean oil/Egg PC/Gal-C4-Chol (70:26:4)	109.7 ± 2.5
Gal 6-emulsion	Soybean oil/Egg PC/Gal-C4-Chol (70:24:6)	110.0 ± 2.6
Gal 9-emulsion	Soybean oil/Egg PC/Gal-C4-Chol (70:21: 9)	110.4 ± 1.7

[a] The mean diameter of emulsions was measured using a dynamic light-scattering spectrophotometer. Each value represents the mean ± S.D. of three experiments.

of prepared emulsions were about 100–110 nm. The particle sizes of the emulsions were kept constant for a period of at least 2 months at 4 °C (data not shown).

3.2. Lectin-induced aggregation of emulsions

The exposure of galactose on the surface of emulsions was confirmed by measurement of amount of aggregation of emulsions caused by the lectin form *Ricinus communis* (RCA120). The aggregation was monitored at room temperature by the time-dependent increase in turbidity as measured by the absorbance at 350 nm. As shown in Fig. 1, there was no lectin-mediated aggregation of bare-emulsions. In contrast, when the emulsions were modified with Gal-C4-Chol, slight lectin-induced aggregation was observed at a mol% of 1 and 3. A mark aggregation was observed at a mol% above 3 that were 4, 6, and 9. At 9 mol% of Gal-C4-Chol, complete aggregation was observed. Furthermore the addition of galactose to the suspension of Gal-emulsions-RCA120 aggregates induced a rapid reduction of turbidity. These results suggest that galactose residues were exposed on the emulsions and the aggregation depended on the amount of galactose residue on the emulsions.

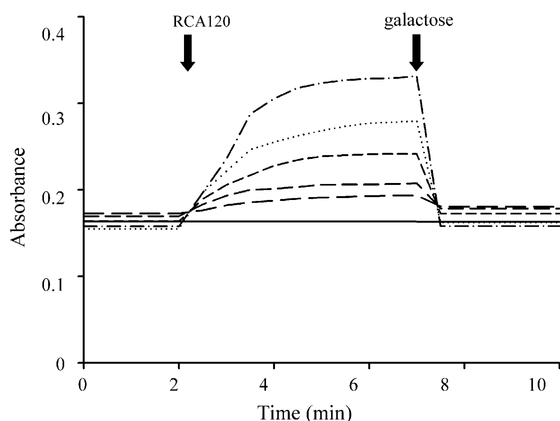

Fig. 1. Time course of the turbidity change of bare (—), Gal 1 (– –), Gal 3 (– – –), Gal 4 (- - - -), Gal 6 (· · ·), and Gal 9 (–·–) emulsions after addition of RCA120 at 25 °C. Emulsions (total lipid conc. 0.1 mg/ml) were added into a cuvette. One hundred microliters of RCA120 (1.0 mg/ml) was added to a cuvette at the appropriate time. After rapid mixing, aggregation of the emulsions was estimated by the time-dependent increase in turbidity as measure by the absorbance at 350 nm in a UV-3100 spectrometer. The reversibility of the aggregation was assessed by the addition of 100 μl (10 mg/ml) free galactose.

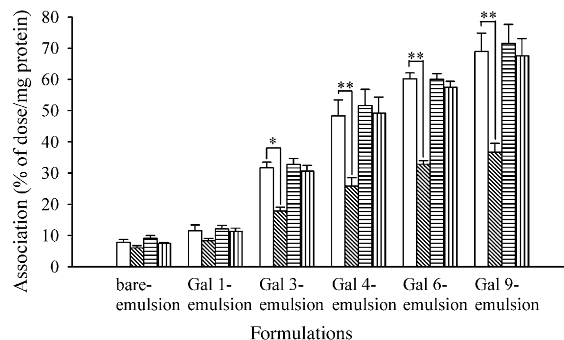

Fig. 2. Uptake of [^{3}H]-labeled emulsions by HepG2 cells. Cells were incubated with each type of [^{3}H]-labeled emulsions without (□) or with an excess amount of Gal-BSA (▧), Man-BSA (▤) or Fuc-BSA (▥). The amount of [^{3}H]-radioactivity associated with the cells was measured following 1 h incubation. Each value represents the mean + S.D. of three experiments. Statistically significant differences ($^*P < 0.05$, $^{**}P < 0.01$) from emulsions incubated without inhibitors.

