

Effect of the Particle Size of Galactosylated Lipoplex on Hepatocyte-Selective Gene Transfection after Intraportal Administration

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The purpose of this study was to examine the effect of the size of galactosylated cationic liposome (Gal-liposome)/plasmid DNA complex (Gal-lipoplex) on hepatocyte-selective gene transfection after intraportal administration. pCMV-Luc was selected as a model plasmid DNA. After intraportal administration of Gal-lipoplex to mice, the hepatic and intrahepatic gene expression was evaluated. To evaluate the effect of size, three different sizes of Gal-liposome were prepared. The mean particle sizes of Gal-lipoplex were about 141, 179, and 235 nm, respectively. The hepatic transfection efficacy was significantly enhanced by increasing the size of Gal-lipoplex. However, the gene expression in liver parenchymal cells (PC) of Gal-lipoplex of about 141 nm in size was significantly higher than that in liver non-parenchymal cells (NPC). In contrast, gene expression in PC of Gal-lipoplex of about 235 nm in size was significantly lower than that in NPC. These results highlight the importance of the Gal-lipoplex size for hepatocyte-selective gene transfer *in vivo*. The information in this study will be valuable for the future use, design, and development of Gal-lipoplex for *in vivo* applications.

Key words gene delivery; hepatocyte; targeting; galactosylated liposome; drug delivery system

Gene transfer to hepatocytes is of great therapeutic potential since hepatocytes are responsible for the synthesis of a wide variety of proteins that play important physiological roles. There has been much interest in *in vivo* gene transfer to the liver, as an alternate to *ex vivo* methods that require invasive surgery. So far, several methods involving the local administration of naked plasmid DNA (pDNA) has been tested in order to achieve gene delivery targeted to the liver.^{1,2)} Compared with these local applications to the liver, systemic application by vascular routes could transfect the gene to a large number of cells in the whole liver. However, the highest gene expression is observed in the lung after the intravenous^{3–5)} and intraportal⁶⁾ administration of cationic liposome/pDNA complex (lipoplex).

The development of targeted gene delivery systems is a promising approach for effective and safe *in vivo* gene transfer to hepatocytes. To achieve targeted gene delivery, galactose has been shown to be a promising targeting ligand for hepatocytes (liver parenchymal cells; PC) because these cells possess a large number of asialoglycoprotein receptors that recognize the galactose units on the synthetic galactosylated carriers.^{7,8)} Recently, we have developed Gal-liposomes containing cholesten-5-yloxy-*N*-(4-((1-imino-2-*D*-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol) for hepatocyte-selective gene transfection after intraportal administration to mice.^{7,9,10)} However, the level of *in vivo* gene expression was not as high as that expected from the *in vitro* results.^{7,11)} This phenomenon could explain the several barriers associated intrinsically with *in vivo* situations; therefore, these *in vivo* barriers need to be investigated to allow the successful development of an effective gene vector. However, little information is available about hepatocyte-selective gene transfer by Gal-lipoplex under *in vivo* conditions.

The passage through the sinusoids is considered an important factor for hepatocyte-selective gene transfection, since the Gal-lipoplex must pass through the endothelial cell barriers to reach the hepatocytes. In this study, therefore, we evaluated the effects of Gal-lipoplex size on hepatic transfection efficacy after intraportal administration. Once the *in vivo*

gene expression is linked with its physicochemical properties including particle size, it is then possible to design a Gal-lipoplex to enable cell-specific *in vivo* gene delivery. *N*-[1-(2,3-Dioleloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA)/cholesterol (Chol)/Gal-C4-Chol liposomes were selected as a Gal-liposome for study because of its hepatocyte-selectivity after intraportal administration.⁷⁾ pCMV-Luc was selected as a model pDNA for evaluating the gene expression by luciferase.

MATERIALS AND METHODS

Materials *N*-(4-Aminobutyl) carbamic acid *tert*-butyl ester and DOTMA were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cholesterol (Chol) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Cholesteryl chloroformate and collagenase type IA were obtained from Sigma Chemicals, Inc. (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available.

Animals Female five-week-old ICR mice (20–23 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

Construction and Preparation of pDNA (pCMV-Luc) pCMV-Luc was constructed by subcloning the *Hind*III/*Xba*I firefly luciferase cDNA fragment from pGL3-control vector (Promega Co., Madison, WI, U.S.A.) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). pDNA was amplified in the *E. coli* strain DH5a, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the pDNA concentration was measured by UV absorption at 260 nm.

