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Intracellular trafficking mechanism of cationic phospholipids including cationic liposomes in HeLa cells

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The development of gene delivery methods is essential for the achievement of effective gene therapy. Elucidation of the intracellular transfer mechanism for cationic carriers is in progress, but there are few reports regarding the intracellular trafficking processes of the cationic phospholipids taken up into cells. In the present work, the trafficking processes of a cationic phospholipid (1,2-dioleoyl-3-trimethylammonium-propane, DOTAP) were investigated from intracellular uptake to extracellular efflux using cationic liposomes *in vitro*. Following intracellular transport of liposomes *via* endocytosis, DOTAP was localized in the endoplasmic reticulum, Golgi apparatus, and mitochondria. Moreover, the proteins involved in DOTAP intracellular trafficking and extracellular efflux were identified. In addition, helper lipids of cationic liposomes were found to partially affect this intracellular trafficking. These findings might provide valuable information for designing cationic carriers and avoiding unexpected toxic side effects derived from cationic liposomal components.

1. Introduction

Nucleic acid medicines and gene therapy have attracted attention as valuable medical technologies for the treatment of congenital genetic disease and cancer (Miele et al. 2012; Nana-Sinkam and Croce 2013; Prickett and Jain 2013; Resnier et al. 2013). The development of gene delivery methods that can achieve safe and high efficiency transgenic results is essential for the establishment of effective gene therapy (Miele et al. 2012; Resnier et al. 2013; Vannucci et al. 2013; Manjila et al. 2013). Gene carriers are mainly classified into viral and nonviral carriers (Vannucci et al. 2013; Manjila et al. 2013). In particular, nonviral carriers have been expected to be useful in clinical applications, as their structure is definitive and their immunogenicity or oncogenic transformation can be controlled (Lundstrom and Boulikas 2003). Gene carriers with cationic properties on their surfaces can be formed by mixing nucleic acid compounds with anionic properties with an excess of cationic nanoparticles (Miele et al. 2012). These nonviral cationic carriers electrostatically associate with cell membranes with anionic properties and, consequently, effective gene transference can be achieved. A number of studies has researched and developed many types of cationic compounds as components of nonviral carriers, such as cationic macromolecules and liposomes (Sato et al. 2008; Opanasopit et al. 2011; Thompson et al. 2012; Abbasi et al. 2012; Oba 2013).

Differing from intracellular delivery of low molecular weight molecules, intracellular uptake by passive diffusion is not involved in the intracellular transfer of nucleic acids. Therefore, intracellular uptake efficiency of carriers is important for intracellular delivery of nucleic acids using cationic carriers. The elucidation of the intracellular uptake mechanism of various types of cationic carriers is in progress (Jung et al. 2009; Thompson et al. 2012; Pang et al. 2012; Choi et al. 2013). Conversely, there are few reports regarding the intracellular trafficking processes of cationic carrier taken up into cells. Fujiwara et al. (2010) reported an intracellular uptake mechanism for cationic liposomes labeled with fluorescent-conjugated neutral phospholipids and indicated that cationic liposomes are transferred to the endosome following liposome intracellular uptake. However, the intracellular fate of cationic phospholipids composing incorporated liposomes has remained unclear.

Our group has previously demonstrated the intracellular trafficking pathways and the proteins involved in the trafficking of block copolymers, a phospholipid (1,2-dioleoyl-*sn*-glycero-3- phosphocholine; DOPC), and cholesterol (Chol); (Sakai-Kato et al. 2012; Un et al. 2012). In addition, we have also studied the intracellular trafficking mechanism of polyethylene-glycol-modified phospholipids, known as functional lipids for improving retention time in blood as well as formulation stability. In the present study, intracellular trafficking mechanisms were investigated as well as the proteins involved in the trafficking of a cationic phospholipid (1,2-dioleoyl-3-trimethylammonium-propane, DOTAP) widely used for nucleic acid transport. Moreover, the effects of cationic liposome helper lipids on intracellular trafficking of cationic phospholipids were also examined.

