Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan

Effect of cationic charge on receptor-mediated transfection using mannosylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice

S. Kawakami, Y. Hattori, Y. Lu, Y. Higuchi, F. Yamashita, M. Hashida

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Prof. Mitsuru Hashida, Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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The purpose of this study was to evaluate the effect of cationic charge of complexes after intravenous administration of cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiomannosyl-ethyl)amino]butyl}formamide (Man-C4-Chol) containing cationic liposomes/pDNA complexes in mice. Transfection efficiency after intravenous administration of complex at a charge ratio ($-:+$) of 1.0 : 2.3 and/or 1.0 : 3.1 in liver and spleen expressing a mannose receptor on the cell surface were higher than those in lung. When complexes were formed at a charge ratio ($-:+$) of 1.0 : 4.7, on the other hand, transfection efficiency in the lung was highest, suggesting a non-specific interaction. Although asialoglycoprotein receptors are expressed on hepatocytes, a liver-selective gene transfection was not achieved by the intravenous administration of pDNA complexed with cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl)-amino]butyl}formamide (Gal-C4-Chol)/DOPE liposomes at a charge ratio ($-:+$) of 1.0 : 2.3. This information supports the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery after intravenous administration.

1. Introduction

The success of *in vivo* gene therapy relies on the development of a vector that achieves target cell-specific, efficient, and prolonged transgene expression following its application. Non-viral vectors are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, attractive for clinical application. Although the cationic liposome/plasmid DNA (pDNA) complex is a useful non-viral vector, it lacks specificity in delivery and transfection. Intravenous administration of pDNA/cationic liposome complexes expressed genes into various tissues, with highest gene expression in the lung (Templeton et al. 1997; Li et al. 1997). The attachment of a ligand that can be recognized by a specific mechanism would endow a vector with the ability to target a specific population of cells. In the search for cationic liposomes-based non-viral vectors, several ligands including asialofetuin (Hara et al. 1995), galactose (Kawakami et al. 1998; 2000a; Fumoto 2003), and mannose (Kawakami et al. 2000b; 2001; Sato et al. 2001) have been used to improve the delivery of pDNA to target cells. Therefore, the incorporation of such ligands into cationic liposomes would improve the cell-specificity of *in vivo* gene transfer by cationic liposome/pDNA complexes.

The mannose receptor is expressed on Kupffer cells, splenic, alveolar, peritoneal macrophages, monocyte-derived dendritic cells, and subsets of vascular and lymphatic endothelial cells (Weis et al. 1998). Recently, we designed cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiomannosyl-ethyl)-amino]butyl}formamide (Man-C4-Chol) to prepare mannosylated cationic liposomes for mannose receptor-mediated gene delivery (Kawakami et al. 2000b). In the previous study, we have reported that the highest gene expression in the liver and spleen after intravenous injection was observed for Man-C4-Chol/dioleoylphosphatidylethanolamine (DOPE) liposome/pDNA complexes via mannose receptor-mediated endocytosis (Kawakami et al. 2000b). However, not only the nature of the ligands grafted to carriers but also the overall physicochemical properties of the complexes need to be optimized for the successful delivery of pDNA under systemic injection (Mahato et al. 1997; Kawakami et al. 2002). Although it is well known that a high cationic charge of cationic liposome/pDNA complexes enhances the transfection efficiency in the lung after intravenous injection because of the non-specific interaction between lung and complex (Templeton et al. 1997; Li et al. 1997), the effect of a cationic charge of ligands grafted cationic liposomes/pDNA complex on cell-selective transfection efficiency is not clear. Once the *in vivo* gene expression is linked with its physicochemical properties, it is then possible to design liposomes or a pDNA/liposome complex to enable cell-specific *in vivo* gene delivery.

In the present study, we tried to elucidate the effect of a cationic charge of complexes after intravenous injection of Man-C4-Chol containing a cationic liposome/pDNA complex in mice. Also, we evaluated the transfection efficiency of Man-C4-Chol containing a cationic liposome/pDNA complex using primary cultured mouse peritoneal

