Table 2. Area under the Blood Concentration-Time Curve (AUC) and Tissue Uptake Clearance of [^{3}H]-Labeled Liposomes after Intravenous Injection into Mice[a]

Liposomes	AUC (% ·h/mL)[a]	Clearance (μL/h)[a]					
		CL_{liver}	CL_{kidney}	CL_{spleen}	CL_{lung}	CL_{heart}	CL_{urine}
Bare-liposomes	9.22	1141	17	227	33	18	20
Gal 1.0-liposomes	7.79	2668	34	458	36	1	14
Gal 2.5-liposomes	6.57	4821	206	585	142	56	25
Gal 3.5-liposomes	2.69	29640	774	1800	347	107	47
Gal 5.0-liposomes	1.13	75980	915	3315	156	48	55
Gal 7.5-liposomes	1.25	68430	1150	3500	501	230	86

An average of three experiments is shown.
[a]AUC and clearances were calculated for the periods up to 10 min after injection.

intravenous injection. Compared with bare-liposomes (PC/NPC *ratio* = 1.1), Gal-liposomes having 1.0 and 2.5 mole% of Gal-C4-Chol distributed to both PC and NPC with a PC/NPC ratio of 1.0 and 0.78 respectively, whereas Gal-liposomes having 3.5, 5.0, and 7.5 mole% of Gal-C4-Chol accumulated selectively in PC with a PC/NPC ratio of 2.8, 15, and 6.0, respectively. However, the highest PC/NPC ratio was observed with Gal-liposomes having 5.0 mole% of Gal-C4-Chol.

DISCUSSION

We synthesized a novel galactosylated cholesterol derivative, Gal-C4-Chol, having bi-functional properties with a lipophilic anchor moiety for stable incorporation into liposomes and a galactose moiety for recognition by the asialoglycoprotein receptors. Since Gal-C4-Chol is stably incorporated into the liposomal membrane under *in vivo* conditions, Gal-liposomes prepared with Gal-C4-Chol are expected to efficiently distribute in liver parenchymal cells. To confirm the exposure of the galactose moiety on the liposomal surface, lectin binding experiments were performed. The aggregation depended on the amount of Gal-C4-Chol to liposomes (Fig. 1), indicating that the addition of Gal-C4-Chol corresponded well to the exposure of galactose on the liposomal surface.

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.[20] Therefore, liposomes with a diameter less than this can readily pass through the fenestration into the Disse space. Accordingly, we prepared liposomes less than 100 nm in diameter in order to allow free access to hepatocytes.

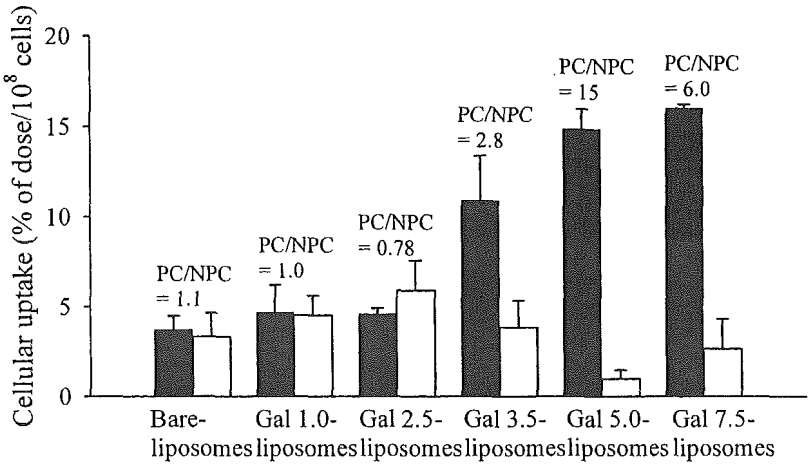

Figure 5. Hepatic cellular localization of [^{3}H]-labeled liposomes after intravenous injection in mice. Radioactivity was determined at 30 min postinjection in PC (■), and NPC (□). Each value represents the mean + SD of three experiments.

In the present study, we have demonstrated that about 80% of the injected dose accumulated in the liver after intravenous administration of Gal-liposomes with both 5.0 and 7.5 mole% Gal-C4-Chol up to 10 min (Fig. 4). Pharmacokinetic analysis demonstrated that the liver uptake clearance of Gal-liposomes with both 5.0 and 7.5 mole% of Gal-C4-Chol is nearly equal to the hepatic plasma flow rate (66000 μL/h), indicating highly efficient uptake by the liver. As far as the uptake efficiency was concerned, the liver uptake of Gal-liposomes having 5.0 or 7.5 mole% Gal-C4-Chol is much higher than that of Gal-liposomes with other groups, including the tri-antennary galactosyl or asialofetuin modified lipids.[21,22] Although Gal-C4-Chol is a mono-antennary lipid, the Gal-C4-Chol moieties might interact with each other on the phospholipid membrane leading to an organization similar to the intramolecular branching of galactose residues.

In order to confirm whether the increased liver uptake of Gal-liposomes resulted from recognition by the asialoglycoprotein receptors on PC, intrahepatic distribution and competitive inhibition studies were performed. The intrahepatic distribution study demonstrated that Gal-liposomes having 3.5, 5.0, and 7.5 mole% Gal-C4-Chol were preferentially recovered from PC (Fig. 5) and the uptake by the HepG2 cells was significantly inhibited by an excess of galactose (Fig. 2), suggesting the asialoglycoprotein receptor mediated uptake on PC. Moreover, the uptake of Gal-liposomes was inhibited by treating the cells with metabolic inhibitors (NaN_3), indicating involvement of endocytosis (Fig. 3) These efficient uptake by the asialoglycoprotein mediated endocytosis of Gal-liposomes agrees with the finding that the liver uptake of Gal-liposomes with 3.5, 5.0, and 7.5 mole% Gal-C4-Chol is much higher than that of Gal-liposomes with 1.0 and 2.5 mole% (Fig. 4).

Kuppfer cells have also been shown to express a galactose-specific receptor (Gal/Fuc-recognizing receptor) different from the receptor on PC.[23,24] Although the recognition mechanism of Gal-liposomes by asialoglycoprotein receptors on PC and Gal/Fuc-recognizing receptors on Kupffer cells remains unclear, some groups have reported that the galactose density on the liposomal surface is an important factor.[21,25] Recently, Sliedregt et al.[21] have reported that liposomes containing 50% tri-antennary galactosides were taken up by Gal/Fuc-recognizing receptors, whereas galactosylated liposomes with a lower degree of tri-antennary galactoside modification (5.0% tri-antennary galactosides) were taken up by the asialoglycoprotein receptors on PC. Since Gal-C4-Chol is a mono-antennary galactoside modified cholesterol derivative, the control of the galactose density on the liposomal surface is easier; therefore such characteristics of Gal-C4-Chol would be an advantage for the preparation of PC-selective Gal-liposomes. Furthermore, the observation by Sliedregt et al.[21] about the effect of the lower degree of tri-antennary galactoside modification of galactosylated liposomes on the asialoglycoprotein receptor uptake on PC agrees with our results showing that Gal-liposomes with 3.5, 5.0, and 7.5 mole% Gal-C4-Chol are taken up by the asialoglycoprotein receptors on PC (Fig. 5).