2. Investigations and results

2.1. Physicochemical properties

The physicochemical properties of cationic liposomes containing fluorescent (nitrobenzoxadiazole, NBD)-labeled DOTAP used in all experiments were evaluated by measurement of particle sizes, polydispersity index (PDI), and voltage (ζ)-potentials. The mean particle sizes and PDI of cationic liposomes used

Table 1: Particle sizes, PDI, and ζ-potentials of liposomes. Values are mean ± S.D. ($n = 3$)

	particle size (nm)	PDI	ζ-potential (mV)
DOTAP:chol:NBD-labeled DOTAP (DOTAP/Chol, 45:50:5 (mol))	98.5 ± 1.78	0.104 ± 0.016	64.03 ± 1.305
DOTAP:DOPC:NBD-labeled DOTAP (DOTAP/DOPC, 45:50:5 (mol))	98.9 ± 3.67	0.112 ± 0.011	63.43 ± 2.329

here were approximately 99 nm and 0.1, respectively (Table 1). Moreover, the ζ-potentials of NBD-labeled cationic liposomes were approximately + 64 mV. The physicochemical properties of cationic liposomes are similar to other previous reports (Yeeprae et al. 2006; Colonna et al. 2008).

2.2. *Intracellular transport*

The intracellular transport mechanism of DOTAP integrated into cationic liposomes was investigated by quantifying intracellular NBD-labeled DOTAP in HeLa cells. Following a confocal microscopy study of intracellular localization of cationic liposomes, NBD-labeled DOTAP was colocalized with endosomes/lysosomes at 1 h after the addition of each type of liposome to HeLa cells (Fig. 1A). Following inhibitory experiments of endocytosis for elucidation of the intracellular transport mechanism, the intracellular DOTAP concentrations contained in cationic liposomes were significantly suppressed at 2 h after the addition of each liposome in the presence of chlorpromazine, an inhibitor of clathrin-mediated endocytosis (Fig. 1B). Moreover, the effects of helper lipids, such as Chol or DOPC for cationic liposomes, on intracellular DOTAP transport were not observed in HeLa cells (Fig. 1).

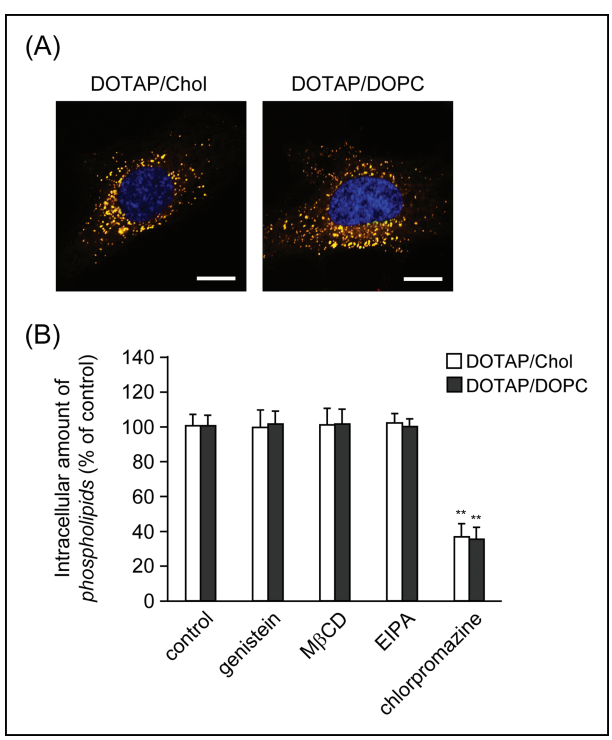

Fig. 1: (A) Confocal images of the intracellular transport of DOTAP/Chol and DOTAP/DOPC liposomes at 1 h after liposome addition to HeLa cells. Liposomes labeled with NBD-labeled DOTAP (green); endosomes/lysosomes labeled with AlexaFluor-594-conjugated transferrin/LysoTracker Red DND-99 (red); and scale bars, 20 μm. (B) DOTAP intracellular transport at 2 h after liposome addition to HeLa cells. Each endocytosis inhibitor added to cells at 30 min before liposome addition; $^{**}p < 0.01$, compared with corresponding control group; and values are mean ± S.D. ($n = 6$).

2.3. *Intracellular localization*

The intracellular localization of DOTAP 24 h after intracellular uptake of cationic liposomes was examined by confocal microscopy observation. Liposomes were labeled with NBD-labeled DOTAP, and the endoplasmic reticulum (ER), Golgi apparatus, and mitochondria labeled with ER-Tracker Red, BODIPY TR C_5-ceramide, and Mito-Tracker Red, respectively. DOTAP was found to be colocalized with ER, Golgi apparatus, and mitochondria at 24 h after the cationic liposome addition in a manner independent of the helper lipid type (Fig. 2).

