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# Effects of fusogenic and DNA-binding amphiphilic compounds on the receptor-mediated gene transfer into hepatic cells by asialofetuin-labeled liposomes

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**Abstract**

Effects of fusogenic and DNA-binding amphiphilic compounds on the receptor-mediated gene transfer using asialofetuin-labeled liposomes (AF-liposomes) were examined with HepG2 cells and rat hepatocytes in primary culture. AF-liposomes were sufficiently taken up by both types of cells through the asialoglycoprotein receptor-mediated endocytosis. In HepG2 cells, bacterial β-galactosidase (β-Gal) gene expression was observed by transfection using AF-liposomes encapsulating plasmid pCMVβ DNA (AF-liposome-pCMVβ). By addition of dioleoylphosphatidylethanolamine (DOPE) to the liposomal lipid composition (AF-liposome(DOPE)-pCMVβ), the transfection efficiency was clearly increased. The effects of DOPE were more conspicuous in the presence of chloroquine in the medium throughout the transfection. When pCMVβ complexed with gramicidin S (pCMVβ(GrS)) was encapsulated (AF-liposome(DOPE)-pCMVβ(GrS)) and was transfected to HepG2 cells, an significantly high β-Gal activity in the cells was observed as compared with that in the cells transfected with AF-liposome(DOPE)-pCMVβ. No effects of GrS were found in the transfection using AF-non-labeled control liposomes. In primary culture of rat hepatocytes, no β-Gal gene expression was observed even though AF-liposome(DOPE)-pCMVβ was introduced into the cells prepared from adult rats. However, following the transfection with AF-liposome(DOPE)-pCMVβ, the β-Gal activity was expressed in the cells from immature rats cultured in the medium supplemented with epidermal growth factor and insulin, and the transfection efficiency was 2-fold higher than that transfected with pCMVβ encapsulated in AF-non-labeled control liposomes. By the complex formation of pCMVβ with GrS, the transfection efficiency of AF-liposome(DOPE)-pCMVβ(GrS) increased according to the increase of GrS in the complex. It was shown that AF-liposome(DOPE)-pCMVβ(GrS) did efficiently introduce and express β-Gal gene in both HepG2 cells and primary hepatocytes in the receptor mediated manner.

*Keywords:* Liposome; Asialofetuin; Amphiphilic compound; Receptor mediation; Gene transfer; Hepatocyte; (Rat)

Abbreviations: AF, asialofetuin; AF-liposomes, AF-labeled liposomes; AF-liposome-pCMVβ, pCMVβ-encapsulated AF-liposomes; DOPE, dioleoylphosphatidylethanolamine; AF-liposome(DOPE)-pCMVβ, pCMVβ-encapsulated AF-liposomes containing DOPE; GrS, gramicidin S; pCMVβ(GrS), pCMVβ complexed with GrS; AF-liposome(DOPE)-pCMVβ(GrS), pCMVβ(GrS)-encapsulated AF-liposomes containing DOPE; AgpR, asialoglycoprotein receptors; RME, receptor-mediated endocytosis; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; Ins, insulin; Chol, cholesterol; EB, ethidium bromide; PC, egg yolk phosphatidylcholine; TMAG, *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; BSA, bovine albumin; DOC, sodium deoxycholate; N-liposome(DOPE)-pCMVβ, pCMVβ-encapsulated non-labeled control liposomes containing DOPE; N-liposome(DOPE)-pCMVβ(GrS), pCMVβ(GrS)-encapsulated non-labeled control liposomes containing DOPE.

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## 1. Introduction

Advancements in gene therapy depend to a large degree on the development of delivery systems capable of efficiently introducing DNA into the target cells. Although significant progress has been made using recombinant retroviruses [1,2] or adenoviruses [3,4] for gene delivery, concern about the possible production of replication competent viruses by the recombination [5] and the oncogenic effects by random insertion into the host genome [6] has encouraged a development of non-viral DNA-mediated gene transfer techniques [7–9].

We are developing a non-viral methods introducing DNA into hepatic parenchymal cells to be a safe and reliable gene delivery system that can be used in gene therapy. Our strategy has been to exploit the function of asialoglycoprotein receptors (AgpR) unique to mammalian hepatocytes and capable of recognizing galactose residue at the terminal of sugar chain [10–12]. This approach uses the combination of asialofetuin (AF) as the specific ligand [13] and liposomes as a vehicle which is able to protect DNA condensed in its internal space. We have demonstrated a successful uptake of AF-labeled liposomes (AF-liposomes) by hepatocytes in vitro [14,15] and their specific distribution into rat liver in vivo through AgpR [16]. Plasmid DNA encapsulated in AF-liposomes was expressed in cultured HepG2 cells [17] and mouse liver parenchymal cells following portal vein administration [18]. In these attempts, it has been clarified that the transgene expression efficiency was increased by pretreatment of cells or animals with EDTA encapsulated in AF-liposomes. We speculated that a large fraction of DNA introduced into cells by the receptor-mediated endocytosis (RME) would be hydrolyzed by nucleases in the lysosome, and that EDTA delivered by the same route as DNA-encapsulated AF-liposomes could reduce lysosomal nuclease activity. The degradation of DNA in the lysosome seems to be a common problem in various gene transfer techniques using RME [19].