We used the acid washing method[26] to evaluate the binding and amount of internalized Gal-liposomes (Fig. 3). The separation of surface-bound liposomes from their internalized counterparts by acid washing shows that there was a difference in the amount of Gal-liposomes internalized, while the surface binding of Gal-liposomes was slightly increased. The difference in amount internalized could be explained by the effect of the galactose density on Gal-liposomes. The way in which galactose moieties are exposed on the liposome surface may be a determinant of the relative affinities of liposomes 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. We previously analyzed the hepatic uptake of galactosylated bovine serum albumin (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 number of the galactose density on the Gal-BSA derivatives.[27] This finding regarding Gal-BSA agrees with the results of galactose density of Gal-liposomes as far as the asialoglycoprotein receptor mediated uptake is concerned; therefore the galactose density of Gal-liposomes is important factor for cell internalization.

After intravenous administration, the uptake PC-selectivity of Gal-liposomes with 7.5 mole% Gal-C4-Chol is lower than that of Gal-liposomes with 5.0 mole% Gal-C4-Chol (Fig. 5). This phenomenon does not agree with our previous observation involving galactosylated macromolecular carriers showing that the targeting efficacy to hepatocytes is dependent on the galactose modification. This might be explained by the difference

in the charge of Gal-liposomes because Gal-C4-Chol possesses an imino group in the spacer part; thus introducing of many Gal-C4-Chol enhances the cationic nature of Gal-liposomes.[9,28] The zeta potentials of bare-liposomes, Gal 5.0-liposomes, and Gal 7.5-liposomes were -3.4 ± 0.3 ($n = 3$), 5.4 ± 0.27 ($n = 3$), and 8.3 ± 0.28 ($n = 3$) mV, respectively. As shown in Figure 2, the degree of inhibition of the cellular uptake of Gal-liposomes with 7.5 mole% Gal-C4-Chol in the presence of excess galactose was insufficient, suggesting enhancement of the nonspecific uptake by the cationic charge. In fact, the NPC accumulation (Fig. 5) and the uptake clearance in kidney, spleen, lung, and heart (Tab. 2) of Gal 7.5-liposomes were higher than that of Gal 5.0-liposomes. Thus, the cationic nature of Gal-liposomes with 7.5 mole% Gal-C4-Chol might reduce the PC-selective uptake after intravenous administration.

In conclusion, we have demonstrated that our Gal-C4-Chol is an effective ligand for hepatocytes targeting and the galactose density of Gal-liposomes prepared by Gal-C4-Chol is important for both efficient recognition by asialoglycoprotein receptors and cell internalization. These observations provide valuable information to help in the design of galactosylated carrier systems in order to optimize their targeting efficiency to hepatocytes.

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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.

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.

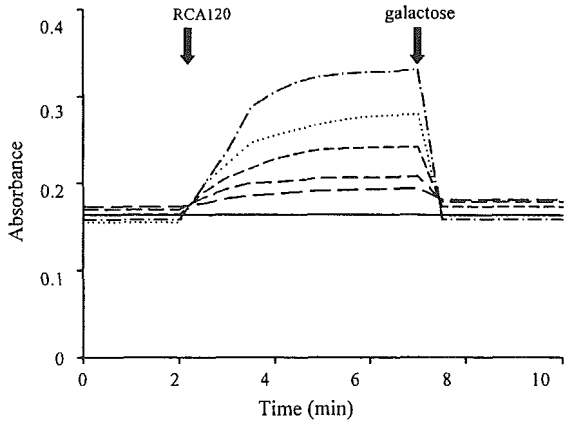

Fig. 1. Time course of the turbidity change of bare (—), Gal 1 (– –), Gal 3 (– – –), Gal 4 (- - - -), Gal 6 (· · ·), and Gal 9 (–·–) emulsions after addition of RCA120 at 25 °C. Emulsions (total lipid conc. 0.1 mg/ml) were added into a cuvette. One hundred microliters of RCA120 (1.0 mg/ml) was added 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.

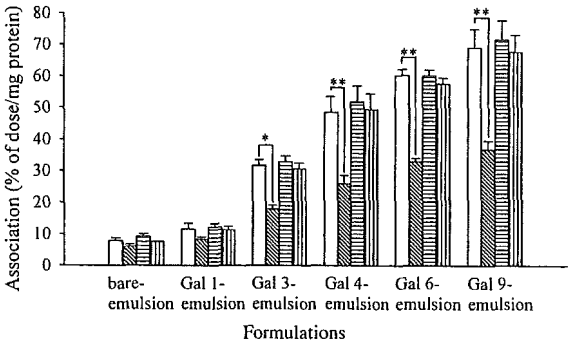

Fig. 2. Uptake of [^{3}H]-labeled emulsions by HepG2 cells. Cells were incubated with each type of [^{3}H]-labeled emulsions without (□) or with an excess amount of Gal-BSA (▨), Man-BSA (▤) or Fuc-BSA (▥). The amount of [^{3}H]-radioactivity associated with the cells was measured following 1 h incubation. 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

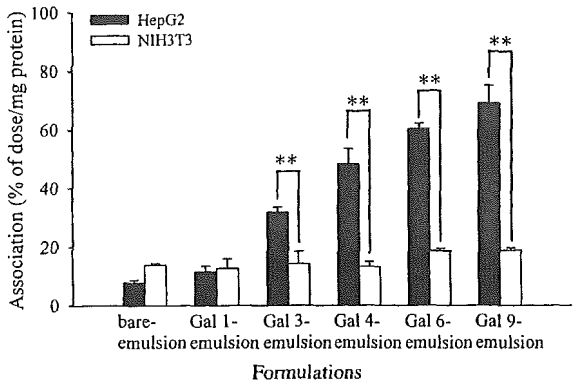

Fig. 3. Uptake of [^{3}H]-labeled emulsions by HepG2 cells (■) and NIH3T3 cells (□). Cells were incubated with each type of bare- or Gal-emulsions. The amount of [^{3}H]-radioactivity associated with the cells was measured following 1 h incubation. Each value represents the mean + S.D. of three experiments.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

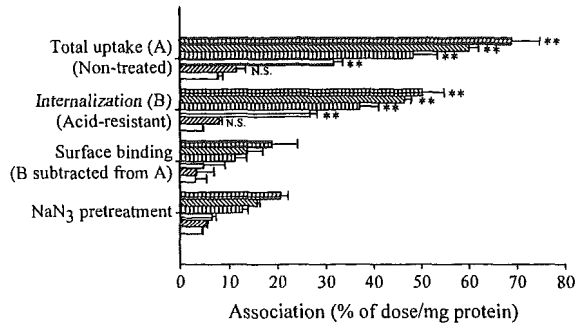

Fig. 4. Amount of [^{3}H]-labeled emulsions associated with HepG2 cells. Cells were incubated with bare-(□), Gal 1-(▨), Gal 3-(▤), Gal 4-(▥), Gal 6-(▧), or Gal 9-(▦) emulsions. At 1 h after incubation, the cells were wash with an acid buffer to separate the surface bound emulsions. The difference in cellular association between acid-treatment and no treatment was regarded as the amount associated with the cell surface. In another group, the cells were pre-incubated 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.