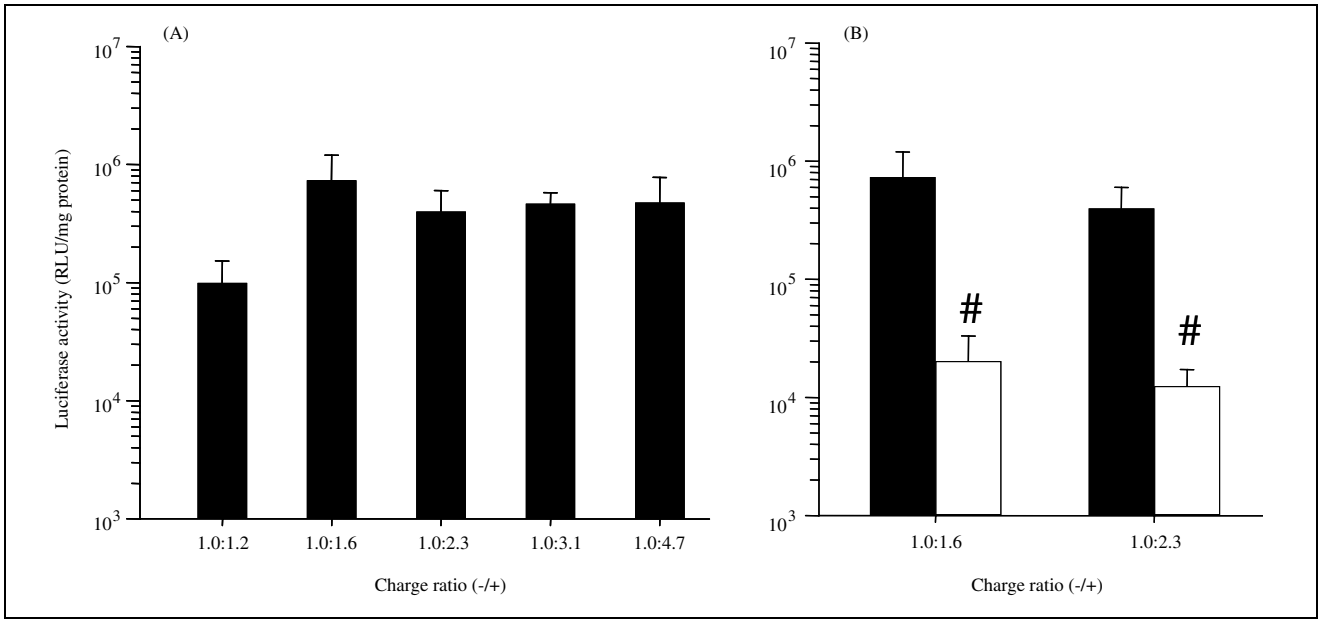

Fig. 1: Transfection activity at various charge ratio (− : +) (A) and the absence (■) or presence (□) of 1mg/ml mannan on transfection activity (B) of pDNA complexed with Man-C4-Chol/DOPE liposomes in cultured mouse peritoneal macrophages. pDNA concentration was fixed at 0.5 μg/ml in all experiments. Each value represents the mean ±S.D. values (n = 3). Stastical analysis was performed by analysis of variance ($^{\#}P < 0.05$)

macrophages which express the mannose receptor, to compare the results with *in vivo* application. pCMV-Luc was selected as a model pDNA because transfected luciferase is easy to detect by fluorescence.

2. Investigations, results and discussion

Complex formation between pDNA and Man-C4-Chol/DOPE complex at a charge ratio (− : +) of 1.0 : 1.2, 1.0 : 1.6, 1.0 : 2.3, 1.0 : 3.1, and 1.0 : 4.7 was determined by agarose gel electrophoresis, followed by gel staining with ethidium bromide and photography under UV light. The addition of any Man-C4-Chol/DOPE liposome to pDNA at these charge ratios resulted in the formation of complexes that did not move towards the positive pole (data not shown).

First, we evaluated the transfection efficiency and its gene expression mechanism on transfection of pDNA complexed with Man-C4-Chol/DOPE liposome at various charge ratios (− : +) in cultured mouse peritoneal macrophages. As shown in Fig. 1, the transfection efficiency was almost the same at charge ratios (− : +) from 1.0 : 1.2 to 1.0 : 4.7. In the presence of 1 mg/ml mannan, the transfection efficiency of pDNA complexed with Man-C4-Chol/DOPE liposomes was significantly reduced. This result suggested that the mannose receptor-mediated endocytosis is involved in the transfection mechanism of pDNA complexed with Man-C4-Chol/DOPE liposomes.

Table: Mean particle sizes of liposome/pDNA (50 μg) complexes in 5% dextrose for *in vivo* experiments

Charge ratio (− : +)	Particle size (nm)
1.0 : 1.2	Aggregated
1.0 : 1.6	Aggregated
1.0 : 2.3	178.1 ± 13.1
1.0 : 3.1	118.6 ± 3.7
1.0 : 4.7	121.3 ± 8.5

Each value represents the mean ± S.D. values (n = 3).

