Non-ionic Surfactant Modified Cationic Liposomes Mediated Gene Transfection in Vitro and in the Mouse Lung

Wuxiao Ding, Tomohiro Izumisawa, Yoshiyuki Hattori, Xianrong Qi, Dai Kitamoto, and Yoshie Maitani

As reported previously, cationic liposomes formulated with dioleoylphosphatidylethanolamine (DOPE) and N,N-methyl hydroxyethyl aminopropyl carbamoyl cholesterol (MHAPC-liposomes) achieved efficient gene transfection in the mouse lung following intratracheal injection. We have studied here the role of surfactants, mannosylerythritol lipid-A (MEL-A) and polysorbate 80 (Tween 80), in affecting gene transfection of MHAPC-lipoplexes (complex with pCMV-luc DNA) in A549 cells and in the mouse lung. MEL-A increased gene transfection of MHAPC-lipoplexes significantly in vitro and slightly in the mouse lung, while Tween 80 decreased it both in vitro and in vivo. As assessed by confocal laser scanning microscopy and fluorescence imaging, MEL-A might facilitate gene dissociation from MHAPC-lipoplexes with fluorescein-labeled oligodeoxynucleotide (FITC-ODN) after internalization into the cells and retained the lipoplexes in the mouse lung for prolonged time, while Tween 80 was inefficient to deliver foreign gene into target cells and in the lung. These results demonstrated that MEL-A is advantageous to Tween 80 in the modification of cationic liposomes as gene delivery vectors in the lung.

Key words: non-ionic surfactant; mannosylerythritol lipid-A; Tween 80; cationic liposome

Airway gene delivery is one of the most noninvasive approaches for gene therapy of cystic fibrosis and non-small cell lung cancer. Efficient and safe gene delivery vectors are necessary to move lung gene therapy from the laboratory to the bedside. Cationic liposomes are currently good candidates due to their safety, high transfection ability, and they can be dosed repeatedly. Many cationic cholesterol derivatives have been formulated into cationic liposomes. We reported previously that cationic liposomes with dioleoylphosphatidylethanolamine (DOPE) and N,N-methyl hydroxyethyl aminopropyl carbamoyl cholesterol (MHAPC, Fig. 1), a cationic cholesterol with a hydroxyethyl group in the cationic part, achieved high gene transfection efficiency in the mouse lung following intratracheal injection. To further increase the gene transfection of MHAPC-liposomes in the lung, we focused on non-ionic surfactant modification due to the reasons addressed below.

Mannosylerythritol lipid-A (MEL-A) is a newly developed biosurfactant, which consists of 4,6-di-O-acetyl-2,3-di-O-alkanoyl-β-D-mannopyranosyl-(1→4)-O-erythritol esterified with two fatty acids and two acetic acids (Fig. 1). With the modification by MEL-A, cationic liposome exhibited high fusion ability with cells, and increased gene transfection in vitro by measurement of the luciferase activity. However, the performance of MEL-A modified cationic liposomes has not been clarified for in vivo gene transfection. Furthermore, Tween 80 is a conventional and widely used surfactant that has been used in many cationic emulsions and cationic nanoparticles as a stabilizer to achieve high gene transfection ability. Although MEL-A and Tween 80 have demonstrated effective roles in cationic liposomes or cationic nanoparticles and emulsions as stated above, they have never been compared in the same formulations for the promotion effect on gene transfection. In this study, we modified MHAPC-liposomes with MEL-A or Tween 80 for the enhancement of gene transfection efficiency in the mouse lung. The in vitro and in vivo gene transfection and the cellular uptake of lipoplexes were compared to evaluate the roles of surfactants in liposomes.

Materials and Methods

Materials

MHAPC was synthesized as reported previously. DOPE and Tween 80 were obtained from NOF Co., Ltd. (Tokyo, Japan), and MEL-A was purified as reported previously. The plasmid p cytomegalovirus (pCMV)-luc (DNA) encoding the luciferase gene under the control of the CMV promoter was constructed as described previously. Twenty-mer randomized oligodeoxynucleotide (5’-CGAGT-GCACACGCTCTCAG-3’, ODN) and fluorescein-labeled ODN with the same base sequences (FITC-ODN) were synthesized with a phosphodiester backbone (Sigma Genosys Japan, Hokkaido, Japan). ddY mice (male, 25 g body weight) were purchased from Sankyo Lab Service (Shizuoka, Japan).