Synthesis of Gal-C4-Chol Gal-C4-Chol was synthesized as reported previously.¹¹⁾ Briefly, cholesteryl chloroformate

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mate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. A solution of trifluoroacetic acid and chloroform was added dropwise and the mixture was stirred for 4 h at 4 °C. The solvent was evaporated to obtain *N*-(4-aminobutyl)-(cholesten-5-yloxy)formamide which was then combined with 2-imino-2-methoxyethyl-1-thiogalactoside and the mixture was stirred for 24 h at 37 °C. After evaporation, the resultant material was suspended in water, dialyzed against distilled water for 48 h (12 kDa cut-off dialysis tubing), and then lyophilized.

Preparation of Gal-liposome Gal-liposomes were prepared as reported previously.^{7,10} The mixtures of DOTMA, Chol, and Gal-C4-Chol were dissolved in chloroform at a molar ratio of 2 : 1 : 1 for Gal-liposomes, vacuum-desiccated, and resuspended in sterile 5% dextrose solution at a concentration of 4 mg total lipids per ml. For small sized liposomes (about 49.6 nm, see Fig. 1), the suspension was sonicated for 3 min and the resulting liposomes were extruded 5-times through 100 nm polycarbonate membrane filters. In the case of medium (about 148 nm, see Fig. 1) and large (about 197 nm, see Fig. 1) sized liposomes, the resulting liposomes were directly extruded 5-times through 200 and 400 nm polycarbonate membrane filters, respectively.

Preparation of Gal-lipoplex Gal-lipoplex was prepared as reported previously.⁷ pDNA in 5% dextrose solution was mixed with an equal volume of Gal-liposomes and incubated for 30 min. The mixing ratio of liposomes and pDNA was expressed as a charge ratio, which is the molar ratio of cationic lipids to pDNA phosphate residues.¹² As far as the charge ratio was concerned, we selected a charge ratio (- : +) of 1.0 : 2.3 for all experiments to obtain the most effective transfection activity for receptor-mediated gene transfer.⁷ The particle size of the Gal-lipoplex was measured using a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics Co., Ltd., Osaka, Japan). The number-fractioned mean diameter was shown.

In Vivo Transfection Experiments Intraportal administration was performed as reported previously.^{7,10} Mice were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg), an incision was made in the abdomen, and the portal vein was exposed. The Gal-lipoplex was injected into the portal vein at a dose of 30 µg, and the abdomen was closed with wound clips. Liver samples were taken 6 h after injection and each sample was homogenized with lysis buffer (0.1 M Tris/HCl containing 0.05% Triton X-

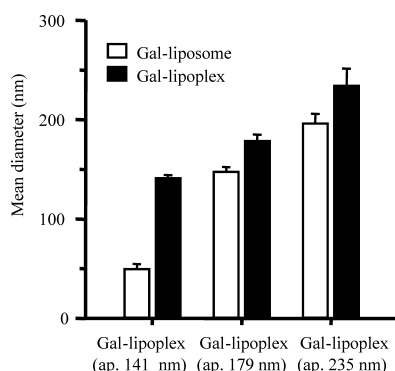


Fig. 1. The Particle Size Distribution of Gal-liposome and Gal-lipoplex

Each value represents the mean ± S.D. of at least three experiments. ap.: approximately.

100 and 2 mM EDTA (pH 7.8)). After three cycles of freezing and thawing, the homogenates were centrifuged at 10000 *g* for 10 min at 4 °C. Twenty microliters of each supernatant was mixed with 100 µl; luciferase assay solution (Picagene, Toyo Ink Mfg. Co., Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, Berthold Technologies, GmbH & Co., Bad Wildbad, Germany). The protein content of the samples was determined using a protein quantification kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, U.S.A.). For evaluation of the intrahepatic localization of gene expression, the luciferase activities in the liver PC and non-parenchymal cells (NPC) were independently determined after centrifugal separation of PC and NPC in collagenase-digested liver as previously described.^{7,9}

Statistical Analysis Statistical comparisons were performed by Student's *t*-test for two groups, Steel-Dwassl test for multiple groups.

RESULTS AND DISCUSSION

Recently, we developed Gal-C4-Chol with bi-functional properties of pDNA binding *via* electrostatic interaction and a high affinity for PC *via* their asialoglycoprotein receptors.¹¹ We have also demonstrated that the galactose density of Gal-liposomes is important for both effective recognition by asialoglycoprotein receptors and cell internalization *in vivo*.¹³ Since Gal-C4-Chol possesses an imino group for binding to pDNA *via* electrostatic interaction, many galactose units could be introduced on the liposomal surface without loss of binding affinity to pDNA.¹¹ These promising properties of our Gal-lipoplex enable PC-selective gene transfer under *in vivo* conditions.^{7,9,10}

In order to analyze the effect of Gal-lipoplex size, three different sizes of Gal-liposomes were prepared using extrusion method. Figure 1 shows the particle sizes of the Gal-liposomes and Gal-lipoplexes prepared. The mean particle sizes of Gal-liposomes prepared using 100, 200, and 400 nm polycarbonate filters were 49.6, 148, and 197 nm, respectively. The mean particle sizes of Gal-lipoplexes were 141, 179, and 235 nm, respectively. Using these Gal-lipoplexes with different particle sizes, the effect of Gal-lipoplex size on hepatic- and hepatocyte-selective gene transfection was stud-