Acknowledgements

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Effect of Low-Molecular-Weight β-Cyclodextrin Polymer on Release of Drugs from Mucoadhesive Buccal Film Dosage Forms

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We investigated the effect of low-molecular-weight β-cyclodextrin (β-CyD) polymer on *in vitro* release of two drugs with different lipophilicities (*i.e.*, lidocaine and ketoprofen) from mucoadhesive buccal film dosage forms. When β-CyD polymer was added to hydroxypropylcellulose (HPC) or polyvinylalcohol (PVA) film dosage forms, the release of lidocaine into artificial saliva (pH 5.7) was reduced by 40% of the control. In contrast, the release of ketoprofen from the polymer film was enhanced by addition of β-CyD polymer to the vehicle. When lidocaine and ketoprofen was incubated with β-CyD polymer in the artificial saliva, concentration of free lidocaine molecules decreased in a β-CyD polymer concentration-dependent manner. The association constant with β-CyD polymer was 6.9±0.6 and 520±90 M^{-1} for lidocaine and ketoprofen, respectively. Retarded release of the hydrophilic lidocaine by β-CyD polymer might be due to the decrease in thermodynamic activity by inclusion complex formation, whereas enhanced release of the lipophilic ketoprofen by the β-CyD polymer might be due to prevention of recrystallization occurring after contacting the film with aqueous solution. Thus, effects of low-molecular-weight β-CyD polymer to the drug release rate from film dosage forms would vary according to the strength of interaction with and the solubility of active ingredient.

Key words β-cyclodextrin polymer; buccal delivery; mucoadehive film dosage form; lidocaine; ketoprofen; hydroxypropylcellulose

Buccal drug delivery systems have been attracting much attention as drug formulations intended for anti-inflammatory and analgesic therapies in the oral cavity. These formulations are expected to increase therapeutic effects and reduce systemic adverse reactions by concentrating the drug in the target tissue. One particular problem associated with the treatment of oral cavity diseases is short duration of therapeutic efficacy due to rapid dilution and washout from the oral cavity by saliva.[1] The use of bioadhesive polymers that can interact with biological membranes is a way of retarding the residence time of drug in oral cavity.[2—6]

Among bioadhesive mucosal dosage forms developed, buccal film are preferable over adhesive tablets in terms of flexibility and comfort, and can circumvent relatively short residence time of oral gels.[7] In order to attain better therapeutic effect, it would be expected to regulate the release rate of drug from the polymer film. Assuming that formation of the inclusion complex might change the release rate of drug, we intended to investigate the effect of β-CyD incorporated in bioadhesive polymer film dosage forms. However, our preliminary experiments indicated that the film containing β-CyD was not elastic enough to be used practically. In the present study, therefore, we investigated the feasibility of low-molecular-weight β-CyD polymer for controlled release of drugs from the film dosage forms. It is known that β-CyD can be readily cross-linked through their hydroxyl groups with epicholohydrin. So far, high-molecular-weight β-CyD polymer has been investigated as a tablet disintegrating agent because of its rapid and high swelling capacity.[8,9] In this study, we selected two drugs varying in lipophilicity (*i.e.*, lidocaine and ketoprofen) as model drugs, prepared the film dosage forms containing water-soluble, low-molecular-weight β-CyD polymer, and evaluated *in vitro* release of lidocaine in artificial saliva. In addition, we investigated interaction of these drugs with β-CyD polymer by solubility measurement following ultra-filtration and competitive inclusion complexation experiment using a fluorescent probe.

MATERIALS AND METHODS

Materials Lidocaine was obtained from Nacalai Tesque Inc., (Tokyo, Japan). Ketoprofen, hydroxypropylcellulose (HPC) and polyvinylalcohol (PVA) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Low-molecular-weight β-CyD polymer was kindly supplied from Nihon Syokuhin Kako Co., Ltd., (Fuji, Shizuoka, Japan). The molecular weight of the β-CyD polymer used was approximately 5000, indicating that four or five units of β-CyD are cross-linked with epichlorohydrin (Fig. 1). 6-(*p*-Toluidino)-2-naphthalenesulfonic acid sodium salt (TNS) was purchased from Sigma Co. (St. Louis, MO, U.S.A.). The other reagents used were of analytical grade.

Preparation of Film Dosage Forms Lidocaine or ketoprofen and β-CyD polymer was mixed at a weight ratio of 1 : 4.85 or 1 : 4.46 (corresponding to guest/host molar ratio of 1 : 1) and kneaded with a little of water. Then, water was added to yield a final lidocaine concentration of 0.5 w/v%,

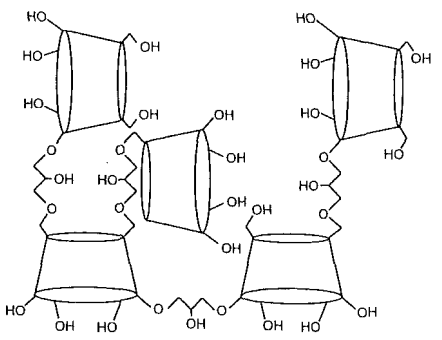

Fig. 1. Structure of Water-Soluble Low-Molecular-Weight β-Cyclodextrin (β-CyD) Polymer

Four or five CyDs were crosslinked by epichlorohydrin.

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together with addition of 5 w/v% HPC or PVA. One hundred and twenty-five microliters of the aqueous solution was placed on a Teflon cell (0.79 cm^2) and put in a desiccator with silica gel at room temperature overnight. Additional 125 μl drug solution was overlaid to the film and dried in the desiccator with silica gel at room temperature completely. The reason why two-step drying was conducted was to minimize the formation of the film edge.

Dissolution Test *In vitro* release of drugs was tested using a modified JP XIV dissolution apparatus. The film dosage form prepared according to the above-mentioned method was attached to the rotatory shaft with an adhesive tape. The shaft was rotated at 50 rpm in 50 ml of artificial saliva in a 50 ml glass beaker thermostated at 37 °C. The artificial saliva was composed of 14.4 mM NaCl, 16.1 mM KCl, 1.31 mM $CaCl_2 \cdot 2H_2O$, 0.545 mM $MgCl_2 \cdot 6H_2O$, and 1.96 mM K_2HPO_4, and the pH was adjusted to 5.7 with HCl. Two hundred and fifty microliters were taken at 1, 3, 5, 7, 10, 15, 20, 30, 45, 60, 90, and 120 min and subjected to further analysis. To keep the volume of dissolution medium constant, an equivalent volume of fresh artificial saliva was added after sampling.

