Comparison between a Multifunctional Envelope-Type Nano Device and Lipoplex for Delivery to the Liver

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The utility of using a multifunctional envelope-type nano device (MEND) for delivering a gene to the liver was examined. Lipotrust, a commercially available transfection reagent whose lipid composition is DC6-14 : DOPE : cholesterol = 4 : 3 : 3, was used as a reference. When Lipotrust was administrated intravenously, luciferase activity of the lung was 25 times higher than that of the liver. The luciferase activity of the lung was greatly reduced when a MEND was administered, even though the lipid composition of the lipid envelope was the same in both devices. Furthermore, the luciferase activity of the liver was 5 times higher than that for lipotrust, suggesting that the encapsulation of plasmid DNA (pDNA) in liposomes is more advantageous for delivering pDNA to the liver than complex formation. The isolation of parenchymal cells (PCs) and non-parenchymal cells (NPCs) showed that the MEND system is capable of expressing the luciferase protein more preferentially in NPCs than the lipoplex system. In addition, when the surface was modified with a pH-sensitive fusogenic peptide (GALA) used as a device for endosomal escape, overall liver luciferase activity was greatly enhanced. This suggests that endosomal escape is a limiting step for the MEND system. In the case of the GALA-modified MEND, the luciferase activity of PCs and NPCs was 18 times and 11 times higher than MEND system, while the transfection efficiency of NPCs was significantly higher compared to that of PCs. Collectively, these data show that a GALA-modified MEND prepared with DC6-14 : DOPE : cholesterol represents a promising device for NPC-targeting gene delivery *in vivo*.

Key words multifunctional envelope-type nano device; lipoplex; liver gene delivery; pH-sensitive fusogenic peptide; non-parenchymal cell

The use of non-viral gene delivery vectors is a promising strategy for curing a variety of intractable diseases. One of the most common non-viral gene delivery vectors are DNA–cationic lipid complexes (lipoplexes) and complexes comprised of DNA and synthetic polycations (polyplexes). Both systems are formed *via* electrostatic interactions between negatively charged nucleic acids and positively charged components. However, these systems have some disadvantages for *in vivo* applications. When administrated intravenously, they tend to accumulate in the lung because blood flow in lung capillaries is reduced by the presence of aggregates of erythrocytes. As a result, they gradually accumulate in the liver.[1)]

To overcome this problem, we developed a Multifunctional Envelope-type Nano Device (MEND), in which plasmid DNA (pDNA) condensed with a polycation is encapsulated by a lipid envelope.[2)] Kogure *et al.* demonstrated that the luciferase activity of a DNA–poly-L-lysine complex (DPC) with a lipid envelope was ten times higher than only DPC in NIH3T3 cells, suggesting that the lipid coating is important and critical for efficient gene delivery.[2)] However, a direct demonstration of the merit of encapsulating pDNA in the lipid envelop is currently available. A comparative study of pDNA-encapsulation type and lipoplex type gene vectors would provide information that would be useful in terms of developing more efficient *in vivo* gene delivery systems.

Lipotrust, a lipoplex gene vector, has been widely used in transfection studies *in vitro*[1,3)] and *vivo*.[4,5)] It contains *O*,*O*′-ditetradecanoyl-*N*-(alpha-trimethylammonio-acetyl) diethanolamine chloride (DC6-14) as a cationic lipid, cholesterol and dioleoylphosphatidylethanolamine (DOPE) at a molar ratio of 4 : 3 : 3. This lipid composition shows high transfection efficiency under conditions where serum is present for *in vitro* and *in vivo* gene delivery.[4,5)] When administered *via* the tail vein, the positively charged DNA–liposome complex (lipoplex) accumulates rapidly in the lung,[1)] a phenomenon that is generally observed in cases where various types of positively charged lipoplexes (or polyplexes) are used.[6,7)] One of the possible mechanisms is that capillaries in the lung may become blocked by aggregates of erythrocytes that are formed *via* electrostatic interactions between blood cells and positively charged liposomes.[1)] Generally, the position of the negatively charged DNA is not controlled in the lipoplex system. Thus, we hypothesized that interactions between negatively charged DNA and positively charged serum components would also result in the formation of large aggregates, and would also result in undesirable lung accumulation. If this hypothesis holds true, encapsulating pDNA in the lipid envelope would be an ideal strategy to decrease the material trapped in the lung by shielding the mutual interactions between DNA and serum proteins.

In this study, we present two findings that show that a MEND system can avoid luciferase activity in the lung, compared to the lipoplex system, and has an advantage in terms of delivering pDNA to the liver. We also tested a pH-sensitive fusogenic peptide (GALA), a functional element for endosomal escape[8,9)] for enhancing liver luciferase activity. The results clearly show that this GALA modified MEND system is more efficient in delivering pDNA to liver non-parenchymal cells compared to parenchymal cells.