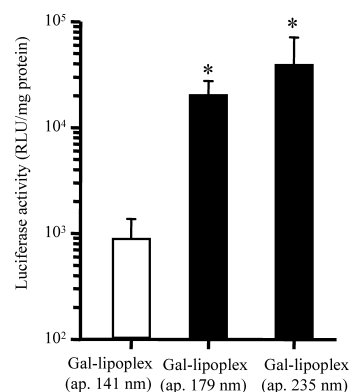


Fig. 2. Hepatic Transfection Activity of Gal-lipoplex after Intraportal Administration in Mice

Luciferase activity was determined 6 h post-injection of lipoplex. Statistically significant differences from galactosylated lipoplex (about 141 nm) (**p* < 0.05). Each value represents the mean ± S.D. of at least three experiments. ap.: approximately.

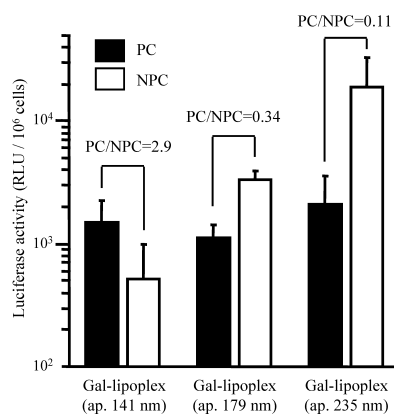


Fig. 3. Intrahepatic Transfection Activity of Gal-lipoplex after Intraportal Administration in Mice

Luciferase activity was determined 6h post-injection of lipoplex. Each value represents the mean \pm S.D. of at least three experiments. ap.: approximately.

ied.

We previously reported that gene expression in the liver was much higher than that in the lung, spleen, kidney, and heart after intraportal administration of Gal-lipoplex.⁷⁾ In the present study, the hepatic and intrahepatic gene expression characteristics were evaluated in relation to the size of Gal-lipoplex. As shown in Fig. 2, the hepatic transfection efficacy was significantly enhanced by an increase in the size of the Gal-lipoplex. To study the intrahepatic transfection activity, PC and NPC in the liver were separated by the collagenase perfusion technique (Fig. 3). The gene expression in PC of Gal-lipoplex about 141 nm in size was significantly higher than that in NPC. In contrast, the gene expression in PC of Gal-lipoplex about 179 and 235 nm in size was significantly lower than that in NPC. This finding might be due to the fact that the endothelial fenestrae measure 150–175 nm in diameter.¹⁴⁾

Although we hypothesized that Gal-lipoplex over 200 nm in size has a limited ability to transfect the gene in liver because of the size limit, total hepatic gene expression was enhanced by increasing the size of the Gal-lipoplex (Fig. 2). This discrepancy may be partly explained by the presence of galactose particle receptors on Kupffer cells in NPC.¹⁵⁾ Kupffer cell would take up the Gal-lipoplex over 200 nm, which could not pass through the endothelial fenestrae. On the other hand, the uptake of the Gal-lipoplex over 200 nm by PC would be limited by the size of fenestrae. In fact, intrahepatic gene expression analysis demonstrated that Gal-lipoplex over 200 nm in size selectively transfect the gene in NPC (Fig. 3). These results highlight the importance of the Gal-lipoplex size for PC-selective gene transfer *in vivo*.

Physicochemical property of Gal-lipoplex is important concerning with PC-selective gene transfer *in vivo*.¹⁶⁾ Previously, we reported that the surface charge of Gal-lipoplex concerned with the interactions with blood components after intraportal injection of Gal-lipoplex¹⁰⁾ and charge of Gal-

lipoplex affected on the gene expression.^{7,17)} In this study, it was revealed that the size of Gal-lipoplex had effect of the hepatic distribution of gene expression. However, Goncalves et al. reported that lipoplex with different size have different gravity, therefore, such factor also might contribute on the *in vivo* gene transfer of Gal-lipoplex.¹⁸⁾

In conclusion, we prepared three different Gal-lipoplex about 141, 179, and 235 nm in size, and demonstrated that the hepatic transfection efficacy was enhanced by increasing the size of the Gal-lipoplex after intraportal administration. However, intrahepatic transfection was altered by the size of the Gal-lipoplex, *i.e.*, PC-selectivity by Gal-lipoplex about 141 nm in size; NPC-selectivity by Gal-lipoplex about 235 nm in size. The information described in this study will be valuable for the future use, design, and development of Gal-lipoplexes for *in vivo* applications.

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