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Comparative Study of Transfection Efficiency of Cationic Cholesterols Mediated by Liposomes-Based Gene Delivery

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Abstract—To develop the efficient non-viral vector for gene delivery, we compared transfection activities of cationic cholesterol derivatives. We found that the stability of the liposome–DNA complex in the presence of endosome deeply related to the transfection efficiency. We also found that the introduction of a hydrophilic group to the amino terminal of the cholesterol derivative decreased stability and facilitated the release of DNA from the endosome, resulting in higher transfection efficiency.

It is needless to say that gene therapy has become a fundamental pillar in modern medicine. However, the establishment of an efficient and safe method for delivering exogenous genes into mammalian cells is still in the formative stage. Among various methods for gene transfection, lipofection using cationic liposomes is one of the most promising ways to introduce a foreign gene to the target cells.[1–3] Cationic cholesterol derivative is composed of three distinct parts: cholesteryl skeleton, cationic amino group and spacer arm between the cholesteryl skeleton and cationic amino group. Such derivatives with different combination of these parts were reported and some of them have high transfection efficiency.[4–6] In addition, we recently developed a novel cationic cholesterol derivative with the hydroxy amino group and found that the compound had very high transfection efficiency.[7] However, the relationships between the structure of the cholesterol derivatives and their characteristics, which are deeply related to transfection efficiency, such as interaction with biomembrane and DNA, have not been well-described. To address this problem, we synthesized several related cationic cholesterol derivatives (Fig. 1) and characterized them in terms of their interaction with biomaterials and transfection efficiency. We found that the hydroxy group affects the stability of the liposome–DNA complex, and reduced stability enhanced transfection efficiency by facilitating the process in which DNA is liberated from the endosome.

Among synthetic cationic cholesterol derivatives, (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol, **1** in Fig. 1) is a typical and commercially available derivative as a lipofection reagent.[8] In addition to DC-Chol, we synthesized seven related derivatives (**2**–**8** in Fig. 1).[7–9] These derivatives are different in

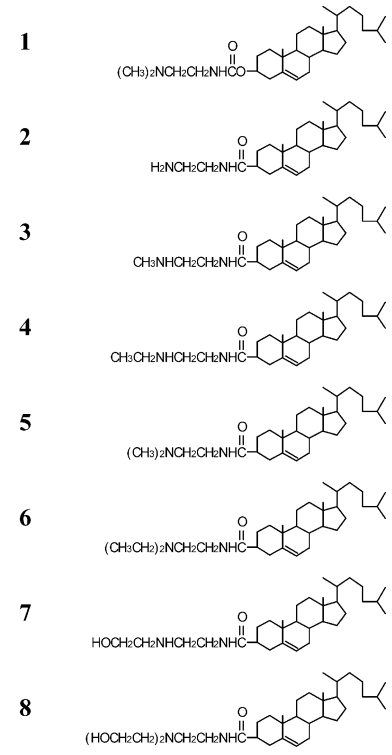

Figure 1. Cationic cholesterol derivatives used in this study.

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number and length of alkyl groups bonded to nitrogen of amino terminal and in number of hydroxy ethyl groups.

Transfection efficiencies of these eight derivatives were compared (Fig. 2). Derivative **7**, which has a hydroxy ethyl group at the amino terminal, showed the highest efficiency among the derivatives tested. Derivative **4** had the highest efficiency among derivatives without a hydroxy ethyl group. These results suggest that derivatives with a primary amine (**2**) and a bulky tertiary amine (**6**) at amino terminal had low transfection activity, while those with secondary (**3**, **4**) and less bulky tertiary (**5**) amine exhibited higher efficiency. The introduction of a hydroxy ethyl group to the amino terminal instead of the ethyl group of derivative **4** greatly enhanced the transfection efficiency. However, the introduction of two hydroxy ethyl groups reduced efficiency to the level of the derivative with a bulky tertiary amine (**6**).

To understand the difference in transfection efficiency of these derivatives, we first examine the interaction between liposomes with cationic cholesterol derivatives and DNA plasmid by ethidium bromide (EtBr) intercalation assay.[10] Electrostatic interaction between cationic liposomes and anionic DNA causes capsulation and compaction of liposome–DNA complexes. This interaction facilitates the uptake of liposome–DNA complexes by target cells and protects plasmid DNA from degradation. When EtBr is added to a solution of DNA, it intercalates between the base pairs of the DNA double helix, emitting an intense fluorescence. This fluorescence is reduced by the formation of liposome–DNA complexes.[10] Using this EtBr intercalation assay, we found that liposomes prepared with derivative **8** had the lowest interaction and no significant differences were observed among derivatives other than **8**. This low interaction with DNA might be one of the reasons derivative **8** had low transfection efficiency.