Since the efficiency of viral infection is in large part due to fusogenic amino acids on viral membrane proteins, we examined whether a fusogenic lipid or membrane destabilizing peptide could be used to improve the efficiency of the transfection using AF-liposomes. Dioleoylphosphatidylethanolamine (DOPE) capable of leading a membrane fusion through a formation of hexagonal II phase in the membrane [20] was added to the lipid composition of AF-liposomes. Gramicidin S (GrS) [21], a membrane binding peptide, which can strongly interact with DNA by charge interactions [22] and destabilize membranes [23,24], was complexed with DNA and encapsulated in AF-liposomes. The ability of AF-liposomes thus modified to transfect both hepatoblastoma cell line, HepG2, and rat hepatocytes in primary culture was examined using a plasmid pCMVβ DNA as a reporter.

## 2. Materials and methods

### 2.1. Materials

AF (type I), *O*-nitrophenyl-β-D-galactopyranoside (ONPG), cytochalasin B, trypsin inhibitor and insulin (Ins) were purchased from Sigma (St. Louis, MO). Cholesterol (Chol), collagenase and ethidium bromide (EB) were from Wako Pure Chemicals (Osaka, Japan). Egg yolk phosphatidylcholine (PC) was obtained from Nippon Fat and Oil (Tokyo, Japan), *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) from Sogo Pharmaceutical (Tokyo, Japan), DOPE from Avanti Polar Lipids (Alabaster, AL) and metrizamide from Nycomed Pharma (Oslo, Norway). Epidermal growth factor (EGF) was obtained from Wakunaga Pharmaceutical (Hiroshima, Japan). Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimum essential medium (EMEM) were from Nissui Pharmaceutical (Tokyo, Japan). DM-160AU medium was purchased from Kyokuto Pharmaceutical (Tokyo, Japan), fetal bovine serum (FBS) from Moregate Exports (Melbourne, Australia) and bovine serum albumin (BSA) from Boehringer-Mannheim (Mannheim, Germany). [$^{14}$C]Cholesteryl oleate and Aquasol-2 were purchased from Du Pont New England Nuclear (Boston, MA). All other reagents were of analytical grade.

The plasmid pCMVβ DNA (7.2 kb) constructed by MacGregor and Caskey [25] was amplified in *Escherichia coli*, isolated by the alkaline method and purified using a pZ523 column (5 Prime-3 Prime Inc., Denver, CO). The DNA was dissolved in an appropriate volume of 10 mM Tris-HCl buffer (THB, pH 8.0) containing 1 mM EDTA. No contamination of bacterial cellular DNA was confirmed by 1.0% agarose gel electrophoresis. The concentration of DNA was determined by the absorbance at 260 nm, and the DNA solution was diluted to 4 μg/μl and stored at 4°C until use.

### 2.2. Preparation of pCMVβ complexes with GrS

pCMVβ and GrS complexes (pCMVβ(GrS)) were prepared according to Legendre et al. [21]. In brief, GrS dissolved in dimethyl sulfoxide (20 mg/ml) was diluted (×1/10) with 10 mM THB (pH 7.5). 20 μl of the diluted GrS solution was mixed with 20 μg of pCMVβ dissolved in 280 μl of 10 mM THB (pH 7.5). The mixture was incubated at room temperature for 30 min, and then pCMVβ(GrS) with the weight ratio of GrS/pCMVβ = 2 was obtained. pCMVβ(GrS)s with the weight ratio of 0.5, 1 and 4 were also prepared using 5, 10 or 40 μl of the diluted GrS solution.

### 2.3. Preparation of AF-liposomes encapsulating pCMVβ

Plasmid pCMVβ DNA or pCMVβ(GrS) was encapsulated in AF-liposomes composed of PC/TMAG/Chol =