Determination of Lidocaine The lidocaine concentrations in the samples obtained from dissolution test were determined using an HPLC system (LC-6A, Shimadzu, Kyoto). A mobile phase composed of water, methanol, and phosphoric acid (79 : 20 : 1) was flowed through an Inersil ODS-2 column (5 mm, 4.6×150 mm, Nacalai Tesque Inc., Tokyo) at the rate of 1.2 ml/min at 36 °C. The detection wavelength for lidocaine was 220 nm.

Determination of Ketoprofen The ketoprofen concentrations in the samples obtained from dissolution test were determined using an HPLC system (LC-10Avp, Shimadzu, Kyoto). A mobile phase composed of 0.06 M KH_2PO_4, acetonitrile, and triethylamine (65 : 35 : 0.1) was flowed through an Inersil ODS-2 column (5 mm, 4.6×150 mm, Nacalai Tesque Inc., Tokyo) at the rate of 1.0 ml/min. The detection wavelength for ketoprofen was 275 nm.

Differential Scanning Calorimetry (DSC) Analysis DSC thermograms of pure drugs, film dosage forms containing drug and β-CyD polymer, and the control films containing drug alone were recorded by DSC system (DSC3200, Mac Science Co,. Tokyo, Japan). All samples containing 1 mg drug were placed in aluminium pans and heated lineally at a scanning rate of 10 °C/min from ambient temperature to 200 °C. Aluminium oxide was used to calibrate the apparatus.

Competitive Inclusion Complexation Assay TNS (20 μM) and β-CyD polymer (β-CyD unit concentration of 200 μM) were dissolved in pH 7.4 phosphate buffered saline (PBS). One milliliter of the mixture and 5 ml of varying drug concentrations in PBS were mixed and adjusted to 10 ml. The final concentrations of TNS and β-CyD units of the polymer were 2 μM and 20 μM, respectively. After the mixture was incubated at 25 °C for 60 min, the fluorescence intensity associated was determined by spectrofluorophotometer (RF-540, Shimadzu, Kyoto), where the excitation and emission wavelengths were 324 and 437 nm, respectively.

Binding Experiment Four hundred milligrams per milliliter lidocaine or 40 mg/ml ketoprofen was incubated with varying amounts of β-CyD polymer in the artificial saliva at 25 °C. Approximately 2 ml aliquot of the incubation medium was subjected to ultra-filtration with a polyethersulfone membrane filter (Vivaspin 2 (5000 MWCO), Vivascience AG, Goettingen, Germany). Five hundred microliters aliquot of the filtrate was taken and diluted by 10 times. The drug concentration in the diluted filtrate was determined by the HPLC method. Assuming that the drug and β-CyD untis of the polymer form a 1 : 1 complex and that the concentration of complex was negligible as compared to the concentration of β-CyD, the association constant (K) was calculated using the following equation:

$$\frac{[\mathrm{Durg}]_{\mathrm{total}}}{[\mathrm{Drug}]_{\mathrm{free}}} - 1 = K \cdot [\beta\text{-CyD}]_{\mathrm{total}} \quad (1)$$

where $[\mathrm{Drug}]_{\mathrm{total}}$, $[\mathrm{Drug}]_{\mathrm{free}}$, and $[\beta\text{-CyD}]_{\mathrm{total}}$ were concentrations of free drug, total drug, and total β-CyD, respectively. The K values and their computer-calcualated standard errors were estimated by linear regression method.

RESULTS

Dissolution of Lidocaine and Ketoprofen from Film Dosage Forms Lidocaine film dosage forms were prepared, using HPC or PVA as a film base. Figure 2 shows the dissolution profiles of lidocaine from the films. When HPC was used as a film base, more than 80% of applied lidocaine was released within 15 min. When β-CyD polymer was added to the film, it took approximately 30 min to release 80% of applied lidocaine. Similar retardation of lidocaine release associated with addition of β-CyD polymer was observed with the PVA base, although the lidocaine release tended to be slower for PVA as compared with HPC. Initial dissolution rates were calculated from the linear portion in early time phase (Table 1). Addition of β-CyD polymer reduced an initial dissolution rate by 40% for both HPC and PVA bases.

Film dosage forms of ketoprofen were also prepared using HPC as a film base. Figure 3 shows the dissolution profiles of ketoprofen from the films. As compared to lidocaine, the release rate of ketoprofen was slower, where it took more than 75 min for 80% of the amount to be released. When β-

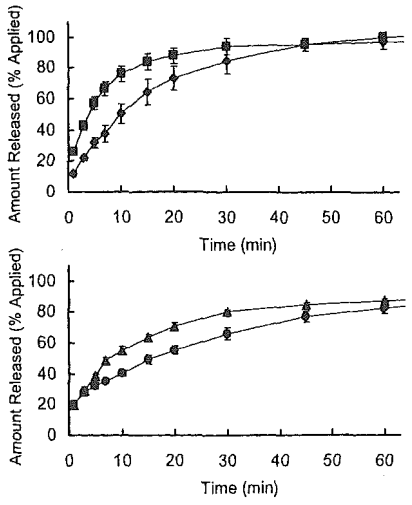

Fig. 2. Dissolution Profiles of Lidocaine from Film Dosage Forms

Key: HPC (■); HPC/β-CyD polymer (◆); PVA (▲); PVA/β-CyD polymer (●). Each data point expresses mean±S.E. of four experiments.

Table 1. Initial Dissolution Rate of Lidocaine from Film Dosage Forms

Formulations	Dissolution rate (%/h)
HPC	6.51±0.13
HPC/β-CyD polymer	3.69±0.03[a]
PVA	4.17±0.06
PVA/β-CyD polymer	2.55±0.03[b]

a) $p<0.05$ *vs.* HPC, *b*) $p<0.01$ *vs.* PVA.

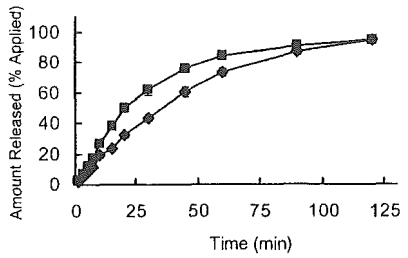

Fig. 3. Dissolution Profiles of Ketoprofen from Film Dosage Forms

Key: HPC (◆); HPC/β-CyD polymer (■). Each data point expresses mean±S.E. of four experiments.

Table 2. Initial Dissolution Rate of Ketoprofen from Film Dosage Forms

Formulations	Dissolution rate (%/h)
HPC	1.47±0.03
HPC/β-CyD polymer	2.12±0.02[a]

a) $p<0.05$ *vs.* HPC.

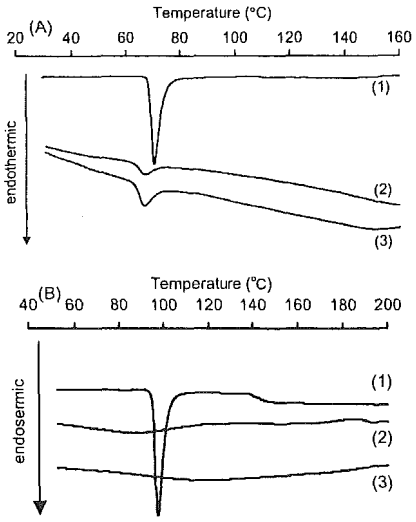

Fig. 4. Differential Scanning Calorimetory Analysis of (A) Lidocaine and (B) Ketoprofen Formulations

(1) Pure drugs, (2) drugs in HPC film, (3) drugs in HPC/β-CyD polymer.