The interaction between cationic liposomes and target cells was also investigated. We measured the number of cells which bear liposome–DNA complexes by flow cytometry. Again cationic liposomes with derivative **8** showed the lowest interaction with target cells, but no significant differences were observed among other derivatives (data not shown).

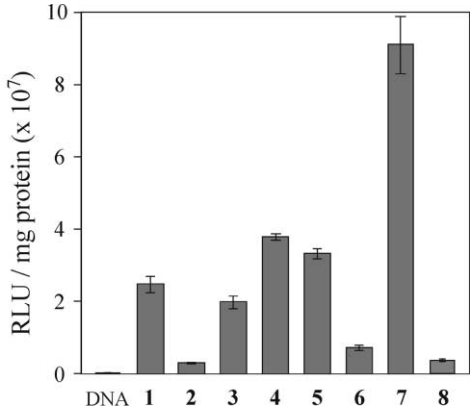

Figure 2. Transfection efficiency of cationic liposomes prepared with various cholesterol derivatives. Transfection activity without lipid is represented as DNA. Each value is the mean ± SE ($n = 3$).

The observation above led us to investigate the intracellular fate of liposme–DNA complexes. Liposome–DNA complexes which were taken up by endocytosis were transported to lysosomes where complexes degrade.[11] Therefore, DNA must be released from liposomes and endosomes before degradation to reach the nucleus where DNA is transcribed.[12,13] To examine the efficiency of the release process, the dissociation of DNA from cationic liposomes caused by the addition of the anionic membrane, which mimics the endosomal membrane, was monitored by the fluorescent resonance energy transfer (FRET) using rhodamine-labeled DNA and NBD-labeled liposomes (Fig. 3). Although the detailed mechanism of the release of DNA from the endosome remains to be elucidated, it requires the membrane fusion between the liposome and anionic endosomal membrane.[13,14] Therefore, it is reasonable to use anionic liposomes to mimic the release of DNA from endosome, and the FRET assay is a proper measure to estimate the kinetics of the release of DNA from the endosomes in vivo. FRET assay is widely used to monitor dynamic changes in the distance between two fluorescent probes.[14] In the case of Figure 3, fluorescence of NBD decreased by the association of liposomes and DNA, since energy transfer from excited NBD to rhodamine occurred. Liposomes prepared with derivative **8** exhibited weaker interaction, even at 15 min after addition of DNA. On the other hand, derivatives other than **8** reached almost the same level at 15 min, although the rate of association was slow in the case of derivative **7**. These data are consistent with the results of the EtBr intercalation assay. After formation of liposome–DNA complexes, dissociation of DNA from liposomes was induced by the addition of anionic liposomes.

The fluorescence intensity of NBD increased, since dissociation caused cancellation of FRET. In the dissociation process, cationic liposomes with different derivatives behaved differently, although the fluorescence

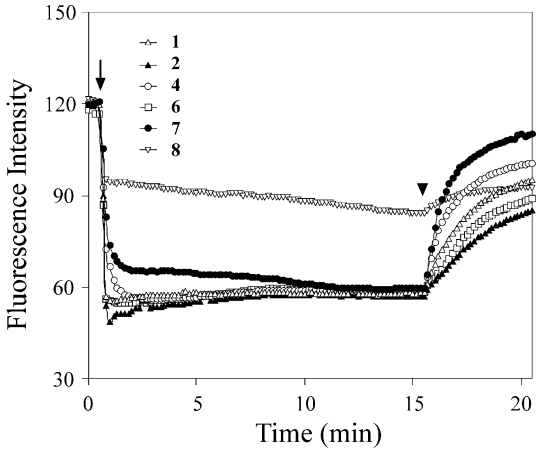

Figure 3. Time course of association and dissociation reaction of cationic liposomes and DNA. Fluorescence intensity of NBD was plotted against time. Rhodamine-labeled DNA and anionic liposomes were added at the time indicated by an arrow and arrowhead, respectively.

intensity of NBD recovered to the initial level by the addition of deoxycholate in all cases. As for liposomes with derivative **8**, DNA dissociated from liposomes very slowly and became steady at low dissociation level. In contrast, DNA dissociated very fast from the liposomes with derivative **7** and reached the highest dissociation level. Interestingly, the orders of the rates and levels of dissociation corresponded with the order of transfection efficiency. These results suggest that the reason for high transfection efficiency of liposomes with derivative **7** is its instability of liposome–DNA complexes in endosomes, resulting in higher efficiency of the translocation of DNA into the nucleus.