5:2:3 (AF-liposome-pCMVβ) or PC/TMAG/DOPE = 2:1:2 (AF-liposome(DOPE)-pCMVβ, AF-liposome(DOPE)-pCMVβ(GrS)) by a tandem combination of the detergent removal and freeze-thaw methods [17]. AF (125 μg) was incubated with 5-fold mol of *N*-palmitoyloxysuccinimide in 25 μl of 10 mM THB (pH 7.5) containing 2% sodium deoxycholate (DOC) at 37°C for 12 h to prepare palmitoyl-AF. 1 μmol of lipids in chloroform were dried under reduced pressure into a thin lipid film on the inner face of a glass tube. Following hydration with 50 μl of 10 mM THB (pH 7.5), the DOC solution of palmitoyl-AF was added and the mixed micelles were prepared by vigorous vortexing. The DOC was dialyzed out to 10 mM THB (pH 7.5) for 24 h, and AF-liposomes encapsulating the buffer were formed. The empty liposomes were sonicated for 5 min in a bath type sonicator (Branson B-2200, Tokyo, Japan). 20 μg of pCMVβ diluted with 10 mM THB (pH 7.5) to 300 μl or pCMVβ(GrS) was added to the liposome suspensions and lyophilized. After rehydration with 20 μl of 10 mM THB (pH 7.5) containing 0.9% NaCl, freeze (at −80°C)-thaw (at 0°C) was repeated. pCMVβ or pCMVβ(GrS)-encapsulated non-labeled control liposomes (N-liposome(DOPE)-pCMVβ, N-liposome(DOPE)-pCMVβ(GrS)) were also prepared without palmitoyl-AF. These liposomes encapsulating DNA were purified by metrizamide-gradient flotation as described previously [17]. The concentration of DNA and phospholipids in the liposomal fractions were determined according to the method of Boer [26] and Chen et al. [27], respectively, following solubilization of liposomes by Triton X-100 (final concentration 0.5%). By this procedure, it was ascertained that more than 85% of used pCMVβ or pCMVβ(GrS) was associated to liposomes and about 65% of them became resistance to DNase I digestion (50 μg/ml, 37°C, 30 min), indicating their encapsulation into the internal aqueous phase of liposomes, irrespective of the liposomal lipid composition and AF-labeling.

### 2.4. Cells

HepG2 cells, human hepatoblastoma cell line [28], were grown in the DM-160AU medium supplemented with 10% FBS, 2 mM L-glutamine, 15 mM $NaHCO_3$ and 60 μg/ml streptomycin in 150 mm plastic culture dishes (Nunc, Roskilde, Denmark) at 37°C under an atmosphere of $CO_2$/air = 5:95. The cells at semi-confluence (4–6 days growth) were scraped from dishes with addition of an aqueous solution of 0.25% trypsin containing 1 mM EDTA, spun down, and washed. The cells were dispersed ($8 \cdot 10^5$/ml) and seeded (5 ml each) in 60 mm plastic culture dishes.

Hepatocytes in primary culture were prepared from male adult (8 week) and immature (3 week) Wistar rats according to the method of Moldeus et al. [29] with a minor modification. A cannula was inserted into portal vein of anesthetized rats and pre-perfusion of the liver was immediately started with a perfusion buffer (8 g of NaCl, 0.4 g of KCl, 0.078 g of $NaH_2PO_4$, 0.151 g of $Na_2HPO_4 \cdot 12\ H_2O$, 2.38 g of Hepes, 0.35 g of $NaHCO_3$/liter, pH 7.4) supplemented with 5 mM glucose and 0.5 mM EGTA at a flow rate of 30 ml/min. When the blood was fully removed, the perfusion buffer was changed to the same buffer containing 0.5 mg/ml collagenase, 50 μg/ml trypsin inhibitor, and 5 mM $CaCl_2$, and the perfusion was continued for another 6 min. At the end of the perfusion, the liver was excised, immersed in ice-cold EMEM supplemented with 0.03% L-glutamine and 0.4% $NaHCO_3$ and dispersed by gentle shaking. The cell dispersion was filtered through a cotton gauze and the cells were collected by centrifugation at $50 \times g$ for 1 min at 4°C. Following washing four times, the cells were dispersed in the normal medium, DMEM containing $10^{-9}$ M Ins, $10^{-9}$ M dexamethasone, 0.25 μg/ml fungizone and 100 μg/ml streptomycin, 1 mg/ml penicillin and 20 mM $NaHCO_3$, or the modified medium, the normal medium not containing dexamethasone but supplemented with $10^{-7}$ M Ins, 20 ng/ml EGF, 90 μg/ml L-alanine, 50 μg/ml L-glutamic acid, 2 μg/ml L-ascorbic acid, 30 μg/ml L-aspartic acid and 30 μg/ml L-proline, at a density of $6 \cdot 10^5$/ml for adult or $5 \cdot 10^5$/ml for immature rat hepatocytes, and seeded (4 ml each) in 60 mm collagen coating culture dishes (KOKEN, Tokyo, Japan).

### 2.5. Uptake of AF-liposomes by hepatic cells

HepG2 cells ($4 \cdot 10^6$) and adult rat hepatocytes ($2.4 \cdot 10^6$) were pre-cultured for 1 day. After the medium was changed to 1 ml of the FBS-free medium, AF-liposomes containing the tracer amount of [$^{14}$C]cholesteryl oleate were added with or without 2 mg of free AF and incubated at 37°C in 5% $CO_2$ for 18 h. At the indicated points of time, the cells were washed with ice-cold medium, lysed with 0.5 ml of 1 N KOH and neutralized with 0.6 ml of 1 N acetic acid. The radioactivity of 1 ml of cell lysates was counted.