2.4. *Elucidation of intracellular trafficking mechanism*

We investigated the intracellular trafficking mechanisms of DOTAP included in cationic liposomes in HeLa cells. In these experimental conditions, when the intracellular amounts of DOTAP are increased by suppression of lipid trafficking-related proteins below-mentioned, it is suggested that the protein is strongly involved in the intracellular trafficking of DOTAP. Under suppressing conditions by metastatic lymph-node gene 64 protein (MLN64), Niemann-Pick C1 protein (NPC1), Niemann-Pick C2 protein (NPC2), and oxysterol-binding protein-related protein 1 (ORP1), which are intracellular transport-related proteins for lipidic molecules (Fig. 3A; Hölttä-Vuori et al. 2005; Koivusalo et al. 2007; Xu et al. 2007; Johansson et al. 2003), intracellular DOTAP concentrations 24 h after the addition of DOTAP/Chol liposomes were increased during ORP1 suppression (Fig. 3B). Moreover, the intracellular DOTAP concentrations at 24 h after the addition of DOTAP/DOPC liposomes were increased under not only with ORP1 but also under NPC1 suppressing conditions (Fig. 3B).

Next, the involvement of ER-to-Golgi transport in intracellular trafficking of DOTAP included in cationic liposomes was evaluated. We selected CERT (ceramide-transfer protein) and sec31A (a component of COPII required for vesicle budding from the ER), which both are ER-to-Golgi transport-related proteins, Fig. 3A; Hanada et al. 2007; Jensen and Schekman 2011). Following knockdown experiments, intracellular DOTAP concentrations 24 h after liposome addition were increased in a manner independent on the helper lipid type (Fig. 3C).

Then, the extracellular DOTAP efflux mechanism was investigated in HeLa cells by evaluation of intracellular transport from the ER/Golgi apparatus to cell membranes. In this study, we focused on the oxysterol-binding protein-related protein 2 (ORP2) (Hsuan and Cockcroft 2001; Hynynen et al. 2001) and phosphatidylinositol transfer protein (PITP), which both are involved in the intracellular transport of lipidic molecules. The intracellular DOTAP concentrations at 24 h after DOTAP/Chol liposome addition were increased with suppression by PITP, an intracellular lipid transport-related molecule (Figs. 3A and 3D; Hsuan and Cockcroft 2001). Moreover, the intracellular DOTAP concentrations at 24 h after DOTAP/DOPC liposome addition were increased not only with PITP suppression but also with ORP2 (Hynynen et al. 2001) suppression (Fig. 3D). Following knockdown experiments of various ATP-binding cassette (ABC) transporters (ABCA1, ABCB1, ABCC1, and ABCG1; Kimura et al. 2007), the intracellular DOTAP concentrations at 24 h after the addition of DOTAP/Chol liposomes were markedly

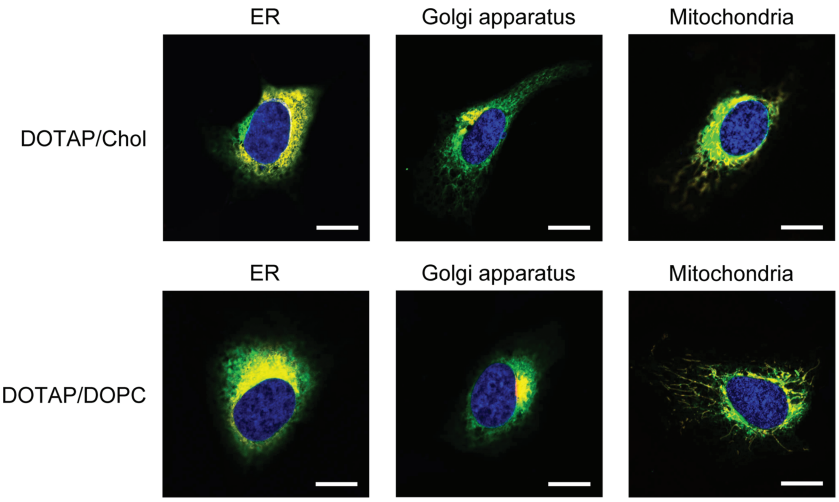

Fig. 2: Confocal images of the intracellular localization of DOTAP at 24 h after liposome addition to HeLa cells. Liposomes labeled with NBD-labeled DOTAP (green); ER, Golgi apparatus, and mitochondria labeled with ER-Tracker Red (red), BODIPY TR C_5-ceramide (red), and Mito-Tracker Red (red); and scale bars, 20 μm.