Ogris et al. reported that mixing complexes at low ion strength prevents aggregation, although, large complexes resulting from aggregation showed high transfection efficiency *in vitro* in the case of DNA/transferrin-PEI complexes (Ogris et al. 1998). Referring to this report, the complexes were prepared with 5% dextrose solution. The Table 1 summarizes the particle sizes of Man-C4-Chol/DOPE liposome/pDNA complexes in 5% dextrose solution. When 50 μg of pDNA was mixed with Man-C4-Chol/DOPE liposomes, complexes at a charge ratio (− : +) of 1.0 : 1.2 and 1.0 : 1.6 were aggregated. In contrast, complexes at a charge ratio (− : +) from 1.0 : 2.3 to 1.0 : 4.7 can be prepared and their size is approximately 120–180 nm. The gene expression in mice following the intravenous injection with pDNA complexed with Man-C4-Chol/DOPE liposomes at charge ratio (− : +) of 1.0 : 2.3, 1.0 : 3.1, and 1.0 : 4.7 is shown in Fig. 2. The transfection efficiency after intravenous administration of

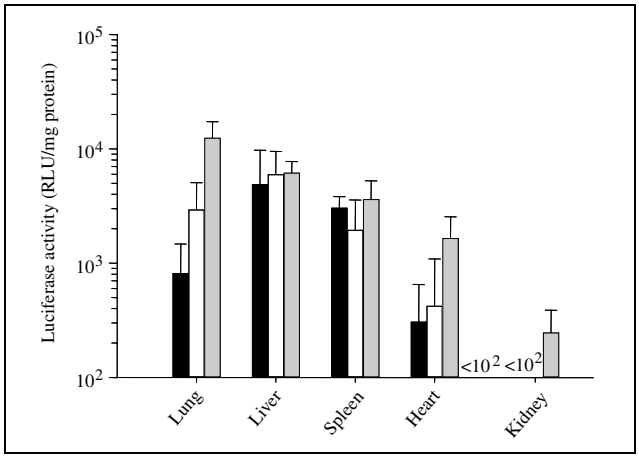

Fig. 2: Transfection activity of pDNA complexed with Man-C4-Chol/DOPE liposomes after intravenous administration in mice. pDNA (50 μg) was complexed with cationic lipids at a charge ratio (− : +) of 1.0 : 2.3 (■), 1.0 : 3.1 (□), and 1.0 : 4.7 (▨). Luciferase activity was determined 6 h post-injection in the lung, liver, spleen, heart, and kidney. Each value represents the mean ±S.D. values (n = 3)

complexes at charge ratios (− : +) of 1.0 : 2.3 and 1.0 : 3.1 in liver and spleen, expressing mannose receptors on the cell surface, were higher than those in lung. When the complex was formed at a charge ratio (− : +) of 1.0 : 4.7, on the other hand, transfection efficiency in the lung was highest, suggesting a non-specific interaction. Also, these results are well congruent with our previous report concerning the asialoglycoprotein receptor-mediated gene transfection following the intraportal administration of pDNA complexed with DOTMA/Chol/cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl)amino]butyl}formamide (Gal-C4-Chol) liposomes in mice (Kawakami et al. 2000a).

Intravenous gene delivery to the lung via pDNA/cationic liposome complexes has been reported. Several parameters have been identified to be important for achieving a high level of gene expression (Huang and Li 1997). Among them, a high cationic charge between a pDNA and cationic lipid is important for efficient intravenous gene delivery; however, these strategies of gene transfection include non-specific adsorption mediated endocytosis. In the present study, we demonstrated that liver and spleen selective gene transfection using mannose receptor mediated endocytosis is achieved by complexes prepared at a charge ratio (− : +) of 1.0 : 2.3 and/or 1.0 : 3.1. An excess cationic charge of complexes resulted in enhanced gene expression in the lung with non-specific interaction.