To confirm this notion, we observed the intracellular distribution of DNA in a living cell. Figure 4a shows the intracellular distribution of rhodamine-labeled DNA at 4 h after introduction of DNA as complexes with cationic liposomes. The fluorescence intensity of the whole cell was almost same; nevertheless, the intensity in the nucleus is higher in the cells transfected by liposomes with derivative **7** than that in the cells transfected by liposomes with derivative **4**, which had the highest efficiency in the derivatives without hydroxy ethylamine. Fluorescence was hardly observed in the nucleus of the cells transfected with derivative **8**. As for **1**, **2**, **3**, **5** and **6**, weak fluorescence was observed in the nucleus but the ratio of the fluorescence intensity in the nucleus to that in the whole cell was less than that in the case of derivatives **4** and **7**.

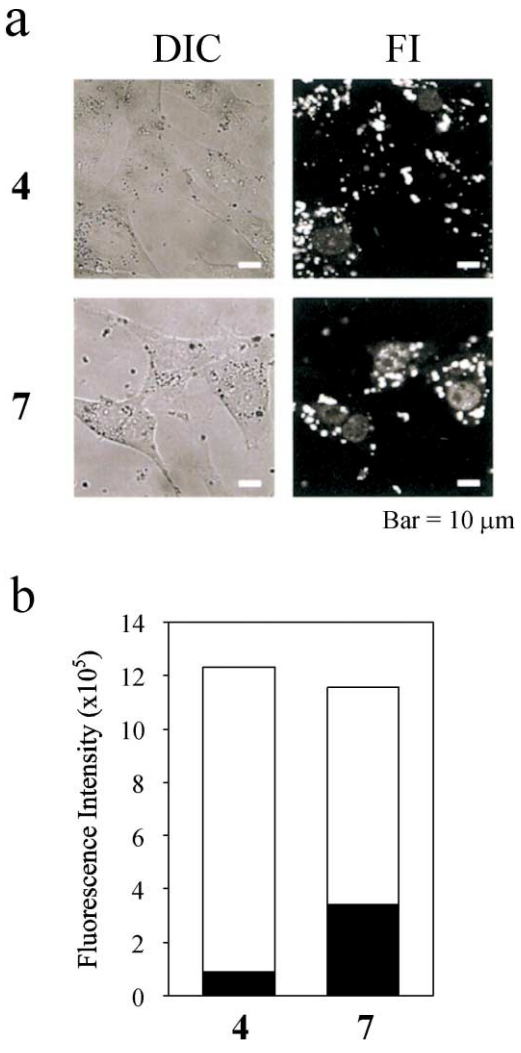

Figure 4. Intracellular distribution of DNA in a living cell. (a) Differential interference contrast image (DIC; left) and fluorescence images (FI; right) of cells at 4 h after transfection with liposomes prepared with derivative **4** (upper) and **7** (lower). (b) Fluorescence intensity of the whole cell (white) and intranuclear region (black) of 100 cells were summed up for each derivative.

In conclusion, the structural differences of cationic cholesterol affected the interaction between liposomes and DNA, and this reflected the differences in transfection efficiency. In particular, the dissociation process of complexes was deeply related to the transfection efficiency. The difference in number and length of alkyl groups bonded to nitrogen of amino terminal affected the dissociation process. The introduction of hydroxy ethyl groups (**7**, **8**) decreased the rate of association. Although the association of DNA and liposomes was very low in the case of derivative **8**, liposomes with derivative **7** still had the same level of association as other derivatives (**1**–**6**). Furthermore, DNA dissociated from liposomes with derivative **7** very fast by the addition of anionic liposomes. These properties of liposomes with derivative **7** are favorable for the gene delivery.