increased under ABCG1 suppressing condition (Fig. 3E). However, intracellular DOTAP concentrations at 24 h after the addition of DOTAP/DOPC liposomes were increased not only under ABCG1 but also ABCB1 suppressing conditions (Fig. 3E).

3. Discussion

Our group has previously reported the intracellular trafficking mechanism of DOPC, a neutral phospholipid included in liposomes (Un et al. 2012). The present study investigated the intracellular trafficking mechanism of DOTAP, a cationic phospholipid widely used for gene transfection and nucleic acid delivery. In addition, intracellular proteins related to the intracellular trafficking of DOTAP have also been examined. Recently, various lipid types have been studied as helper lipids in cationic liposomes for stability improvement and endosomal escape efficacy (Farhood et al. 1995; Hui et al. 1996; Huang et al. 2012). Here, DOPC and Chol were selected as helper lipids and their effects in cationic liposomes investigated in terms of intracellular DOTAP trafficking.

The cationic liposomes containing DOTAP used in this study were taken up by cells *via* clathrin-mediated endocytosis independent of the helper lipid type (Figs. 1 and 4). Following confocal microscopy examination of intracellular DOTAP localization 24 h after cationic liposome addition, DOTAP was distributed to the ER, Golgi apparatus, and mitochondria in a manner independent of the helper lipid type (Figs. 2 and 4). Moreover, DOTAP included in DOTAP/Chol liposomes was transferred intracellularly by ORP1 from the endosome/lysosome to cytoplasm, transported by PITP to cell membranes, and then, extracellularly effluxed by ABCA1 and ABCG1 (Figs. 3 and 4). In addition, the involvement of ER-to-Golgi transport by CERT and sec31A was observed in the intracellular transport of DOTAP included in DOTAP/Chol liposomes. Except for transport to mitochondria, intracellular trafficking mechanism of DOTAP included in DOTAP/Chol liposomes corresponded to that observed for DOPC (Un et al. 2012). These findings suggested that cationic properties did not affect intracellular transport of liposomal phospholipids except for trafficking to mitochondria.

Recently, various lipids have been studied as helper lipids in cationic liposomes for stability improvement and endosomal escape efficacy (Farhood et al. 1995; Hui et al. 1996; Huang et al. 2012). In this study, we selected DOPC and Chol as helper lipids, and the effects of helper lipids in cationic liposomes were investigated on DOTAP intracellular trafficking by evaluating intracellular trafficking of DOTAP included not only in DOTAP/Chol liposomes but also DOTAP/DOPC liposomes. Different from DOTAP/Chol liposomes, intracellular transport of DOTAP included in DOTAP/DOPC liposomes from the endosomes/lysosomes to the cytoplasm was controlled not only by ORP1 but also NPC1 (Figs. 3B and 4). The transport to cell membranes of DOTAP included in DOTAP/DOPC liposomes was controlled not only by PITP but also ORP2 (Figs. 3D and 4). Furthermore, ABCA1 involvement was decreased and that of ABCG1 increased in the extracellular efflux of DOTAP included in DOTAP/DOPC liposomes (Figs. 3E and 4). Our group has investigated intracellular trafficking mechanism of Chol included in liposomes and demonstrated the intracellular Chol trafficking is mainly controlled by NPC1, ORP2, ABCA1, and ABCB1 (Un et al. 2012). Since these proteins related to liposomal Chol trafficking were also observed to be involved in the intracellular trafficking of DOTAP included in DOTAP/DOPC liposomes, these results suggested that intracellular trafficking of DOTAP by NPC1, ORP2, and ABCA1 competed with Chol included in DOTAP/Chol liposomes. These findings suggest that the intracellular trafficking of DOTAP is partly controlled by proteins involved in Chol trafficking; however, the affinity of Chol for each protein is assumed to be higher than that observed for DOTAP. Therefore, it is considered that the intracellular trafficking of DOTAP might be affected by Chol when Chol is one of the liposomal components.