### 2.6. Transfection and β-Gal assay

After HepG2 cells ($4 \cdot 10^6$) and adult ($2.4 \cdot 10^6$) and immature rat hepatocytes ($2 \cdot 10^6$) were pre-cultured for 1 day, the medium was removed and the cells were washed three times with FBS-free medium. Liposomes encapsulating pCMVβ or pCMVβ(GrS) were diluted up to 1 ml with FBS-free medium, and then added to the cells and incubated at 37°C in 5% $CO_2$ for 16 h. When the liposomes were added together with chloroquine or cytochalacin B, incubation was shortened to 6 h. At the end of the incubation, the medium was removed and the cells were further cultured for 48 h in the fresh medium containing 10% FBS. The cells were washed twice and were scraped from dishes into ice-cold 20 mM THB (pH 7.5) containing 150

mM NaCl and 1 mM EDTA and centrifuged at 2500 × *g* for 5 min. The cells, resuspended in 0.4 ml of 0.1 M PBS (pH 7.5), were disrupted by five freeze-thaw cycles using dry ice/methanol and a 37°C water bath. Following centrifugation at 14000 × *g* for 15 min, a portion of the supernatant was assayed for protein content by the Bradford microprotein assay (Bio-Rad, Lichmond, CA). β-Gal activity was assayed according to Miller [30] as follows: the supernatants containing 100 μg of protein were diluted up to 231 μl with 0.1 M PBS (pH 7.5), mixed with 3 μl of 0.1 M $MgCl_2$ containing 4.5 M β-mercaptoethanol, and 66 μl of 0.4% ONPG in 0.1 M PBS (pH 7.5), and incubated at 37°C for 30 min. The reaction was stopped by addition of 500 μl of 1 M $Na_2CO_3$. The optical density of the reaction mixture was measured at 420 nm. The enzyme activity was expressed as a unit (one nmol of *O*-nitrophenol liberated from ONPG per min) per mg protein.

## 3. Results

### 3.1. AgpR-mediated uptake of AF-liposomes by hepatic cells

AF-liposomes containing a tracer amount of [$^{14}$C]cholesteryl oleate were added to HepG2 cells or adult rat hepatocytes in primary culture and incubated at 37°C. At the indicated points of time, the radioactivity associated with the cells was determined as uptake indicating the sum of the AF-liposomes adsorbed to and incorporated into the cells. As shown in Fig. 1, the cell-associated radioactivity increased with lapse of time and reached about 50% of the added dose in HepG2 cells and about 74% in primary hepatocytes for 18 h. By addition of excess free AF, the cell-associated radioactivities were reduced by about 80% in HepG2 cells and by 60% in primary hepatocytes.

Table 1
Effects of DOPE on the transfection of hepatocytes by AF-liposomes

| DNA (μg/dish) | β-Galactosidase activity (units/mg protein) [a] | | |
|---|---|---|---|
| | HepG2 cells | | primary hepatocytes [b] |
| | AF-liposome-pCMVβ [c] | AF-liposome (DOPE)-pCMVβ [d] | AF-liposome (DOPE)-pCMVβ [d] |
| 0 | 2.36 ± 0.22 | 2.36 ± 0.22 | 1.89 ± 0.26 |
| 1.25 | 2.81 ± 0.31 | 4.01 ± 0.46 * | ND |
| 2.50 | 3.65 ± 0.24 | 5.61 ± 0.63 ** | ND |
| 5.00 | 6.89 ± 0.52 | 13.0 ± 1.18 *** | 2.10 ± 0.31 |
| 10.0 | 11.0 ± 1.21 | 15.4 ± 2.07 * | ND |
| 15.0 | 12.8 ± 1.06 | 20.0 ± 2.14 ** | 2.37 ± 0.25 |

[a] The mean from three experiments ± S.D.
[b] Hepatocytes from adult rats were cultured in normal medium (DMED supplemented with 10% FBS).
[c] AF-liposome-pCMVβ (23.6 μg DNA/μmol lipids) was added.
[d] AF-liposome (DOPE)-pCMVβ (26.3 μg DNA/μmol lipids) was added.
ND, not determined.
Statistically significant differences from AF-liposome-pCMVβ: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

### 3.2. Effects of DOPE on the transfection efficiency of AF-liposomes encapsulating pCMVβ

Hepatic cells were transfected with various amounts of AF-liposome-pCMVβ or AF-liposome(DOPE)-pCMVβ. In HepG2 cells, β-Gal gene expression was observed for both AF-liposome-pCMVβ and AF-liposome(DOPE)-pCMVβ, and β-Gal activity in the cells increased with increasing DNA added to the cells (Table 1). Transfection efficiency of AF-liposome(DOPE)-pCMVβ was 1.4–1.9-fold higher than that of AF-liposome-pCMVβ at any amount of DNA added. But, no increase in β-Gal activity was observed in adult rat hepatocytes in primary culture transfected with AF-liposome(DOPE)-pCMVβ.