CyD polymer was added to the film, the release rate of ketoprofen was increased, in contrast to the case of lidocaine. The initial dissolution rate was increased by 1.4-folds by addition of β-CyD polymer in the polymer film (Table 2).

DSC Analysis of Lidocaine and Ketoprofen Film Dosage Forms Figure 4 shows the DSC thermograms of pure lidocaine or ketoprofen, the HPC film, and the HPC/β-CyD polymer film. In the case of lidocaine, a small melting peak of lidocaine was observed at 68—69 °C for HPC and HPC/β-CyD polymer film. The endothermic peak for

Table 3. Data of Differential Scanning Calorimetory Analysis of Lidocaine or Ketoprofen and These Film Dosage Forms

	Melting point (°C)	Peak area (cal/g)
Lidocaine	70.5	4.15
Lidocaine/HPC	67.3	0.154
Lidocaine/HPC/β-CyD	67.0	0.268
Ketoprofen	95.0	7.10
Ketoprofen/HPC	—	—
Ketoprofen/HPC/β-CyD	—	—

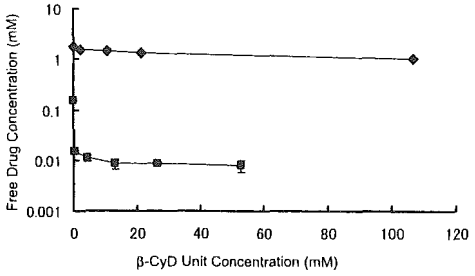

Fig. 5. Effect of β-CyD Polymer on Concentration of Free Lidocaine (◆) or Ketoprofen (■)

Polymer concentration was calculated with their β-CyD units of the polymer. Each data point expresses mean±S.E. of four experiments.

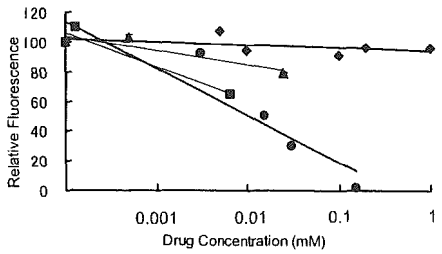

Fig. 6. Effect of Drugs on Fluoresence Associated with TNS–β-CyD Polymer Inclusion Complex

Key: lidocaine (◆); ketoprofen (●); progesterone (■); quercetin (▲).

HPC/β-CyD polymer film was slightly larger than that for the HPC film, indicating that crystallinity of lidocaine would be slightly increased by the addition of β-CyD polymer.

In contrast, the film dosage forms of ketoprofen did not exhibit any endothermic peak irrespectively of the addition of β-CyD polymer film. Thus, it was indicated that ketoprofen be in the amorphous state in the polymer films.

Confirmation of Drug-β-CyD Polymer Complexation by Ultrafiltration Method To confirm the interaction of drugs with β-CyD polymer, we performed binding experiments using ultra-filtration method. Figure 6 shows the relationship between free drug concentration and the concentration of β-CyD polymer. The concentration of free drug molecules in the artificial saliva decreased significantly in a β-CyD polymer concentration-dependent manner, while the effect of β-CyD polymer was much more significant with ketoprofen. The association constants calculated assuming that the β-CyD forms 1 : 1 complex with the guest molecule were 6.9±0.6 and 520±90 M^{-1} for lidocaine and ketoprofen, respectively. Here, it should be noted that the association constant was estimated with the concentration of β-CyD monomer units.

Detection of Lidocaine-β-CyD Polymer Inclusion Com-

plexes Using a Fluorescent Probe As one of convenient methods to investigate formation of inclusion complex, competitive experiments using a fluorescent probe such as TNS have been proposed.[9,10] The fluorescence associated with TNS increases markedly in a non-polar environment. When TNS forms an inclusion complex with β-CyD, the compound emits strong fluorescence due to hydrophobic environment in the cavity of β-CyD and restricted molecular motion. By using the fluorescent probe, we investigated whether or not lidocaine forms an inclusion complex with β-CyD polymer (Fig. 5). When TNS was incubated with β-CyD polymer, strong fluorescence was observed as reported with monomeric β-CyD.

Ketoprofen significantly reduced fluorescence associated with TNS-β-CyD polymer complex in a concentration-dependent manner, where relative fluorescence was decreased up to approximately 2% when 20 times higher concentration of ketoprofen than that of β-CyD unit of the polymer was added. Competitive inhibition effect of ketoprofen was comparable to that of progesterone[11] known to be strongly associated with β-CyD. In contrast, no effect of lidocaine was observed even when its concentration was increased up to 1 mM, being 50 times as high as that of β-CyD unit.

DISCUSSION

In the present study, β-CyD polymer retarded the release of lidocaine from mucoadhesive polymer films. Miyoshi *et al.*[12] reported that β-CyD forms an inclusion complex with lidocaine and improves solubility and stability of the drug. Dollo *et al.*[13] revealed in their NMR studies that significant changes of chemical shifts assigned to inner protons of β-CyD (H3 and H5), as well as proton near the cavity (H6 on the rim of the torus), due to the addition of lidocaine were observed whereas no appreciable shifts detected for protons located outside the cavity of β-CyD. Taking together with the results of DSC and infrared spectroscopy analyses, they concluded that lidocaine molecule may interact with the cavity of β-CyD.[13] Although lidocaine was not associated with β-CyD polymer as strongly as to inhibit the formation of TNS–β-CyD complex (Fig. 6), free concentration of the drug was significantly decreased in β-CyD polymer-concentration dependent manner (Fig. 5). Therefore, it is likely that decreased thermodynamic activity by complex formation might be one of the reasons why β-CyD polymer retarded the release of lidocaine from the film dosage form. Based on the relationship between β-CyD polymer concentration and free concentration of lidocaine, the association constant for lidocaine–β-CyD polymer complex was estimated to be $6.9 \pm 0.6\ \mathrm{M}^{-1}$. Taking into account that the association constant for binding of progesterone and quercetin with monomeric β-CyD was 5000[11] and 130[14] M^{-1}, respectively, it was indicated that the binding between lidocaine and β-CyD polymer was very weak.

The endothermic peak for HPC/β-CyD polymer film was slightly larger than that for the HPC film, indicating that crystallinity of lidocaine would be increased by the addition of β-CyD polymer. Since the amount of β-CyD polymer was almost a half of HPC in the film dosage form, it is not surprising that the structure of polymer network might be different between β-CyD polymer-containing film and the control. Thus, crystallinity of lidocaine in the films might be changed by the addition of β-CyD polymer. It cannot be ruled out that such effect might alter the release rate from the film dosage forms.