DOTAP were localized in the mitochondria 24 h after cationic liposome addition (Fig. 2), a phenomenon not observed with neutral phospholipids (DOPC) and Chol but apparent with the addition of cationic properties to phospholipids (Un et al. 2012). Recently, some researchers have reported that the drug-lipid conjugates was developed to improve intracellular drug uptake efficiency and enhance pharmaceutical effects (Hwang et al. 2007; Huan et al. 2009). We have previously reported that lipidic molecules, such as phospholipids or cholesterol, are transported by various types of intracellular proteins (Un et al. 2012). Taking this into consideration, drug-lipid conjugates might not only affect intracellular uptake of conjugated drugs but also their intracellular localization. In particular, there are a few reports regarding drug intracellular delivery to mitochondria. In this study, DOTAP was shown to partially accumulate in mitochondria (Fig. 2); therefore, the delivery of various molecules to mitochondria might be achieved by the use of drug-DOTAP conjugates.

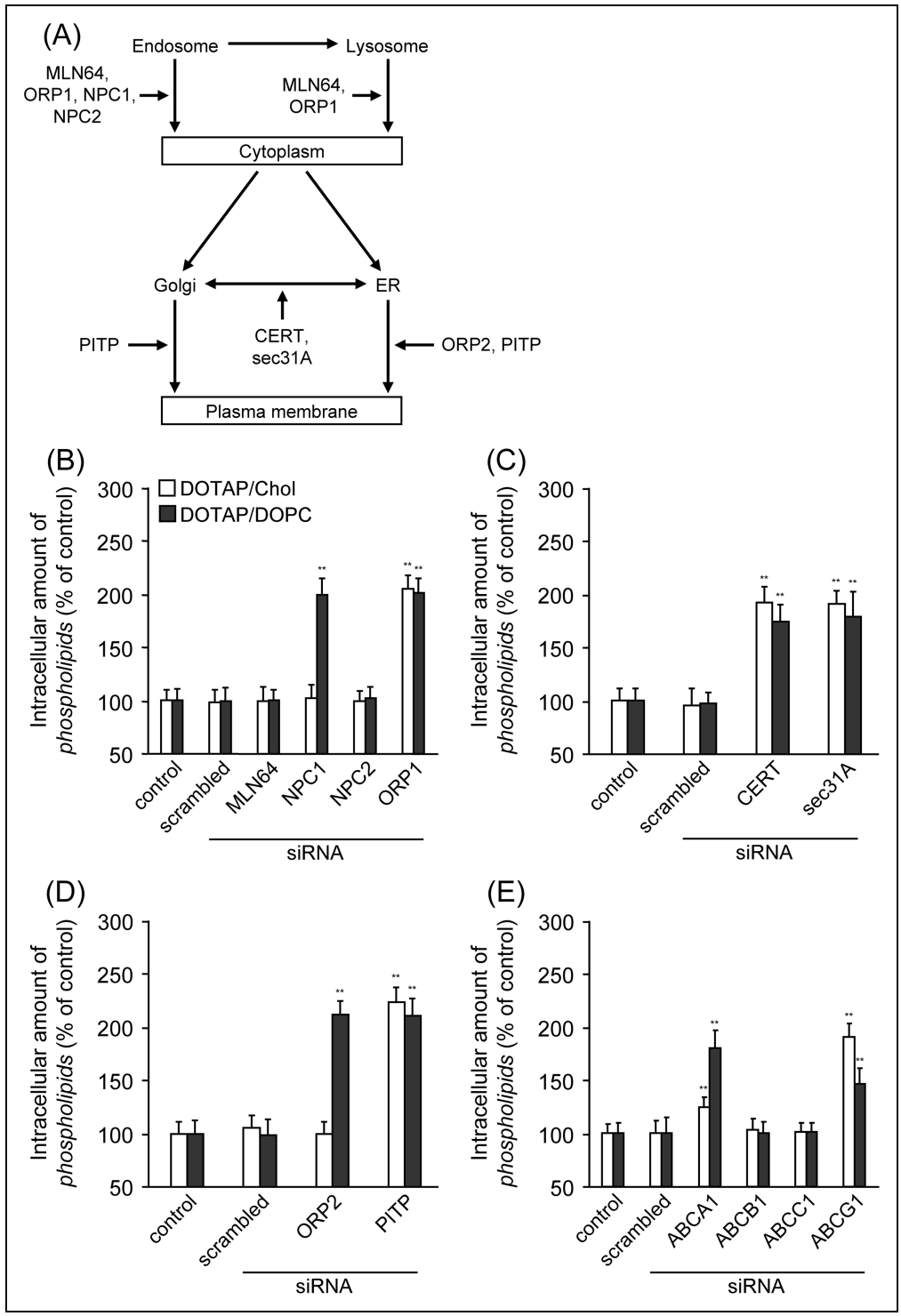

Fig. 3: Elucidation of intracellular DOTAP trafficking mechanism in HeLa cells. (A) Schematic of phospholipids trafficking-related intracellular proteins. Intracellular DOTAP under suppressing conditions for various proteins at 24 h after liposome addition to HeLa cells. Proteins involved in (B) intracellular trafficking from the endosome/lysosome to the cytoplasm (MLN64, NPC1, NPC2, and ORP1), (C) ER-to-Golgi transport (CERT and sec31A), (D) intracellular