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MATERIALS AND METHODS

Materials *O*,*O'*-Ditetradecanoyl-*N*-(alpha-trimethylammonio-acetyl) diethanolamine chloride (DC6-14) was purchased from Sogo Pharmaceutical (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Lipotrust was purchased from Hokkaido System Science (Sapporo, Japan). Chol-GALA was synthesized as described previously.[8,9] Protamine sulfate salmon mint was purchased from Calbiochem (Darmstadt, Germany). The reporter plasmid pcDNA3.1(+)-luc (7037 bp) encoding the firefly luciferase gene was purified with a Qiagen Endofree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). All other chemicals used were commercially available reagent-grade products.

Animals 5—6-Week-old male ICR mice were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals. Animals were used without fasting in all experiments.

Preparation of the MENDs and Lipotrust MENDs were prepared by the lipid hydration method as reported previously.[2] In a typical experiment, 412.5 and 420.8 nmol lipids composed of DC6-14/DOPE/Chol (4 : 3 : 3) and DC6-14/DOPE/Chol/Chol-GALA (4 : 3 : 3 : 0.2), respectively were dissolved in chloroform and ethanol (1 : 1) and the solution evaporated in a round-bottom glass tube. The pDNA was condensed with protamine in 10 mM Hepes buffer (pH=7.4) at a nitrogen/phosphate (N/P) ratio of 1 and used for hydrating the dry lipid film for 5—10 min. The sample was then sonicated for approximately 1 min in a bath-type sonicator. Lipotrust was prepared following the manufacture's recommendations. In brief, 100 μg pDNA was mixed with 1 μmol of total lipid (DC6-14/DOPE/Chol=4 : 3 : 3 molar ratio), followed by incubation for 5 min at room temperature. The average diameter and the zeta-potential of the particles were determined using a Zetasizer Nano ZS (Worchestershire, U.K.).

***In Vivo* Gene Expression** 5—6-Week-old male ICR mice were treated with the MEND or lipotrust (tail vein injection) containing 40 μg pDNA in a 400 μl injection volume. Mice were killed 6 h after the treatment, and the liver, lungs, and spleen were collected and used in luciferase gene expression assays, as described previously.[10]

Isolation of Parenchymal Cells (PC) and Non-parenchymal Cells (NPC) Six hours after intraveneous administration of a MEND equivalent to 60 μg of pDNA in a 400 μl injection volume, the mice were anesthetized with pentobarbital sodium (approximately 250 mg/kg) and cell suspensions were prepared by the 2-step collagenase perfusion method[11] and centrifuged twice at 70×g for 1 min (the pellet was collected as a PC fraction). The supernatant was centrifuged 3 times at 70×g for 1 min to remove hepatocytes and cell debris. The resulting supernatant was precipitated by centrifugation at 600×g for 4 min (the pellet was collected as a NPC fraction). The Percoll gradient method[11,12] was then used to remove damaged cells and debris. Briefly, the PC fraction, resuspended in 5 ml phosphate buffered salin (PBS), was layered on a 70% Percoll PBS solution and centrifuged at 110×g for 10 min. The pellet was collected as an intact PC. NPC fraction, resuspended in 2.5 ml PBS, was layered on a 25%/50% Percoll PBS solution and centrifuged at 110×g for 30 min. The intermediate layer between 25% and 50% percoll solution was collected as an intact NPC and diluted with PBS and centrifuged at 110×g for 10 min to pellet the cells.

Cell Cultures of PC and NPC The PCs were resuspended in Willium's E medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 1 nM dexamethazone, and 1 nM insulin. The NPCs were resuspended in Clonetics EBM-2 medium with EGM-2 supplements, penicillin (100 units/ml) and streptomycin (100 μg/ml). The PCs and NPCs were seeded on gelatin coated dish and fibronectin coated dish, respectively. These cells were incubated in a CO_2 incubator.

Luciferase Assay of PC and NPC One hour after CO_2 incubation, non attached cells were washed with PBS. The attached PCs were isolated with Accutase (San Diego, U.S.A.) and centrifuged at 18×g for 5 min. The attached NPCs were treated with a 0.5% trypsin solution for 10 min and gently isolated with a cell scraper and centrifuged at 18×g for 5 min. The supernatant was removed and mixed well by pipetting. Cell aggregates were then removed using a 40 μm cell strainer. After checking cell viability and cell numbers, the cell suspension was centrifuged at 600×g for 4 min and the supernatant removed. The pellets were lysed using 200 μl of reporter lysis buffer (Promega, Madison, WI, U.S.A.) and used in a luciferase gene expression assay as described previously.[10] The conversion of RLU/mg protein into RLU/1×10^5 cells used the following relationship calculated in these experiments; the average amount of mg protein in 1×10^5 cells is 0.523±0.097 for PCs and 0.024±0.005 for NPCs (n=8).