Preparation and Characterization of Liposomes and Lipoplexes

MHAPC was formulated into liposomes with DOPE by modified ethanol injection method, namely MHAPC-liposomes (MHAPC/DOPE = 1/1, m/m). For MEL-A: mannosylerythritol lipid-A; MHAPC: N,N-methyl hydroxyethyl aminopropyl carbamoyl cholesterol iodide.

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A and Tween 80 modified MHAPC-liposomes, 20% (molar percent in liposomes) of these surfactants were included in the lipids prior to preparation, they were named as MEL-A and Tween 80, respectively. The liposomes contained 4.5 mm MHAPC concentration for all the experiments. For fluorescence labeling, 0.2% (molar ratio to total lipids) of N-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (rhodamine-DHPE) was included in the lipids.

The MHAPC-lipoplexes (complex with pCMV-luc DNA) for in vitro gene transfection at various charge ratios (+/−) of cationic lipids to DNA were formed by addition of each lipid to 20 μg of DNA (20 and 33.34 μl for the charge ratio (+/−) of 3/1 and 5/1, respectively, in which liposomes were diluted to a cationic lipid concentration of 0.9 mm). They were left at room temperature for 10 min before transfection. For in vitro study, the lipoplexes at charge ratios (+/−) of 3/1 and 5/1 were prepared by the addition of 40 and 66.5 μl of liposomes, respectively to 20 μg of DNA in 25 μl of MilliQ water.

The particle size and zeta potential of liposomes and lipoplexes were measured by dynamic light scattering method (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan). The particle size and zeta potential of lipoplexes at a charge ratio (+/−) of 3/1 were measured both in Milli Q water (Elix® equipment, Millipore Corporate, MA, U.S.A.) and 1/10 phosphate-buffered saline (pH=7.4, 1/10 PBS).

Transfection Protocol and Luciferase Activity Measurement
The human lung adenocarcinoma A549 cell line was obtained from ATCC (VA, U.S.A.). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and kanamycin (100 μg/ml) at 37 °C in a 5% CO2 humidified incubator. Cells at a confluence level of 70% in a 35-mm culture dish were transfected with each lipoplex. For transfection, the prepared lipoplexes were diluted in 1 ml of culture medium and then incubated with the cells for 24 h. Luciferase expression in A549 cells was measured as counts per second (cps)/μg protein using the luciferase assay system (Picagene, Tokyo Ink Mfg. Co., Ltd., Tokyo, Japan) and the cps value was normalized to the protein concentration, as determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.).

Intratracheal injection was employed to study gene transfection in the mouse lung as reported previously. Briefly, the mice were positioned in a vertical position and complex solutions were bolusly injected into the lung through opened trachea while anesthetized. In vivo jet-PEI (Polypus-transfection, NY, U.S.A.) was widely used as a golden standard for in vivo gene transfection, its complex with DNA was prepared at a charge ratio (+/−) of 5/1 according to the manufacturer’s instructions. Luciferase in the lung was measured 24 h after intratracheal injection. The lung was harvested and immediately homogenized in 500 μl of cold lysis buffer (Promega Co., WI, U.S.A.). The homogenate samples were centrifugated at 15000 rpm for 5 min at 4 °C and the luciferase in the supernatant was measured as described above. The cps value was normalized to the total protein concentration as cps/mg protein.

Flow Cytometry A549 cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each diluted liposome (20 μl of liposomes with a cationic lipid concentration of 0.9 mm) was mixed with 2 μg of FITC-ODN or rhodamine-labeled liposome mixed with 2 μg of ODN at a charge ratio (+/−) of 3/1, and then diluted in 1 ml of medium. Cells were incubated with the lipoplexes at 37 °C for 2 h and 24 h. After incubation, the cells were washed 2 times with PBS and detached with 0.25% trypsin and centrifuged at 1500 rpm for 3 min. The supernatant was discarded and the cell pellets were resuspended with PBS containing 0.1% BSA and 1 mm EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, CA, U.S.A.). Data for 10000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and green and red fluorescence. Mean intensity values of FITC or rhodamine inside cells were calculated to compare the uptake amount of lipoplexes.