trafficking from ER/Golgi apparatus to cell membrane (ORP2 and PITP), and (E) extracellular efflux (ABCA1, ABCB1, ABCC1, and ABCG1). The siRNAs against targeted proteins transfected using Lipofectamine RNAiMAX according to recommended protocols; $^{**}p < 0.01$ compared with corresponding control group; and values are mean + S.D. ($n = 6$).

Sahay et al. (2013) reported that siRNA delivery efficiency using cationic nanoparticles is controlled by endocytotic recycling and that the design of cationic carriers that can escape the recycling pathways is important in improving siRNA delivery efficiency to the cytoplasm. As the endosome interior has a low pH, the endosomal escape of nucleic acids is essential for suppression of nucleic acid degradation and increased pharmaceutical activities in gene delivery (Tseng et al. 2009). Various types of endosomal escape mechanisms have been reported, and endosomal escape, based on disassembly, is partially induced by the association of lipids and endosomal components (Tseng et al. 2009). We have previously shown that DOTAP, DOPC, and Chol included in liposomes are transferred by ORP1 or NPC1 from the endosomes/lysosomes to the cytoplasm (Fig. 3B; Un et al. 2012). It is considered that the association of these liposomal lipids and endosomal proteins might induce interactions between liposomal cationic and endosomal membrane anionic lipids and thus trigger disassembly (Tseng et al. 2009). Consequently, the endosomal escape of nucleic acids associated with cationic liposomes might be enhanced. Therefore, the liposomes composed