Assay

Materials

1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (DOPS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cationic derivatives of cholesterol were synthesized as described previously.[7–9] Plasmids coding luciferase (pGL3) was purchased from Promega (Madison, WI, USA). NBD-PE was obtained from Molecular Probes (Eugene, OR, USA). Rhodamine or FITC labeled *c-myc* antisense nucleotides were obtained from Nihon Gene Research Lab's (Miyagi, Japan). Deoxycholate was purchased from Wako Pure Chemicals (Tokyo, Japan).

Preparation of cationic liposomes

DOPE was mixed with a cationic cholesterol derivatives (**1**–**8**) in chloroform [DOPE/Chol = 20:30 (mol/mol)], and the mixture was evaporated under reduced pressure to give a thin film of lipids. To the thin film, phosphate-buffered saline (PBS) was added, and the mixture was allowed to stand for 60 min in the dark. The samples were then sonicated using a bath-type sonicator (Bransonic Model B1200, Branson) for 5 min, followed by sonication with a probe-type sonicator (Sonifier 250, Branson) for 10 min to form small unilamellar vesicles (SUVs). The diameter of these cationic liposomes was measured with a multi-angle light-scattering instrument (Otsuka Electronics, Japan). The average diameter of the liposomes was 200 nm.

Cell culture and gene transfection

NIH3T3 cells were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Rockville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Trace Scientific, Melbourne, Australia), penicillin (100 units/mL) and streptomycin (100 μg/mL).

For transfection with luciferase plasmids using cationic lipids, 5.4 μg of pGL3 was complexed with liposomes consisting of 20 nmol of DOPE and 30 nmol of cationic cholesterol derivatives (**1**–**8**) in SFM-101 (Nissui Pharmaceutical, Tokyo, Japan) and incubated for 15 min at 37 °C to form liposome–DNA complexes. Liposome–DNA complexes were incubated with the cells (1×10^6 cells/dish) in SFM-101 in a culture dish (60×15 mm) for 4 h at 37 °C, and then the cells were washed and cultured in growth medium (DMEM) for another 40 h at 37 °C, followed by a luciferase assay.

Luciferase assay

The luciferase assay was carried out using a picagene luciferase assay kit (Toyo Ink, Tokyo, Japan). The transfected cells were washed three times with PBS and lysed in a cell lysis buffer. The lysate was centrifuged at 12,000*g* at 4 °C for 3 min and the supernatant was subjected to a luciferase assay. The intensity of chemiluminescence was measured with a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA) and values were normalized to the amount of protein determined by BCA assay.

EtBr intercalation assay

Fluorescence of EtBr at 595 nm (excited at 520 nm) was continuously monitored by a spectrofluorometer (RF-5300PC; Shimadzu, Japan) to investigate the effect of various additives on the extent of EtBr intercalation into DNA.

Flow cytometry

To evaluate the interaction of cells with liposome–DNA complexes, cells loaded with FITC-labeled DNA were detected by a flow cytofluorometer. After cells were incubated FITC-labeled DNA for 4 h, they were washed and resuspended in PBS. The fluorescence intensity of 10,000 cells was measured with a FACScan cytometer (Becton Dickinson).

FRET assay

To monitor the association and dissociation between DNA and liposomes, liposomes and DNA were labeled with NBD as a donner and rhodamine as an acceptor, respectively. Fluorescence intensity of NBD was monitored with a spectrofluorometer (ex. 465 nm, em. 525 nm). To start the association reaction, rhodamine-labeled DNA was added to the solution of NBD-labeled cationic liposomes. To induce the dissociation reaction, liposomes prepared from DOPC, DOPE and DOPS (DOPC/DOPE/DOPS = 1:2:1; molar ratio) were added in an anionic liposomes to NBD-labeled cationic liposomes ratio of 3:1 (molar ratio).

Imaging with confocal laser scanning microscopy

Confocal fluorescent images were obtained by a confocal laser scanning microscope (LSM-510, Zeiss) equipped with an argon ion laser (548 nm). For the imaging of rhodamine-labeled *c-myc* antisense nucleotides was excited by a He–Ne laser and their fluorescence was collected through a long-pass filter (>560 nm). The observation chamber was kept at 37 °C throughout the imaging experiments. Fluorescent intensity of the whole cell and of the nucleus were quantified with NIH Image 1.61 based on the confocal images obtained in the same conditions.

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