Since ketoprofen has a much larger association constant with β-CyD polymer than lidocaine, it was likely that retardation of drug release by β-CyD polymer be more significant with ketoprofen. Nevertheless, the release of ketoprofen from mucoadhesive polymer films was rather increased by addition of β-CyD polymer. There have also been controversial reports regarding the effect of β-CyD on dissolution of drugs in tablet formulations.[15—19] Horiuchi *et al.*[15] demonstrated that dissolution rate of diltiazem from the tablets was decreased by the addition of diethylated and triethylated β-CyD. In addition, ethylated β-CyD was reported to retard *in vitro* release of buserelin acetate from oily suspensions.[16] On the contrary, increased dissolution by inclusion complexation with β-CyD or its derivatives has been observed for nifedipine,[17] naproxen,[18] and famotidine.[19] Enhanced dissolution by β-CyD tends to be observed with water-insoluble drugs, presumably since dissolution of water-insoluble drugs in the crystal form would be slower than that from inclusion complexes. Although ketoprofen was in the amorphous state in the polymer films, recrystallization would occur easily after contacting with aqueous solution. When an inclusion complex with β-CyD polymer was being formed, however, the phase transition to water-insoluble crystal forms would not occur. Hence, the release rate of ketoprofen from β-CyD polymer-containing film dosage forms would be higher than that from the control film.

In conclusion, effect of low-molecular-weight β-CyD polymer to the drug release rate from film dosage forms appears to vary according to the strength of interaction with and the solubility of active ingredient. However, this study clearly demonstrated that addition of low-molecular-weight β-CyD polymer to film dosage forms could control *in vitro* dissolution rate of the drug.

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Review

Lipid Carrier Systems for Targeted Drug and Gene Delivery

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For effective chemotherapy, it is necessary to deliver therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial effects and side effects. The use of lipid dispersion carrier systems, such as lipid emulsions and liposomes, as carriers of lipophilic drugs has attracted particular interest. A drug delivery system can be defined as a methodology for manipulating drug distribution in the body. Since drug distribution depends on the carrier, administration route, particle size of the carrier, lipid composition of the carrier, electric charge of the carrier and ligand density of the targeting carrier, these factors must be optimized. Recently, the lipid carrier system has also been applied to gene delivery systems for gene therapy. However, in both drug and gene medicine cases, a lack of cell-selectivity limits the wide application of this kind of drug and/or gene therapy. Therefore, lipid carrier systems for targeted drug and gene delivery must be developed for the rational therapy. In this review, we shall focus on the progress of research into lipid carrier systems for drug and gene delivery following systemic or local injection.

Key words drug delivery system; liposomes; lipid emulsion; gene delivery; targeting

1. Introduction

For effective chemotherapy, it is necessary to deliver therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial effects and side effects. In general, the lack of selectively of most conventional drugs is closely related to their pharmacokinetic properties. The *in vivo* fate of a drug given by a particular administration route is determined by both the physicochemical properties of drug and the anatomical and physiological characteristics of the body. Most conventional drugs diffuse freely throughout the body and show relatively even tissue distribution due to their low molecular weight.[1)]

The use of lipid dispersion carrier systems, such as lipid emulsions and liposomes, as carriers of lipophilic drugs has attracted particular interest. A drug delivery system can be defined as a methodology for manipulating drug distribution in the body. Since the drug distribution of loaded lipid carriers varies depending on; i) administration route (*i.e.*, local or systemic injection) of the carrier, ii) drug release from the carrier, iii) lipid composition and electric charge of the carrier, and iv) particle size of the carrier, these factors must be considered.

Recently, lipid carrier systems have also been applied to gene delivery systems for gene therapy.[2)] The most important factor for successful gene therapy is the development of novel gene vectors; therefore, various viral vectors and non-viral vectors have been developed. Although the gene transfer efficacy of the current non-viral vector systems is lower than that of viral vectors, the approach seems useful for many applications that require gene expression from the viewpoint of safety. Among the various types of non-viral vectors, cationic liposome mediated gene transfection seems to be one of the most promising approaches because of its relatively high transfection efficiency.

In both drug and gene medicine cases, however, lack of site (or cell)-selectivity limits the wide application of this kind of drug and/or gene therapy. In this review, we shall focus on the progress of research into lipid carrier systems for drug and gene delivery following systemic or local injection.

2. Lipid Emulsions for Drug Delivery Following Local Injection

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 because lipid emulsions possess an oil phase in particulate form, so they can dissolve large amounts of highly lipophilic drugs.[3)] An important prequisite for success in the application of pharmacologically active drugs is site-specificity. Local injection into the diseased tissues is one promising approach. This is particularly applicable to cancer chemotherapy, in which the supply of antitumor drugs to non-diseased tissue leads to serious side effects.

The local retention of anticancer agents injected intratumorally is very low because of the large diffusion capability due to their small molecular size. In our series of studies, we have demonstrated an increased transport and prolonged supply of antitumor drugs to lymphatics with water-in-oil (W/O) emulsions.[4,5)] In addition, the intratumoral injection of antitumor drugs is one of the most promising approaches for solid

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local tumors, to minimize the side effects and maximize cytotoxicity at the tumor site.[6—8] To enhance the retention and/or distribution in the lymph or tumor, lipid emulsions have been used because of their favorable characteristic as a biodegradable drug reservoir. In this section, we shall focus on lipid emulsions for local injection.

2.1. Distribution Characteristics of Lipid Emulsions or Liposomes Following Intramuscular or Intragastric Injection Figure 1 represents a model of drug transfer to the lymph after topical injection of lipid carrier formulation. As shown in this model, the drug injected into interstitial spaces of tissues is transported away from the injection site by the circulating blood, but reaches the regional lymph nodes to varying degrees, depending on the site of injection.

The lymphatic transport of bleomycin in different formulations; oil-in-water (O/W) and W/O emulsions was investigated.[4] When O/W and W/O emulsions were utilized as the delivery system, the W/O emulsion was effective in increasing the lymph level, in both cases of intraperitoneal and intramuscular injection. In the emulsion system, the hydrophilic anticancer drug is predominantly located not in the oily phase, but in the aqueous phase; consequently, bleomycin is distributed in the outer phase in the case of the O/W emulsion, and it is encapsulated in the inner phase in the case of the W/O emulsion. Although the utilization of an emulsion seems promising for the facilitation of drug transportation into the lymph, the instability of the emulsion is one of the problems from the viewpoints of pharmaceutical technology. In order to enhance the stability, gelatin sphere-in-oil (S/O) emulsions were developed as a new formulation for anticancer drugs. The greatest enhancement of the drug delivery and successful prevention of lymphatic metastasis was obtained with S/O emulsions following intramuscular and intragastric injection.[5,9,10] The lymphatic transport of the S/O emulsion was accelerated by the increase of injection volume and massage of the injection site, suggesting that hydrostatic tissue pressure plays a role in lymphatic delivery.[11] Furthermore, sphere-in-oil-in-water (S/O/W) multiple emulsions were developed to reduce the viscosity and improve the storage stability of multiple emulsions.[12] Both S/O and S/O/W emulsions an exhibited enhanced lymphatic transfer of bleomycin following injection into the appendices of rabbits (Fig. 2).