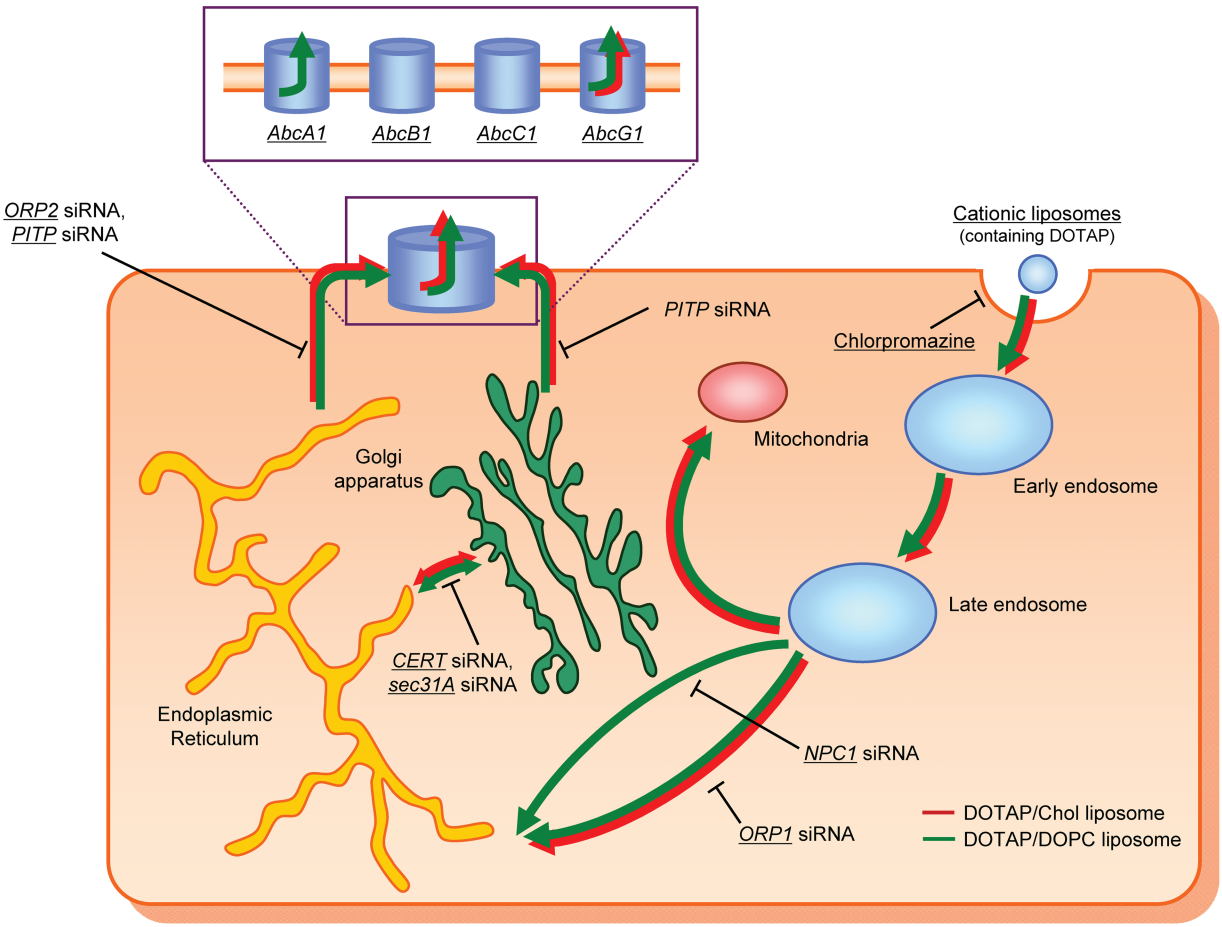

Fig. 4: Predicted mechanism of intracellular DOTAP trafficking derived from DOTAP/Chol and DOTAP/DOPC liposomes.

of DOTAP, DOPC, and Chol used in this study might be suitable for gene delivery applications in respect to endosomal escape. In conclusion, the trafficking processes of DOTAP from intracellular uptake to extracellular efflux were demonstrated using cationic liposomes *in vitro*. Following intracellular transport of liposomes *via* clathrin-mediated endocytosis, DOTAP included in DOTAP/Chol liposomes was localized in the ER, Golgi apparatus, and mitochondria in HeLa cells. Moreover, proteins involved in intracellular trafficking and extracellular efflux of DOTAP were identified. In addition, cationic liposome helper lipids were shown to partially affect cationic phospholipid intracellular trafficking.

4. Experimental

4.1. Materials and cells

Chol and DOPC were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). DOTAP and NBD-labeled DOTAP were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Dulbecco's-modified Eagle Medium (D-MEM), penicillin/streptomycin, phosphate buffer saline (PBS), and Opti-MEM I were purchased from Life Technologies, Inc. (Carlsbad, CA, USA), and fetal bovine serum (FBS) obtained from Nichirei Biosciences Inc. (Tokyo, Japan). All other chemicals were of the highest purity available. HeLa cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in D-MEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Cells were grown in a humidified incubator at 37 °C and under 5% CO_2.

4.2. Preparation of liposomes

Liposomes were prepared according to the Bangham method (Bangham 1965). Briefly, DOPC, DOTAP, and Chol were mixed in chloroform at predetermined ratio (Table 1), and the mixture dried by evaporation and vacuum desiccation. The resultant lipid film was resuspended in PBS (pH 7.4) under mechanical agitation. After hydration for 30 min at room temperature, the dispersion was sonicated for 10 min in a bath-type sonicator (Sharp Manufacturing Systems Corp., Osaka, Japan) and for 3 min in a tip-type sonicator (Sonics & Materials, Inc., Newtown, CT, USA). Then, the liposome solution was sized by repeated extrusion through 100 nm pore-sized polycarbonate membrane filters (Avestin Inc., Ottawa, Canada). Particle sizes, PDIs, and ζ-potentials of liposomes were determined using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK).