3.3. In vitro uptake of [^{3}H]CHE-labeled emulsions by HepG2 cells and NIH3T3 cells

Fig. 2 shows the in vitro uptake of [^{3}H]CHE-labeled emulsions by HepG2 cells. Gal-emulsions having 1 and 3 mol% of Gal-C4-Chol were taken up by HepG2 cells to an extent that was comparable with that of bare-emulsions. On the other hand, the uptake of Gal-emulsions having 4, 6, and 9 mol% of Gal-C4-Chol, was much higher than that of bare-emulsions. In the presence of 20 mM Gal-BSA, the uptake of Gal-emulsions having 4, 6, and 9 mol% of Gal-C4-Chol was significantly inhibited (Fig. 2), suggesting uptake by the asialoglycoprotein receptors. The involvement of asialoglycoprotein receptor-mediated endocytosis was confirmed in NIH3T3 cells, which are not expressing the asialoglycoprotein receptors. Gal-emulsions were taken up by NIH3T3 cells to an extent that was comparable with that of bare-emulsions (Fig. 3), suggesting uptake by the asialoglycoprotein receptor-mediated endocytosis.

The amount of surface binding and internalization of Gal-emulsions were evaluated using an acid-wash procedure. As shown in Fig. 4, the surface binding of both bare-emulsions and Gal-emulsions having 1 and 3 mol% of Gal-C4-Chol was similar. Very little amounts of bare-emulsions and Gal-emulsions with 1 and 3 mol% of Gal-C4-Chol were internalized into the cells. In contrast, Gal-emulsions having 4, 6, and

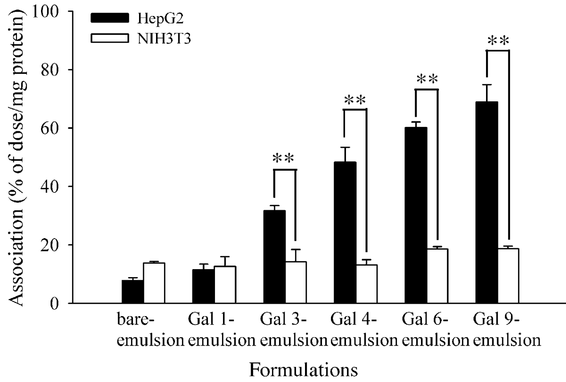

Fig. 3. Uptake of [^{3}H]-labeled emulsions by HepG2 cells (■) and NIH3T3 cells (□). Cells were incubated with each type of bare- or Gal-emulsions. The amount of [^{3}H]-radioactivity associated with the cells was measured following 1 h incubation. Each value represents the mean + S.D. of three experiments. Statistically significant differences ($^{**}P<0.01$) from NIH3T3 cells.

9 mol% of Gal-C4-Chol showed a slight increase in surface binding and exhibited extensive uptake and internalization into HepG2 cells. These results suggest that the galactose density on the emulsion surface affects the ligand-receptor interaction that results in the different internalization of these Gal-emulsions into the cells.

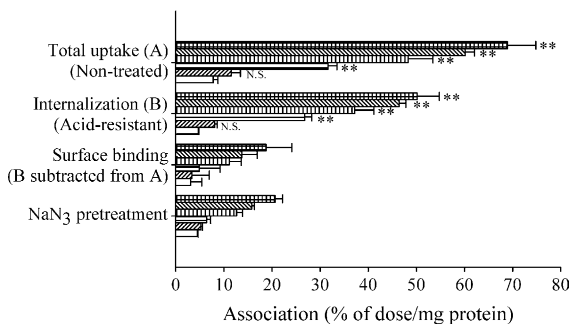

Fig. 4. Amount of [^{3}H]-labeled emulsions associated with HepG2 cells. Cells were incubated with bare-(□), Gal 1-(▨), Gal 3-(▤), Gal 4-(▥), Gal 6-(▧), or Gal 9-(▦) emulsions. At 1 h after incubation, the cells were wash with an acid buffer to separate the surface bound emulsions. The difference in cellular association between acid-treatment and no treatment was regarded as the amount associated with the cell surface. In another group, the cells were preincubated with HBSS containing 10 mM NaN_3 for 20 min prior to the addition of emulsions. Each value represents the mean + S.D. of three experiments. Statistically significant differences ($^{**}P<0.01$) from bare-emulsions. N.S., not significant.