Confocal Laser Scanning Microscopy (CLSM) A549 cells were prepared by plating in a 35-mm glass dish 24 h prior to each experiment (30% confluence). Each type of rhodamine-labeled liposomes (20 μl of liposomes with a cationic lipid concentration of 0.9 mm) was mixed with 2 μg of FITC-ODN at a charge ratio (+/−) was 3/1, and then diluted in 1 ml of medium. The cells were incubated with the lipoplex at 37 °C for 2 h. After incubation, the dishes were washed 2 times with PBS and mounted in Aqua Poly/mount. Observation was performed with a Radiance 2100 confocal laser-scanning microscope (BioRad, CA, U.S.A.). FITC-ODN and rhodamine-DHPE were excited by an argon ion laser (488 nm) and a He–Ne laser (543 nm), respectively.

Fluorescence Imaging Rhodamine-DHPE (0.2%, molar ratio to total lipids) labeled MHAPC-liposomes and MEL-A and Tween 80 modified MHAPC-liposomes were complexed with 20 μg FITC-ODN. The lipoplexes were intratracheally injected into the mouse lung, after 2 h and 24 h, the lung was harvested and cryosectioned into 12 μm sections before mounting on glass slides. The sections were then embedded in Aqua Poly/mount (Polysciences Inc., Warrington, PA, U.S.A.), and the fluorescence levels of rhodamine and FITC were observed by fluorescence microscopy (Nikon Eclipse TS 100, Nikon, Tokyo, Japan). The exposure times were set as the same for each image.

Statistical Analysis The statistical significance of the data was evaluated with Student’s t-test. A p value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

In Vitro Gene Transfection of MEL-A and Tween 80 Modified Lipoplexes As reported previously, the gene transfection of cationic liposomes in the presence of 10% serum was increased with elevated molar ratio of MEL-A in liposomes (from 10 to 30% of MEL-A in liposomes), and even 30% MEL-A modified liposomes did not show significant cytotoxicity at transfection concentration. Therefore, we used 20% molar ratio of MEL-A or Tween 80 in this study. As MHAPC-liposomes exhibited much higher gene transfection at a charge ratio (+−) of 3/1 than 1/2, we examined charge ratios (+−) of 3/1 and higher. As shown in Fig. 2, for all the formulations, a charge ratio (+−) of 3/1 was much more effective than 5/1 in gene transfection. MEL-A increased gene transfection of MHAPC-liposome at both charge ratios (+−) of 3/1 and 5/1, while Tween 80 signifi-
cantly decreased them without significant cytotoxicity (data not shown). With a notion that DNA dissociation from lipoplexes after endocytotic internalization is a key step for effective gene transfection,\textsuperscript{21} excess cationic liposomes at a charge ratio (+/−) of 5/1 might hamper DNA dissociation, thereby decreasing gene expression as compared to 3/1.

The increased gene transfection of MHAPC-liposome by MEL-A was in accordance with OH-Chol-liposomes (OH-Chol = cholesteryl-3β-carboxyamidoethylene-N-hydroxy-ethyamine).\textsuperscript{12,13} Although Tween 80 was reported to be very effective in nanoparticles and emulsions for gene transfection, it did not work for cationic liposomes.\textsuperscript{16–21} We suppose that Tween 80 plays different roles in nanoparticles, emulsions and liposomes. In nanoparticles and emulsions, Tween 80 is an important stabilizer and emulsifier to maintain their stability, which is essential for their excellent transfection ability.\textsuperscript{16,18} Since the liposome itself is stable system due to the lipid bilayer, the addition of Tween 80 forms a steric barrier and may prevent cellular association of the lipoplexes, thereby decreasing gene transfection.\textsuperscript{17}