Statistical Analysis Statistical comparisons were performed by one-way ANOVA, followed by Student's t-test for two groups.

RESULTS AND DISCUSSION

The physical characteristics of lipotrust and the MEND used in this study are shown in Table 1. The polydispersity is similar in both carriers, but the MEND is smaller in size and has a higher Z-potential. The higher Z-potential of the MEND can be attributed to the more efficient packaging of

Table 1. Physical Properties of Lipotrust and MEND

	Lipid composition (molar ratio)	Size (nm)	Z-potential (mV)	Polydispersity
Lipotrust	DC6-14/DOPE/Chol (4 : 3 : 3)	203±11	34±2	0.19±0.03
MEND (N/P ratio=1.0)	DC6-14/DOPE/Chol (4 : 3 : 3)	168±2	46±3	0.18±0.02

Values are the mean of 3—4 independent experiments; the error represents the mean±S.D.

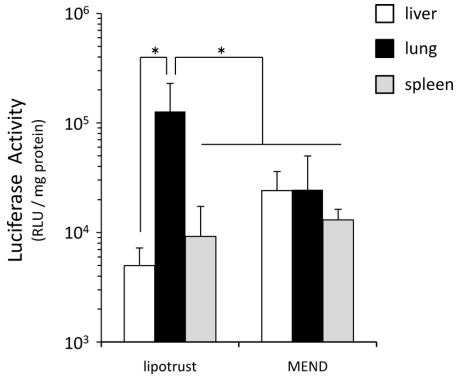

Fig. 1. Luciferase Activity in the Liver, Lungs and Spleen 6 h after Intravenous Administration

Each bar represents S.D. $n=3$. $*p<0.05$.

the negatively charged pDNA than the lipoplex. The topology of pDNA (encapsulated or complexed) reflects the activity of the lung and liver. As shown in Fig. 1, the lung luciferase activity of lipotrust is 25 times higher than the corresponding value for the liver, which can be explained by the fact that positively charged DNA-liposome complexes (lipoplex) of this lipid composition had accumulated in the lung.[1)] On the other hand, the lung luciferase activity of the MEND is similar to that for the liver, suggesting that the procedure used to encapsulate pDNA into the lipid envelope results in a particle that aggregates less in the lung. In addition, the merit of the MEND structure is reflected in the liver luciferase activity. When comparing the MEND to lipotrust, the liver luciferase activity of the MEND is 5 times higher. Collectively, these data support our hypothesis that the encapsulation of pDNA in the lipid envelope has a distinct advantage for delivering pDNA to the liver. To the best of our knowledge, this represents the first direct comparison of *in vivo* transfection activity between a complex system (lipoplex) and a pDNA-encapsulated system (MEND) on the basis of the same lipid composition. If pDNA could be encapsulated efficiently by a lipid envelope, a more efficient liver gene delivery would be possible, even in a positively charged surface. A similar tendency was found at 24 h, however, the value was low (data not shown), around 10^3, in both systems. Therefore, the assays were conducted at 6 h in all subsequent experiments.

A determination of the contribution of parenchymal cells (PCs) and non-parenchymal cells (NPCs) to overall gene expression in the liver would provide useful information concerning the determinant factors for the sorting of carriers into PCs or NPCs (*i.e.* ligand, size, and charge).[13—15)] To address this issue, we isolated PCs and NPCs using a combination of the 2-step collagenase perfusion method and Percoll centrifugation.[11,12)] The latter is a highly potent method for separating cell populations into damaged cells, leukocytes and erythrocytes. As shown in Fig. 2a, the morphology of these two cell fractions was clearly different, suggesting that this isolation procedure is appropriate. In this experiment, we used a GALA peptide as a device for endosomal escape.[8,9)] In terms of physicochemical properties, the GALA modified MEND was similar in size (171 nm) and had a lower Z-potential (34 mV). In the case of luciferase activity of the whole liver, the use of the MEND resulted in a 6 times higher value