4. Discussion

The use of lipid dispersion carrier systems, such as lipid emulsions and liposomes, as carriers of lipophilic drugs has attracted particular interest. In a series of investigations, we have developed galactosylated liposomes to deliver lipophilic drugs to hepatocytes after intravenous administration (Kawakami et al., 2000b; Hattori et al., 2000; Kawakami et al., 2001; Managit et al., 2003), which show superior liver targeting via asialoglycoprotein-receptor mediated endocytosis. We have confirmed that not only the distribution profiles of galactosylated lipid carriers but also the controlled release and solubilizing capacity of incorporated drugs need to be optimized for the hepatic targeting (Hattori et al., 2000; Ishida et al., 2004); accordingly galactosylated lipid carriers must be selected by considering the physicochemical properties of incorporation drug. Since lipid emulsions possess an oil phase in particulate form, lipid emulsions are considered to be superior to liposomes due to the fact that they have a high solubilizing capacity. Taking these into considerations, we previously developed Gal-emulsions as an alternative drug carrier for hepatocyte-selective drug delivery (Ishida et al., 2004). In this study, we evaluated the uptake characteristics of Gal-emulsions using cultured HepG2 and NIH3T3 cells.

Since the lipid emulsions (oil-in-water) surface exhibits an aqueous property, galactose moiety is expected to display on the surface of emulsion by addition of the Gal-C4-Chol. In order to confirm the existence of galactose on Gal-emulsions, Gal-emulsions were incubated with a lectin from RCA120 (Fig. 1). The recognition of Gal-emulsions with RCA120 increased with respect to the increased Gal-C4-Chol of Gal-emulsions; suggesting that galactose moiety is covered the surface of Gal-emulsions according to the amounts of Gal-C4-Chol added. This phenomenon is corresponding to Gal-liposomes (Managit et al., in press).

Then in vitro uptake experiment using HepG2 cells, which is rich in asialoglycoprotein receptors, was performed, to investigate targeting efficiency of. Gal-emulsions. Over 3 mol% of Gal-C4-Chol, Gal-emulsions were efficiently taken up by the HepG2 cells according to the amount of Gal-C4-Chol added (Fig. 2). These results were corresponding to our previous in vivo distribution data following the intravenous administration (Ishida et al., 2004); thus, galactose density

of Gal-emulsions is important for the recognition by asialoglycoprotein receptor. In the presence of excess Gal-BSA, which contained a ligand of asialoglycoprotein receptors, the uptake of Gal-emulsions was significantly inhibited (Fig. 2); however, the inhibition effect was not observed in the presence of excess Man- and Fuc-BSA. In order to confirm the involvement of asialoglycoprotein receptor-mediated endocytosis, the uptake experiments by NIH3T3 cells, which are not express the asialoglycoprotein receptors, were performed. As shown in Fig. 3, the uptake amount between bare-emulsion and Gal-emulsion having various Gal-C4-Chol was the almost the same. Taking these data into considerations, Gal-emulsions were taken up by the asialoglycoprotein receptors on PC.

We used the acid washing method to evaluate the binding and amount of internalized Gal-emulsions (Fig. 4). The separation of surface-bound emulsions from their internalized counterparts by acid washing shows that there was a difference in the amount of Gal-emulsions internalized, while the surface binding of Gal-emulsions was slightly increased. The difference in amount internalized could be explained by the effect of the galactose density on Gal-emulsions. The way in which galactose moieties are exposed on the emulsions surface may be a determinant of the relative affinities of emulsions towards asialoglycoprotein receptors. The appropriate amount of galactose might improve the exposure of the galactose moiety and possibly provide an optimal configuration for interaction with asialoglycoprotein receptors. From these results, at least 4 mol% of Gal-C4-Chol was necessary for the recognition with receptors and 6 mol% provided the extensive asialoglycoprotein receptors mediated uptake in vitro. Ogawara et al. (1998) analyzed the hepatic uptake of Gal-BSA with varying numbers of galactose units in isolated, perfused rat liver, and found that the internalization rate of these Gal-BSA was different and depended on the galactose residue on Gal-BSA derivatives. These findings supported our phenomenon that galactose density is the determining factor of ligand-receptor interaction that affects the rate and amount of the ligands internalized into PC.

In the present study, we showed that Gal-emulsions could be prepared by incorporation of Gal-C4-Chol into emulsions. These current results provide evidence that introduction of ligand-grafted lipids, such as mannose (Kawakami et al., 2000c; Opanasopit et al., 2002), fucose (Kawakami et al., 2000d; Higuchi et al., 2004), folate (Ni et al., 2002; Reddy et al., 2002), and transferrin (Ishida et al., 2001) to emulsions also allows cell-selective targeting.

In conclusion, the present study strongly suggested that the Gal-emulsions are taken up by the asialoglycoprotein receptor-mediated endocytosis and galactose density of Gal-emulsions is important for effective recognition and cell internalization.

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