Physiochemical Properties of Liposomes and Lipoplexes

The physiochemical properties of liposomes and lipoplexes had a significant effect on their in vitro and in vivo gene transfection ability, therefore, zeta potential and particle size of liposomes alone and lipoplexes at a charge ratio (+/−) of 3/1 were measured in Milli Q water and PBS (Table 1). For particle size, MHAPC-liposomes and its modified formulations had a mean particle size of about 70 nm. Modification with MEL-A and Tween 80 significantly reduced the size of their lipoplexes. MHAPC-liposomes and its modified formulations were strongly positively charged. Although MEL-A and Tween 80 didn’t change the zeta potential of liposomes very much, Tween 80 significantly decreased the zeta potential of lipoplexes to 11.1 mV as compared to non-modified MHAPC-lipoplexes in 1/10 PBS. As seen from lipid-polyethylene glycol (lipid-PEG),\textsuperscript{24} Tween 80 might also decrease zeta potential of lipoplexes by the steric layer of oxyethylene groups.

**Cellular Association of MHAPC-Lipoplexes in A549 Cells**

To clarify the different gene transfection levels conferred by the surfactants, we examined the cellular association of lipoplexes using FITC-ODN as the DNA source and liposomes labeled with rhodamine-DHPE. In Fig. 3A, MEL-A modification inhibited the association of MHAPC-lipoplexes at 2h from both the FITC and rhodamine intensities; however, the associated amount of liposomes was significantly increased by MEL-A at 24 h as compared to non-modified ones. This phenomenon suggested that MEL-A could gradually increase the association of MHAPC-lipoplexes within 24 h.\textsuperscript{15} Tween 80 modification significantly reduced the association of lipoplexes both at 2 and 24 h (Fig. 3A). Subsequently, we carried out confocal microscopy experiment to compare the effect of MEL-A and Tween 80 on MHAPC-liposome-mediated gene delivery. In Fig. 3B, red fluorescence represents the location of rhodamine-labeled lipoplexes within 24 h.15 Tween 80 modification greatly reduced the uptake of lipoplexes (Fig. 3A), and green fluorescence was barely observed in the cells (Fig. 3B(c)). In Fig. 3B(b)), suggesting that MEL-A may promote gene dissociation from MHAPC-liposomes during the internalization step into A549 cells. MEL-A modified cationic liposomes carried out gene transfection through different process from conventional ones.\textsuperscript{13,14} MEL-A promoted fusion between MEL-A-containing liposomes and the plasma membrane of target cells, the foreign DNA probably dissociated from the liposomes rapidly and delivered into the cytoplasm and nucleus.\textsuperscript{13,14} Modification of MHAPC-L with Tween 80 greatly reduced the uptake of lipoplexes (Fig. 3A), and green fluorescence was barely observed in the cells (Fig. 3B(c)). The decreased cellular association of lipoplexes by Tween 80 was probably caused by the steric barrier of polyoxyethylene, the decreased zeta potential (Table 1) and the wetting surface of lipoplexes,\textsuperscript{25} which were supposed to be disadvantageous in the cellular association of lipoplexes.

![Fig. 2. Gene Transfections of MHAPC-Lipoplexes in A549 Cells at 24 h](image)

Each result represents the mean±S.D. (n=3). **p<0.01 and *p<0.05 compared with values of non-modified lipoplexes at the corresponding charge ratios.

Table 1. Physiochemical Properties of Liposomes and Lipoplexes at a Charge Ratio (+/−) of 3/1

<table>
<thead>
<tr>
<th>Liposome/Lipoplex</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milli Q</td>
<td>1/10 PBS</td>
</tr>
<tr>
<td>MHAPC-L Liposome</td>
<td>78.9</td>
<td>—</td>
</tr>
<tr>
<td>MHAPC-L Lipoplex</td>
<td>291.8±8.2</td>
<td>294.1±11.3</td>
</tr>
<tr>
<td>MEL-A Liposome</td>
<td>71.5</td>
<td>75.9</td>
</tr>
<tr>
<td>MEL-A Lipoplex</td>
<td>162.8±4.1**</td>
<td>173.8±7.3**</td>
</tr>
<tr>
<td>Tween 80 Liposome</td>
<td>67.0</td>
<td>—</td>
</tr>
<tr>
<td>Tween 80 Lipoplex</td>
<td>153.8±8.5**</td>
<td>164.3±9.1**</td>
</tr>
</tbody>
</table>