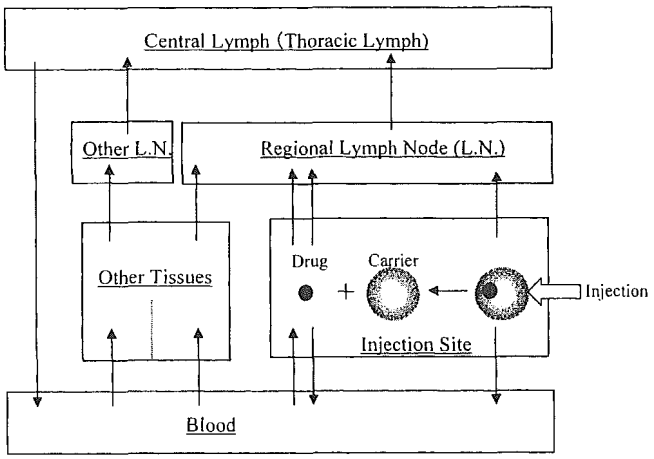

Fig. 1. Schematic Diagram of a Model of Drug Transfer Following the Injection of Lipid Carrier Formulations

In clinical trials, 27 of 33 patients received a bleomycin S/O emulsion injected directly into the tumors with satisfactory results. Comparative studies of treatments between the bleomycin S/O emulsion and surgery indicated that injection therapy of the bleomycin S/O emulsion would be more beneficial than surgical excision.[13]

As for the O/W emulsion, large logPCoct values or a high lipophilicity are required for drugs in order to keep them in the O/W emulsion. One of the most interesting potential approaches to prolong the retention time in emulsions after local injection is to increase the lipophilicity of the drug by chemical modification, leading to a prodrug.[14,15] That is, the combined application of lipophilic prodrug to the lipid carrier should achieve controlled drug release.[16] In fact, the lipophilic prodrug mitomycin C[17] and 5-fluorouracil[18] were more stably incorporated into the O/W emulsion and liposomes after intramuscular injection. This approach, the combined application of lipophilic prodrug to lipid carrier, could be applied to the liposomes. After intramuscular injection, liposomes appeared to accumulate at the lymph nodes to a greater degree than O/W emulsions[19]; accordingly, liposome formulation is an effective approach for the lymph-selective drug delivery carrier. However, a distribution study of liposomes with incorporated drugs demonstrated that hydrophilic drugs were rapidly released from the liposomal formulations after intramuscular injection. In contrast, nonyloxycarbonyl mitomycin C was completely incorporated in the liposomes. In addition, we confirmed that nonyloxycarbonyl mitomycin C incorporated liposomes enhanced drug delivery to the regional lymph nodes after intramuscular injection. Drug incorporation efficacy into the liposomes depends not only on the lipophilicity of drugs but also on the type used in the lipid of liposomes[20]; therefore, both the physicochemical properties of the drug and the lipid formulation should be considered with liposomal drug delivery systems.

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Mitsuru Hashida

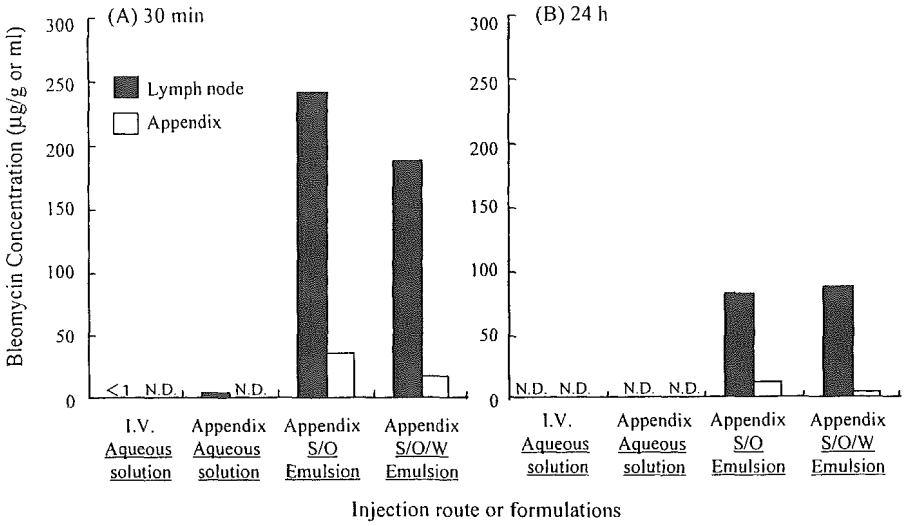

Fig. 2. Bleomycin Concentration in the Lymph Node and Appendix after Injection in Rabbits Using Various Formulations
Each result is the mean value of three experiments. N.D.; not detected. I.V.; intravenous.

2.2. Distribution Characteristics of Lipid Emulsions Following Intratumoral Injection Efficient cancer chemotherapy requires a high degree of selective localization of antitumor drug in the tumor. In this context, various drug delivery systems have been proposed and extensively investigated for their potential therapeutic application. Among the various routes of administration, the intratumoral route is a promising approach for solid local tumors to minimize the side effects and maximize cytotoxicity at the tumor site.[6—8] However, in most cases, the retention of antitumor drugs injected intratumorally is considered to be very low because of their low molecular size. In order to improve the retention of these drugs, lipid carriers could be used as a drug reservoir.

In order to clarify the distribution characteristics of injected drugs or carrier formulations after direct intratumoral injection, a Walker 256 tissue-isolated tumor perfusion system was employed.[21—23] This is a unique system, composed of a solid tumor with a pair of supplying arteries and a draining vein, and enabled us to study the intratumoral pharmacokinetics of a variety of materials, independent of the systemic circulation. The pharmacokinetics of anticancer drugs, macromolecular prodrugs and drug carriers following intra-arterial infusion or direct injection into the tumor have been studied with this system.

Using tumor-perfusion systems, the effect of the size of emulsions was studied.[24] A large emulsion (250 nm in diameter) and a small emulsion (85 nm in diameter) were prepared. Each formulation was labeled with [^{3}H]cholesteryl hexadecyl ether. In the case of the small emulsion formulations, a large fraction of the injected dose appeared in the venous outflow, 35—50% of the dose was recovered in the first minute after injection, on the tumor surface, and only about 10—40% of the injected dose remained in local tumor tissue. On the other hand, the large emulsion formulations remained in the tumor for a considerably longer time, and about 70% of the injected dose remained in the tumor 2 h after intratumoral injection. These results indicate that particle size is an important determinant of the retention in the tumor after intratumoral injection.

3. Lipid Emulsions for Drug Delivery Following Systemic Injection

O/W lipid emulsions and liposomes, as carriers of lipophilic drugs, have attracted particular interest following systemic injection. In particular, lipid emulsion formulations are considered to be superior to others due to the fact that they can be produced on an industrial scale, are stable during storage, and are highly biocompatible. In fact, emulsion formulations of lipophilic drugs, such as prostaglandin E_1, diazepam and non-steroidal anti-inflammatory drugs, have already been developed and marketed.[25—27] With recent pharmaceutical and therapeutic developments, lipid emulsions have been introduced as parental drug carriers offering sustained release and organ targeting. In this section, we shall focus on the progress of research into passive and active targeting systems of emulsions after systemic injection.