Effects of chloroquine on the transfection efficiency of AF-liposome-pCMVβ and AF-liposome(DOPE)-pCMVβ were examined in HepG2 cells. As shown in Fig. 2,

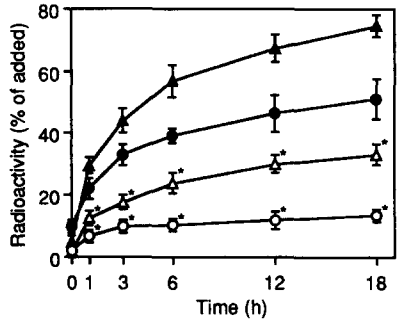


Fig. 1. Uptake of AF-liposomes by hepatic cells. HepG2 cells (circle) or adult rat hepatocytes in primary culture (triangle) were incubated with [$^{14}$C]AF-liposomes (20 nmol lipid/$10^6$ cells) at 37°C for indicated time in the absence (closed symbol) or presence (open symbol) of free AF (0.5 mg/$10^6$ cells). Each point presents the mean from three experiments ± S.D. statistically significant differences from the absence of free AF: $P < 0.001$(*).

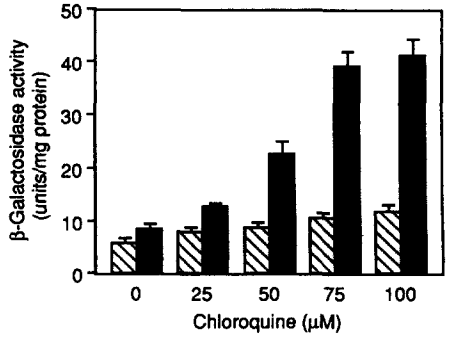


Fig. 2. Effects of chloroquine on the transfection efficiency of AF-liposomes encapsulating pCMVβ. HepG2 cells were transfected with AF-liposome-pCMVβ (4.0 μg DNA/0.17 μmol lipid, sparsely hatched column) or AF-liposome(DOPE)-pCMBβ (4.0 μg DNA/0.18 μmol lipid, closely hatched column) in the medium containing indicated concentration of chloroquine for 6 h. Each column presents the mean from three experiments ± S.D.

Table 2
Transfection of primary hepatocytes with AF-liposome (DOPE)-pCMVβ in the presence of chloroquine

| DNA [a] (μg/dish) | β-Galactosidase activity (units/mg protein) [b] | | |
|---|---|---|---|
| | adult DMEM [c] | immature DMEM [d] | immature modified DMEM [e] |
| 0 | 1.78±0.28 | 2.07±0.22 | 1.87±0.18 |
| 1.0 | ND | ND | 2.24±0.38 |
| 2.0 | ND | ND | 3.08±0.49 * |
| 4.0 | 2.18±0.29 | 2.64±0.28 | 3.41±0.26 ‡ * * |
| 8.0 | 2.06±0.24 | 2.83±0.17 # * * | 3.61±0.19 ‡‡ * * * |
| 16 | 1.74±0.33 | 2.69±0.23 # | 2.81±0.21 * * |

[a] AF-liposome (DOPE)-pCMVβ (28.6 μg DNA/μmol lipids) were added with 25 μM chloroquine.
[b] The mean from three experiments ± S.D.
[c] Hepatocytes from adult rats were cultured in normal medium (DMEM supplemented with 10% FBS).
[d] Hepatocytes from immature rats were cultured in normal medium (DMEM supplemented with 10% FBS).
[e] Hepatocytes from immature rats were cultured in modified medium (DMEM supplemented with 10% FBS, EGF and Ins).
ND, not determined.
Statistically significant differences from without DNA: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***); between adult and immature: $P < 0.05$ (#); between normal and modified medium: $P < 0.05$ (‡), $P < 0.01$ (‡‡).

transfection efficiencies of both AF-liposome-pCMVβ and AF-liposome(DOPE)-pCMVβ were enhanced depending upon the concentration of chloroquine in the medium, and almost the maximum effect was observed at 75 μM or more. However, the degree of enhancement by chloroquine was markedly different between the transfection by AF-liposome-pCMVβ and AF-liposome(DOPE)-pCMVβ, and a greater effect was observed in the latter with about 5-fold increase in β-Gal activity at 75 μM chloroquine, compared with only 2-fold increase in the former.

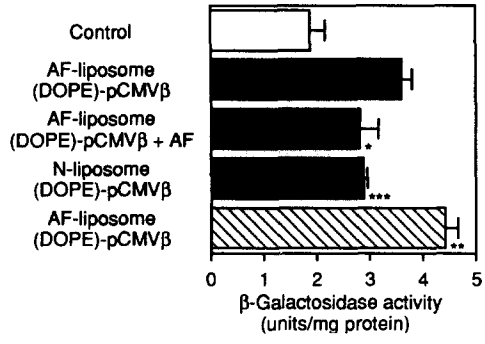


Fig. 3. Transfection of primary hepatocytes with AF-liposome(DOPE)-pCMVβ. Immature rat hepatocytes in primary culture were transfected with AF-liposome(DOPE)-pCMVβ (8.0 μg DNA/0.28 μmol lipid) with or without free AF (2 mg) or N-liposome(DOPE)-pCMVβ (8.0 μg DNA/0.28 μmol lipid) in the presence of chloroquine (25 μM). β-Galactosidase activity was assayed at day 2 (closely hatched column) or at day 6 (sparsely hatched column). Each column presents the mean form three experiments ± S.D. statistically significant differences from AF-liposome(DOPE)-pCMVβ at day 2: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.01$ (***).