** p<0.01 compared with the values of non-modified MHAPC-lipoplexes. — is not measured.
In Vivo Gene Transfection and Retention of MEL-A and Tween 80 Modified Lipoplexes

As shown in Fig. 4A, in contrast to in vitro gene transfection results, a charge ratio (+/-) of 3/1 was almost equal to 5/1 in gene transfection in the lung. The effect of surfactants on gene transfection in the mouse lung had the same trend with that in vitro (Fig. 2). MEL-A modified increased gene transfection more than 2-fold higher than the positive control, in vivo jet-PEI, but Tween 80 significantly decreased the gene transfection level. The effect of MEL-A and Tween 80 on gene transfection of MHAPC-lipoplexes in the mouse lung was well reflected on the retention ability of lipoplexes (Fig. 4B). In Fig. 4B, the intensity of green fluorescence (FITC) and red fluorescence (rhodamine) indicated the amount of FITC-ODN and rhodamine-labeled liposomes in the lung sections, respectively. After intratracheal injection, the lipoplexes firstly contacted with the mucus layer covering the epithelial cells, and may be taken up by the epithelial cells if they are not quickly cleared by the mucus clearance system. At 2 h after injection of lipoplexes, both MEL-A and Tween 80 modified MHAPC-L showed similar rhodamine and FITC intensities, indicating the same amount of lipoplexes was retained into the mouse lung. At 24 h, MEL-A modified lipoplexes however showed higher FITC intensity than Tween 80 modified lipoplexes, which rarely showed any FITC fluorescence. Although MEL-A and Tween 80 modified MHAPC-liposomes similarly exhibited strong interaction with DNA by circular dichroism, MEL-A has adhesive ability to cells and did not decrease the zeta potential of MHAPC-lipoplexes as compared to Tween 80 (Table 1). These information suggested that MEL-A has higher mucoadhesion ability than Tween 80 modified formulation and could retain the lipoplexes on mucus layer.

Fig. 4. Gene Transfections (A) and Retention (B) of MHAPC-Lipoplexes in the Lung

In A, jet-PEI was complexed with DNA at a charge ratio (+/-) of 5/1 according to manufacture’s instructions. Each result represents the mean±S.D. (n=3). **p<0.01 and *p<0.05 compared with values of non-modified lipoplexes for the corresponding fluorescence and time point. In B, the retention of MEL-A or Tween 80 modified lipoplexes (complex with FITC-ODN, +/-=3/1) was evaluated at 2 and 24 h. The middle lane is rhodamine and the right lane is FITC. Representative images are shown for each sample. 40×magnification for each image.
and cells of the lung for longer period than Tween 80. The prolonged retention ability of MEL-A modified MHAPC-lipoplexes might increase the association of lipoplexes with epithelial cells and gene transfection in the lung. From these results, MEL-A was superior to Tween 80 in increasing gene transfection of cationic liposomes by retaining the lipoplexes in the lung for prolonged period.

**CONCLUSIONS**

This study demonstrated that the gene transfection and cellular association of MHAPC-lipoplexes were differently affected by the modification of MEL-A and Tween 80 both in vitro and in the mouse lung. MEL-A increased gene transfection of MHAPC-lipoplexes significantly in vitro and slightly in the mouse lung, while Tween 80 was not effective both in vitro and in vivo. CLSM experiment demonstrated that MEL-A might induce different mechanism in the cellular uptake of lipoplexes compared with Tween 80, and facilitated foreign gene dissociation from cationic liposomes after internalization into A549 cells. MEL-A could retain the lipoplexes in the mouse lung for prolonged time while Tween 80 did not. These results provided some useful information of non-ionic surfactant modified cationic liposomes for gene transfection in the lung.

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