It has been reported that hepatocytes exclusively express large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior. Referring to the results presented in Fig. 2, pDNA/galactosylated liposome complexes were prepared at a charge ratio (− : +) of 1.0 : 2.3. Sinusoids in the liver lobules are invested with a unique type of endothelial lining consisting of endothelial cells with flattened processes perforated by small fenestrae of about 200 nm in size. Therefore, pDNA/liposome complexes with a diameter less than this can readily pass through the fenestration into the Disse space and we prepared pDNA complexed with Gal-C4-Chol/DOPE liposomes having a size of approximately 150 nm in diameter for free to access to hepatocytes. Although the size of the liposomes was approximately 150 nm, selective gene targeting to the liver was not achieved (Fig. 3). For the target cells of mannosylated liposome/pDNA complexes, there is not need to pass through fenestrae; therefore, selective gene targeting in liver and spleen on macrophages may be achieved by intravenous administration.

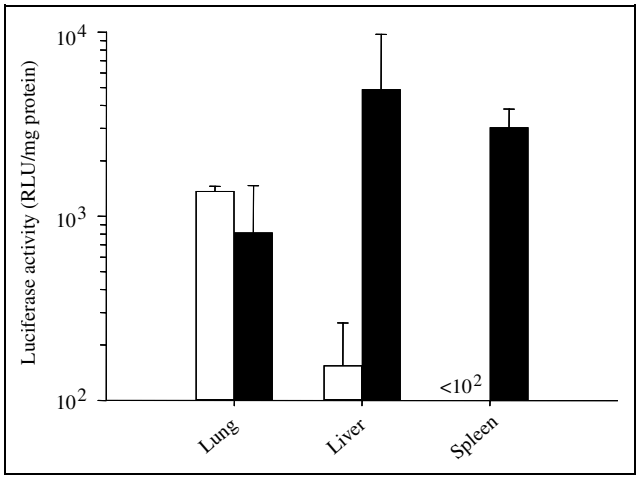

Fig. 3: Transfection activity of pDNA complexed with Gal-C4-Chol/DOPE liposomes (□) or Man-C4-Chol/DOPE liposomes (■) after intravenous administration in mice. pDNA (50 μg) was complexed with cationic lipids at a charge ratio (− : +) of 1.0 : 2.3. Luciferase activity was determined 6 h post-injection in the lung, liver, and spleen. Each value represents the mean ±S.D. values (n = 3)

The difference of transfection between galactosylated liposomes and mannosylated liposomes suggested that the size of complexes at a charge ratio (− : +) of 1.0 : 2.3 might be increased over 200 nm after intravenous administration. Thus, not only the introduction of a ligand to the cationic liposomes but also a controlled size of complexes is important for an efficient targeted gene delivery. We previously reported that the pDNA/cationic liposome complexes are interacting with erythrocytes after intravenous administration (Sakurai et al. 2001). Recently, Eliyahu et al. (2002) also characterized the interaction of blood components with pDNA/cationic liposome complexes under conditions relevant to *in vivo* intravenous administration. In their report, the selection of a medium (i.e. plasma and serum) and/or modification of the cationic liposomes with 1% polyethyleneglycol lipids reduced the aggregation of pDNA/cationic liposome complexes in the presence of erythrocytes; accordingly, such approaches may enhance the cell-specificity of pDNA complexed with galactosylated and mannosylated cationic liposomes. Further studies on the interaction with blood components and/or the synthesis of polyethyleneglycol-grafted glycosylated lipids for cell-selective gene delivery are required. These information supports the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery under *in vivo* conditions.

3. Experimental

3.1. Materials

N-(4-Aminoethyl) carbamic acid *tert*-butyl ester and *N*-(4-aminobutyl) carbamic acid tert-butyl ester, *N*-(4-aminohexyl) carbamic acid tert-butyl ester were obtained from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate and DOPE were obtained from Sigma Chemicals (St. Louis, MO) and Avanti Polar-Lipids (Alabaster, AL), respectively. Fetal bovine serum (FBS) was obtained from Biowhittaker (Walkersville, MD). Opti-MEM I and other culture reagents were obtained from Gibco BRL (Grand Island, NY). All other chemicals were of the highest purity available.

3.2. 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, Madison, WI) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA). pDNA was amplified in the E. coli strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

3.3. Synthesis of Man-C4-Chol and Gal-C4-Chol

As reported previously (Kawakami et al. 2000c), *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide was obtained from cholestery chloroformate and *N*-(4-aminobuthyl) carbamic acid tert-butyl ester. The product was reacted with 5 equivalents of 2-imino-2-methoxyethyl-1-thiomannoside or 2-imino-2-methoxyethyl-1-thiogalactoside (Lee et al. 1976) in pyridine containing 1.1 equivalents of triethylamine for 24 h. After evaporation of the reaction mixture *in vacuo*, the resultant material was suspended in water and dialyzed against water for 48 h. Then, the compounds were lyophilized.