### 3.3. Transfection of primary hepatocytes

Hepatocytes from adult and immature rats in primary culture were transfected with AF-liposome(DOPE)-pCMVβ in the presence of 25 μM chloroquine. As shown in Table 2, no increase in β-Gal activity was observed in adult rat hepatocytes, while β-Gal activity of immature rat hepatocytes increased with increasing DNA added to the cells irrespective of their culture medium, and the maximum transfection efficiency was obtained in the immature rat hepatocytes cultured in the modified medium containing EGF and Ins when 8 μg DNA of AF-liposome(DOPE)-pCMVβ was introduced.

In immature rat hepatocytes cultured in the modified medium, the transfection efficiency of AF-liposome(DOPE)-pCMVβ was compared with that of N-liposome(DOPE)-pCMVβ. As shown in Fig. 3, the β-Gal gene expression levels in the cells transfected with AF-liposomes were about 2-fold higher than that in the cells transfected with N-liposomes. By addition of free AF, the transfection efficiency with AF-liposomes was significantly reduced to the same level as that of N-liposomes. Surprisingly, β-Gal activity at day 6 in the cells transfected with AF-liposome(DOPE)-pCMVβ was significantly higher than at day 2.

### 3.4. Effects of pCMVβ complex with GrS on the transfection efficiency

Plasmid pCMVβ DNA (20 μg) was complexed with GrS (40 μg), encapsulated in liposomes and introduced into HepG2 cells. In the case of AF-liposomes, β-Gal activity was considerably enhanced by the transfection with AF-liposome(DOPE)-pCMVβ(GrS), and showed

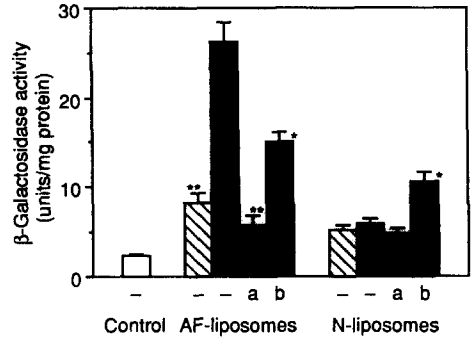


Fig. 4. Effects of GrS on the transfection efficiency of liposomes. HepG2 cells were transfected with liposomes encapsulating pCMVβ (sparsely hatched column) or pCMVβ(GrS) (closely hatched column) in the presence of free AF (2 mg/ml, a) or cytochalasin B (5 μg/ml, b). AF-liposome(DOPE)-pCMVβ (4.0 μg DNA/0.16 μmol lipid), AF-liposome(DOPE)-pCMVβ(GrS) (4.0 μg DNA/0.18 μmol lipid), N-liposome(DOPE)-pCMVβ (4.0 μg DNA/0.13 μmol lipid), N-liposome(DOPE)-pCMVβ(GrS) (4.0 μg DNA/0.25 μmol lipid). Each column presents the mean from three experiments ± S.D. statistically significant differences from the cells transfected with pCMVβ(GrS)-encapsulated liposomes: $P < 0.01$ (*), $P < 0.001$ (**).

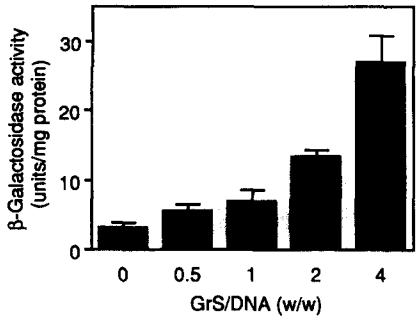


Fig. 5. Effects of GrS content on the transfection efficiency of AF-liposome(DOPE)-pCMVβ(GrS). Immature rat hepatocytes in primary culture were transfected with AF-liposome (DOPE)-pCMVβ(Grs) (0, 4.0 μg DNA/0.14 μmol lipid; 0.5, 4.0 μg DNA/0.17 μmol lipid; 1, 4.0 μg DNA/0.16 μmol lipid; 2, 4.0 μg DNA/0.15 μmol lipid; 4, 4.0 μg DNA/0.16 μmol lipid). Each column presents the mean from three experiments ± S.D.