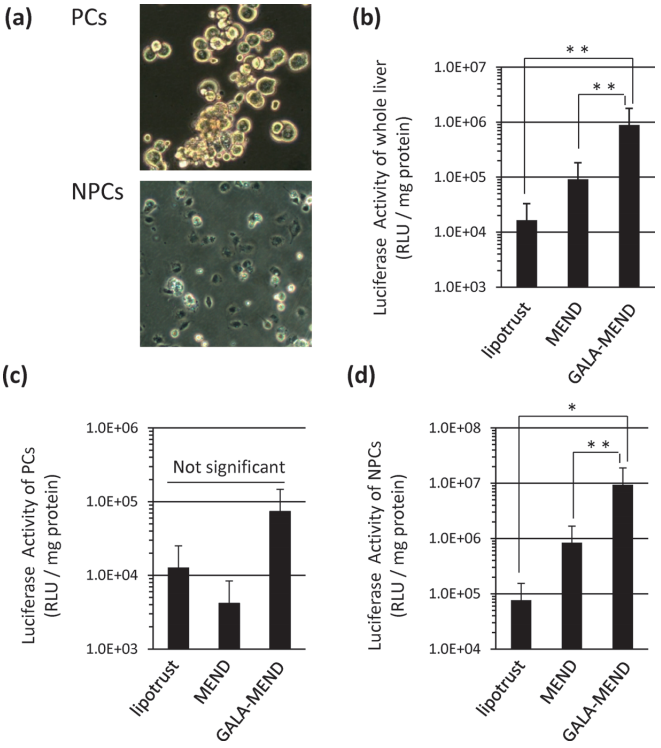

Fig. 2. Intrahepatic Luciferase Activity of Lipotrust, MEND and GALA-MEND

(a) Morphology of parenchymal cells (PCs) and non-parenchymal cells (NPCs) 1h after CO_2 incubation at 37 °C. The isolation procedures are described in material and methods. Luciferase activity per mg protein for (b) the whole liver, (c) PCs and (d) NPCs is shown. Each bar represents S.D. $n=3$—4. $*p<0.05$, $**p<0.01$.

than lipotrust, and modification of the surface of the MEND with GALA further improved the transfection activity by 10-fold (Fig. 2b), suggesting that endosomal escape is a limiting step for the MEND system. We next examined the luciferase activity in parenchymal cells (PCs) and non-parenchymal cells (NPCs). As shown in Figs. 2c and d, the use of the MEND resulted in a 3 times lower in luciferase activity in PCs and was 11 times higher in NPCs, compared to lipotrust, suggesting that the MEND system has a preference for expressing luciferase protein in NPCs. On the other hand, the use of the GALA modified MEND resulted in a significant improvement in luciferase activity compared to the MEND (18 times in PCs and 11 times in NPCs). The luciferase activity was then normalized by the number of cells (as described in Material and Methods) to compare the transfection efficiency of PCs and NPCs. The results showed that the transfection efficiency of NPCs is significantly higher (6 times) than PCs in the case of the GALA modified MEND (Fig. 3). It is noteworthy that this tendency is almost the same when low doses were used (10 μg of pDNA) (data not shown). These results clearly indicate that the GALA modified MEND has the capacity to deliver pDNA into NPCs.

In addition to PCs, NPCs, which mainly include liver sinusoidal endothelial cells (LSEC) and Kupffer cells contribute to a wide range of liver pathophysiologies. For example, microcirculatoy disturbances of LSEC have been observed in cases of alcoholic liver damage and fulminant hepatic failure.[16,17)] In addition, altered immunoresponses of LSECs are closely related to the pathogenesis of viral hepatitis, fulminant hepatic failure, and transplant rejection.[18)] Inflammatory

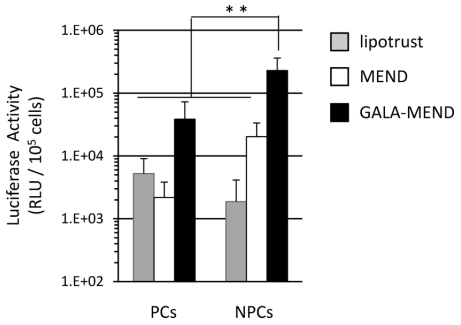

Fig. 3. A Comparison of Luciferase Activity between PCs and NPCs

Luciferase activity was normalized by the number of cells (per 1×10^5 cells). Each bar represents S.D. $n=3$—4. ** $p<0.01$.

cytokines produced by Kupffer cells are associated with hepatic failure. In particular, the inhibition of nuclear factor (NF)-κB in Kupffer cells, which regulates inflammatory cytokine production, has been reported to prevent liver injury.[19] Therefore, a gene delivery system for NPCs is a potentially useful strategy for treating such liver diseases.

In conclusion, the findings reported herein, indicate that a MEND system can be used to avoid the luciferase activity in the lung, compared to the lipoplex system, and has advantage in terms of delivering pDNA to the liver. Luciferase activity in the whole liver was greatly improved when the MEND was modified with a GALA peptide, and this increase contributes to NPCs, but not PCs. The findings reported in this study promise to be useful for the development of an efficient NPC (in particular, liver sinusoidal endothelial cells or Kupffer cells) gene delivery for *in vivo* applications.

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