3.4. Particle size measurements

The particle sizes of liposome/pDNA complexes were measured in a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan).

3.5. Harvesting and culture of macrophages

Male ICR mice weighing 20–25 g were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. Elicited macrophages were harvested from mice 4 days after intraperitoneal injection of 1 ml 2.9% thioglycolate medium (Nissui Pharmaceutical Co. LTD., Tokyo, Japan). The washed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum

(FBS, Flow Laboratories, Irvine, U.K.), penicillin G (100 U/ml), and streptomycin (100 μg/ml) and were plated on 6- or 12-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) at a density of 3×10^5 cells/cm^2. After incubation for 24 h at 37 °C in 5% CO_2 – 95% air, non-adherent cells were washed off with culture medium and cells were cultivated for another 48 h.

3.6. Preparation of pDNA/liposome complexes for in vitro experiments

Man-C4-Chol was mixed with DOPE at a molar ratio of 3 : 2 in chloroform and the mixture was dried, vacuum desiccated, and resuspend in 1 ml sterile 20 mM HEPES buffer (pH 7.8) in a sterile test-tube. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes. It was passed through a 0.45 μM filter for sterilization. The lipid concentration was determined by phosphorus analysis and was adjusted (1 mg/ml) (Bartlett 1959). Cationic liposomes and pDNA (1.0 μg) in a 12 × 75 mm polystyrene tube were diluted with Opti-MEM at various charge ratios before carrying out the transfection experiment. The theoretical charge ratio of cationic lipid/pDNA was calculated as a molar ratio of Man-C4-Chol (monovalent) to a nucleotide unit (average molecular weight 330). Complex formation was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

3.7. In vitro transfection experiment

Macrophages were seeded in 10.5 cm^2 dishes at a density of 1.1×10^6 cells/cm^2 in RPMI 1640 supplemented with 10% fetal calf serum. After 3 days in culture, the culture medium was replaced with Opti-MEM I containing 0.5 μg/ml pDNA and cationic liposomes. Six hours later, the incubation medium was replaced again with RPMI 1640 supplemented with 10% FBS and incubated for an additional 18 h. Then, the cells were scraped and suspended in 200 μl pH 7.4 phosphate-buffered saline (PBS). One hundred microliters the cell suspension were subjected to three cycles of freezing (liquid N_2 for 3 min) and thawing (37 °C for 3 min), followed by centrifugation at 10,000 *g* for 3 min. The supernatants were stored at −20 °C until the luciferase assay was performed. Ten microliters of supernatant were mixed with 100 μl luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured in a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as the relative light units per mg protein. The protein content of the cell suspension in PBS was determined by the modified Lowry method using BSA as a standard (Wang and Smith 1975).

3.8. Preparation of pDNA/liposome complexes for in vivo experiments

Man-C4-Chol or Gal-C4-Chol was mixed with DOPE at a molar ratio of 3 : 2 in chloroform and the mixture was dried, vacuum desiccated, and resuspend in 5 ml sterile 5% dextrose in a sterile test-tube. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes. It was passed through a 0.45 μM filter for sterilization. The lipid concentration was determined by phosphorus analysis and was adjusted (3 mg/ml) (Bartlett 1959). Equal volumes of pDNA (50 μg) and stock liposome solution diluted with 5% dextrose solution to produce various ratios of pDNA/liposomes were mixed in 1.5 ml Eppendorf tubes at 4 °C. Then, the DNA solution was added rapidly to the surface of the liposome solution using a Pipetman and the mixture was agitated rapidly by pumping it up and down twice in the pipet tip and was stored at 4 °C for 12 h.

3.9. In vivo transfection experiment

Five-week-old ICR mice were injected intravenenously with 300 μl of pDNA/liposome complexes using a 30-gauge syringe needle. Six hours after injection, mice were killed and lung, liver, kidney, spleen and heart were removed and assayed for gene expression. The organs were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1M Tris, pH 7.8). The lysis buffer was added in a weight ratio of 5 μl/mg for liver samples or 4 μl/mg for other organ samples. After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C and 20 μl supernatant was analyzed to determine the luciferase activity using a luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany). The protein concentration of each tissue extract was determined by the modified Lowry method (Wang and Smith 1975). Luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

3.10. Statistical analysis

Statistical comparisons were performed by analysis of variance. $P < 0.05$ was considered to be indicative of statistical significance.

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