about 3-fold higher β-Gal activity than that of AF-liposome(DOPE)-pCMVβ (Fig. 4). By addition of excess free AF, the transfection efficiency of AF-liposome(DOPE)-pCMVβ(GrS) was decreased to the same level as that of N-liposome(DOPE)-pCMVβ(GrS). Although the addition of cytochalasin B in the medium also decreased the transfection efficiency of AF-liposome(DOPE)-pCMVβ(GrS), the degree of inhibition was smaller than that by AF. In N-liposomes, no increase in β-Gal activity was observed even when the cells were transfected with N-liposome(DOPE)-pCMVβ(GrS). The addition of AF exhibited no effect and that of cytochalasin B brought about a significant increase in the transfection efficiency of N-liposome(DOPE)-pCMVβ(GrS).

pCMVβ(GrS) complexes with various GrS contents were encapsulated and the effects of GrS content on the transfection with AF-liposome(DOPE)-pCMVβ(GrS) were examined using immature rat hepatocytes. As shown in Fig. 5, the transfection efficiency of AF-liposome(DOPE)-pCMVβ(GrS) was increased with increasing GrS content in the complex. An extremely high β-Gal activity was found in the cells transfected with AF-liposome(DOPE)-pCMVβ(GrS) with the GrS/pCMVβ weight ratio of 4, and the activity was about sevenfold of that in the cells transfected with AF-liposome(DOPE)-pCMVβ without GrS.

## 4. Discussion

HepG2 cells and rat hepatocytes in primary culture were used as transformed and non-transformed cells, respectively, in this report. These cells are quite different in various properties such as the cell surface expressed proteins and proliferation activity. With primary cells in culture, including hepatocytes, successful transfection and expression of foreign DNA have been generally difficult to achieve [31], and there is no report concerning the transfection of primary hepatocytes using receptor-mediated gene transfer techniques.

As clearly shown in Fig. 1, AF-liposomes were sufficiently taken up by both HepG2 cells and adult rat hepatocytes in primary culture. The strong inhibition in the uptake by free AF indicates that interactions with the AgpR are largely involved in the association of AF-liposomes with both types of cells. In this experiment, it was determined beforehand that the association constant ($K_a$) and number ($n$) of AgpR on the primary hepatocytes were $1.8 \cdot 10^9$ $M^{-1}$ and $1.1 \cdot 10^5$/cell, respectively. These values are almost the same as those reported for isolated rat hepatocytes [32] and are somewhat higher than those of HepG2 cells previously determined as $K_a = 2.8 \cdot 10^8$ $M^{-1}$ and $n = 2.9 \cdot 10^4$/cell in our laboratory [33]. As predicted from their binding parameters, the uptake by primary hepatocytes were slightly higher than that by HepG2 cells (Fig. 1). However, in the transfection with AF-liposome(DOPE)-pCMVβ, a substantial β-Gal gene expression was observed only in HepG2 cells (Table 1). These results indicate that the reason for no transgene expression in adult rat hepatocytes in primary culture could not be attributed to their ability to take up AF-liposome-pCMVβ. Moreover, plasmid pCMVβ DNA contains cytomegalovirus immediate early promoter, which has been reported to exhibit a high promoter activity in adult rat and human hepatocytes [34,35].

It is known that DOPE is a hexagonal II phase-forming lipid under the physiological conditions and is able to promote membrane fusion [20]. Therefore, DOPE has been widely used in various cationic liposomes for transfection such as Lipofectine [36]. The significantly higher transfection efficiency of AF-liposome(DOPE)-pCMVβ in HepG2 cells than that of DOPE deficient AF-liposome-pCMVβ (Table 1) may be attributed to the fusogenic activity of DOPE, and the importance of liposome-cell fusion, whether with plasma membrane or endosome/lysosome membrane, was suggested in the efficient transfection using AF-liposomes. The DOPE effect was considerably enhanced by chloroquine (Fig. 2), a lysosomotropic agent [37]. It has been reported that a ligand endocytosed via AgpR is transferred to the lysosome and degraded rapidly [38]. In the presence of chloroquine, lysosomal function could be inhibited and AF-liposome (DOPE)-pCMVβ could be retained in the endosome/lysosome for a long period. The fusion with the membrane of the organella could occur and encapsulated pCMVβ could be released into the cytoplasm.

In this experiment, cells were transfected in an FBS-free medium. It has been reported that a considerable reduction of transfection efficiency by the serum components was observed in cationic liposomes [39]. In our previous report [17], transfection of HepG2 cells using AF-liposomes slightly inhibited by addition of negatively charged fetuin but not by bovine serum albumin, indicating that the

inhibition may due to the reduction of non-specific charge interaction of AF-liposomes with cells. Hence, addition of FBS would somewhat lower the transfection efficiency of AF-liposomes but the transfection via the specific interaction with AgpR may not be affected

The order of transgene expression efficiency was as follows; HepG2 cells > immature rat hepatocytes cultured in the modified medium containing EGF and Ins > immature rat hepatocytes cultured in the normal medium and no expression in adult rat hepatocytes (Tables 1 and 2). EGF and Ins are able to promote the cells in G1 to S and facilitate cellular proliferation [40]. Therefore, the order was consistent with that of their proliferation activities. It is speculated that exogenous DNA molecules might migrate into the nucleus during the disappearance period of the nuclear membrane, because their volume is too large to diffuse through nuclear pores [41]. It may be considered as another possibility that the transport of some endogenous nuclear proteins occurs at a high level when the cells are proliferating and exogenous DNA interact with the endogenous nuclear proteins and is co-introduced into the nucleus by means of active process [42–44]. Since adult liver consists of $G_0$ cells, it is important to elucidate the transport mechanism of foreign DNA into the nucleus to achieve in vivo gene transfer into hepatocytes.