3.1. Distribution Characteristics of Lipid Emulsion Following Systemic Injection In order to clarify the distribution characteristics of emulsions after systemic injection, a pharmacokinetic study was performed using a [^{14}C]-labeled cholesteryl oleate labeled emulsion. After intravenous injection, the large emulsion (about 280 nm in diameter) rapidly disappeared from the blood and about 60% of the dose was recovered in the liver within 10 min of its intravenous injection in mice.[28] On the other hand, small emulsions (about 100 nm in diameter) showed a reduced hepatic uptake and a prolonged blood circulation time. A pharmacokinetic analysis revealed that the small emulsion has an 8- to 100-times smaller organ distribution clearance by the liver, spleen and lungs and about a 4-times greater area under the plasma concentration–time curve (*AUC*) than the large emulsion. Single-pass rat liver perfusion experiments have shown that more than 70% of the large emulsion was extracted by the liver, indicating extensive uptake of the large emulsion during a single passage.[29] In addition, the large emulsion was predominantly recovered from liver non-parenchymal cells (NPC), including Kupffer cells, and showed a higher accumulation in the NPC fraction.

Sphingomyelin (SM) is known to stabilize the membrane structure of liposomes and the addition of SM to liposomes

has been reported to be effective in reducing their clearance by the RES.[30—32] In order to develop a stable emulsion in blood, we developed novel emulsions composed of soybean oil and egg yolk SM (SM emulsion).[28] After intravenous injection, the SM emulsion showed a prolonged retention in the blood circulation. The uptake clearance of the SM emulsions in the liver was about 4-times less than that of conventional emulsions, suggesting reduced clearance by the RES.

3.2. Galactosylated Emulsions for Asialoglycoprotein Receptor-Mediated Drug Delivery to Hepatocytes 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 hepatocyte targeting.[33] These apo E associated emulsions are reported to be selectively taken up by liver parenchymal cells (PC) and are useful for the delivery of antiviral drugs, such as iododeoxyuridine, to hepatocytes. However, the introduction of apo E to the carrier is rather complicated, and so there can be problems as far as the reproducibility and stability of apoE emulsions are concerned.

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 non-reducing terminal of sugar chains. The lipid emulsion (oil-in-water) surface exhibits aqueous properties; thus a galactose moiety could cover the emulsion surface. It was reported that a lipophilicity exceeding logPCoct=8[34] or 18[35] was required for the stable entrapment of drugs in O/W emulsions after intravenous injection; accordingly, ligand modified lipids should possess a high lipophilicity for efficient delivery *in vivo*.

Our strategy for the efficient targeting of lipid carriers 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[36,37] and only mono-galactoside was introduced to the lipid as a ligand because the introduction of many hydrophilic galactose moieties to a lipid anchor would result in their removal from liposomes by interacting with lipoproteins and/or other lipid compartments under *in vivo* conditions.[38] 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 lipid carriers with galactose moieties for hepatocyte targeting.[39] When Gal-C4-Chol was added to O/W emulsions, a hydrophilic galactose moiety was fixed to the particle surface. Figures 3A and B show the scheme of ligand modified lipid carriers for cell-selective drug delivery.

After intravenous injection, galactosylated emulsions (Gal-emulsions) were rapidly eliminated from the blood and accumulated in the liver, in contrast to the bare-emulsions.[40] The liver uptake clearance of the Gal-emulsions was 3.2-times greater than that of the bare-emulsions. The uptake ratio in liver PC and NPC of the Gal-emulsions was higher than that of the bare-emulsions, suggesting that Gal-emulsions are effective PC-selective carriers. The hepatic uptake of Gal-emulsions, but not that of bare-emulsions, was significantly inhibited by predosing not only with lactoferrin but also Gal-liposomes, suggesting an asialoglycoprotein receptor-mediated endocytosis. Thus, Gal-emulsions have been proven to be an alternative carrier for hepatocyte-selective drug targeting after intravenous injection.

4. Liposomes for Drug Delivery Following Local Injection

Similar to emulsions, the intratumoral injection of antitumor drugs or genes with liposomes is one of the most promising approaches for solid local tumors to minimize side effects and maximize cytotoxicity at the tumor site. Liposomes have also been used because of their favorable characteristics as a biodegradable drug or gene medicine reservoir. In this section we shall focus on the local distribution characteristics of liposome formulations after intratumoral injection have become an important issue in drug or gene delivery.

4.1. Distribution Characteristics of Liposomes Following Intratumoral Injection Since drugs incorporated in liposomes are distributed with liposomes, the distribution characteristics of liposomes after intratumoral administration are important. To investigate the effect of size or charge on distribution, neutral liposomes (120-nm in diameter), and cationic liposomes (125-nm in diameter) were prepared.[24] Each formulation was labeled with [^{3}H]cholesteryl hexadecyl ether. The zeta potentials of neutral liposomes and cationic liposomes were −5.4 and 47.6 mV. The pharmacokinetic properties of the gene were studied after direct intratumoral injection using a Walker 256 tissue-isolated tumor perfusion system. After intratumoral injection, approximately 90% of the administered cationic liposomes remained in the tumor while the corresponding figure for neutral liposomes was 18%. Since the size of each liposome was almost the same, cationic liposomes may remain in the tumor due to the electrostatic interaction after intratumoral injection.

4.2. Distribution and Gene Expression Characteristic of pDNA and Its Complex with Cationic Liposomes after Intratumoral Injection Wolff *et al.* reported that naked pDNA in the skeletal muscle after intramuscular injection is specifically located in T tubules and/or caveolae specific to striated muscle, and these structures may play an important role in the uptake rather than physical disruption of the membrane of myotubes with direct injection.[41] We applied this method to the pharmacokinetic evaluation of naked pDNA and its cationic liposome complexes.[42] The pharmacokinetic properties of the gene were studied after direct intratumoral injection using a Walker 256 tissue-isolated tumor perfusion system.[21—23] Approximately 50% of the naked pDNA was eliminated from the tumor 2 h after injection and intact pDNA was found in the venous outflow, while more than 90% of the pDNA was retained in the tumor when complexed with cationic liposomes (Lipofectin®), suggesting that the cationic liposomes increase the retention of pDNA in the tumor tissue due to electrostatic interaction which results in less appearing in the venous outflow.

Gene expression was assessed in three types of solid mouse tumors after the direct injection of naked pDNA encoding the luciferase gene (pCMV–Luc) and its DC–Chol liposome complexes.[43] The intratumoral injection of naked pCMV–Luc into subcutaneously inoculated mouse colon tumor (CT-26), fibrosarcoma (MCA-15) and bladder carcinoma (MBT-2) resulted in significant gene expression regardless of the rapid clearance from the injection site. Sur-