Gene transfer and expression by AF-liposome (DOPE)-pCMV$\beta$ observed in primary hepatocytes were demonstrated to occur partially via AgpR (Fig. 3). The expression in the presence of free AF remained at the same level as that of N-liposome (DOPE)-pCMV$\beta$, and these were probably caused by the charge interaction between liposomes possessing positive charge and cells having negative surface charge. Surprisingly, $\beta$-Gal activity in the cells showed a higher level on day 6 rather than on day 2 following transfection with AF-liposome(DOPE)-pCMV$\beta$ (Fig. 3). However, whether the expression is transient or stable is a matter of great importance and remains to be elucidated.

GrS, an amphipathic cyclic decapeptide having positively charged two ornithine residues, can interact with DNA [22] or destabilize biological membranes [23,24]. Legendre et al. reported an excellent transfection technique using DNA-GrS complex, and this complex was non-specifically adsorbed on the cells in culture and directly penetrated into the cytoplasm and the maximum transfection efficiency was obtained at an equivalent charge ratio between the DNA and GrS [21]. The transfection efficiency of AF-liposomes was also improved by GrS (Fig. 4), and the highest transfection efficiency was observed when pCMV$\beta$(GrS) with the weight ratio of GrS/pCMV$\beta$ = 4 corresponding to the charge ratio of GrS/pCMV$\beta$ = 2 was encapsulated (Fig. 5). The discrepancy may come from the difference in the uptake mechanism. A significant reduction in the transfection efficiency of AF-liposome(DOPE)-pCMV$\beta$(GrS) was observed by treatment with cytochalasin B (Fig. 4), which is well known to inhibit phagocytosis through the microfilament function [45] and has been demonstrated to inhibit incorporation of AF [46] or AF-liposomes [17] into hepatocytes, suggesting that GrS may exhibit its membrane destabilizing effect after on going endocytosis is over. The detailed mechanism is considered as follows; (1) AF-liposome(DOPE)-pCMV$\beta$(GrS) taken up by RME and incorporated into the endosome is transferred to the lysosome, (2) liposomes are degraded and pCMV$\beta$(GrS) complex is released from liposomes, (3) pCMV$\beta$(GrS) complex penetrates the lysosomal membrane by destabilizing the membrane, and (4) the complex is transferred into the cytoplasm before its hydrolysis. This possible mechanism is also supported by the results that the transfection was almost completely inhibited by addition of free AF and that GrS had no effects on the transfection using N-liposomes (Fig. 4), which were not incorporated into hepatocytes by endocytosis as was apparent from no inhibition of the uptake by cytochalasin B [17]. The reason for an incomplete inhibition of the transfection with AF-liposome(DOPE)-pCMV$\beta$(GrS) and an enhancement in the transfection with N-liposome(DOPE)-pCMV$\beta$(GrS) by cytochalasin B is unclear but a few direct penetration of the liposomes through the plasma membrane into the cytoplasm may possibly be enhanced. It was reported that cytochalasin B promote the transfection efficiency of lipopolylysine containing cationic liposomes [47].

There is an excellent work to improve the efficiency of receptor-mediated gene transfer by exploiting the fusogenic and endosomolytic activity of replication defective adenovirus particles [48]. This technique allowed DNA to escape efficiently from the lysosomal degradation and could enhanced delivered gene expression in a variety of cultured cells, but could not be used in in vivo gene transfer because of a high antigenicity of the viral particles and their capture and degradation by reticuloendothelial systems [49]. AF-liposome(DOPE)-pCMV$\beta$(GrS) described here contains no viral components and is relatively easy to prepare. Immunogenisity of the complexes of DNA and GrS has not been cleared, but it would be masked by encapsulation them into liposomal lipid bilayers. Leakage of pCMV$\beta$ from AF-liposome(DOPE)-pCMV$\beta$(GrS) was less than 15% in mouse plasma at 37°C for one day in vitro. Since the transfer of AF-liposomes from the blood circulation into the liver is very rapid ($t_{1/2} < 2$ min) [16], intravenously injected AF-liposome(DOPE)-pCMV$\beta$(GrS) could be incorporated into hepatocytes as an intact form. These emphasize the possibility of AF-liposomes as a liver specific gene transfer vector for in vivo gene therapy.

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