4.3. Evaluation of intracellular trafficking of liposomes

Intracellular concentrations of DOTAP taken up into the cells were evaluated by exposing cells to liposomes labeled with NBD-labeled DOTAP at a molar ratio of 5%. HeLa cells (2.5×10^4) were plated in 12-well microplate in D-MEM containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for 48 h at 37 °C and under 5% CO_2, the cells were exposed to liposomes (final conc. 50 μg total lipids/mL) in culture medium. After an additional 48 h incubation, the cells were washed three times in PBS and then trypsinized with 0.25% trypsin-ethylenediamine tetraacetic acid (Invitrogen Corp., Carlsbad CA, USA), washed with Hanks' Balanced Salt Solution (HBSS; Life Technologies, Inc.) three times, and suspended in lysis buffer (1% Triton X-100 in HBSS). The cell suspension was shaken, centrifuged at 15,000 × g and 4 °C for 10 min, and the fluorescence intensity of resultant supernatant measured in a fluorescence spectrophotometer (F-7000; Hitachi High-Technologies Corp., Ltd., Tokyo, Japan). The fluorescence intensity was normalized with respect to cellular protein content, as determined using a Protein Quantification Kit-Wide Range (Dojindo Laboratories, Inc., Kumamoto, Japan).

4.4. Endocytosis inhibitory experiments

Endocytosis was inhibited by chlorpromazine (50 μM) as a clathrin-mediated endocytosis inhibitor, genistein (200 μM), and methyl-β-cyclodextrin (2.0 mM) as caveolae-mediated endocytosis inhibitors, and 5-(N-ethyl-N-isopropyl) amiloride (50 μM) as a macropinocytosis inhibitor

Table 2: Sequences of siRNA

target gene	sense strand	antisense strand
MLN64	5′-GCUGA AGGAU UAAAC AAUGA CUUCA-3′	5′-UGAAG UCAUU GUUUA AUCCU UCAGC-3′
ORP1	5′-GCACC UCUGA GGAGU UGGAU GAAAU-3′	5′-AUUUC AUCCA ACUCC UCAGA GGUGC-3′
NPC1	5′-CCCUC GUCCU GGAUC GACGA UUAUU-3′	5′-AAUAA UCGUC GAUCC AGGAC GAGGG-3′
NPC2	5′-GACGU UCGCA AUGAC UGGGA AACAA-3′	5′-UUGUU UCCCA GUCAU UGCGA ACGUC-3′
CERT	5′-ACGUC AGAAG UUGGC UGAAA UGGAA-3′	5′-UUCCA UUUCA GCCAA CUUCU CACGU-3′
sec31A	5′-CCAGG CCAAU AAGCU GGGUC UCUAA-3′	5′-UUAGA CACCC AGCUU AUUGG CCUGG-3′
ORP2	5′-GAGAG GAGAG GUGAC CACCU GAGAA-3′	5′-UUCUC AGGUC GUCAC CUCUC CUCUC-3′
PITP	5′-GGAUA UUUAC AAACU UCCAU CGCCA-3′	5′-UGGCG AUGGA AGUUU GUAAA UAUCC-3′
ABCA1	5′-UUUAG AUGCU GGACA CUGCC AAGGC-3′	5′-GCCUU GGCAG UGUCC AGCAU CUAAA-3′
ABCB1	5′-UCCCG UAGAA ACCUU ACAUU UAUGG-3′	5′-CCAUA AAUGU AAGGU UUCUA CGGGA-3′
ABCC1	5′-CCGGU CUAUU CCCAU UUCAA CGAGA-3′	5′-UCUCG UUGAA AUGGG AAUAG ACCGG-3′
ABCG1	5′-UCUCG CUGAU GAAAG GGCUC GCUCA-3′	5′-UGAGC GAGCC CUUUC AUCAG CGAGA-3′

(Rejman 2005; Perez 2011). Each endocytosis inhibitor was added to culture medium 30 min before liposome addition.

4.5. Confocal microscopy study

For observation of colocalization of markers in endosomes and lysosomes, cells were washed with HBSS and labeled with AlexaFluor-546 conjugated transferrin and LysoTracker Red DND-99 (Life Technologies, Inc.) in accordance with the manufacturer's instructions, respectively. For labeling of ER, Golgi apparatus, and mitochondria, cells were labeled with ER-Tracker Red, BODIPY TR C_5-ceramide complexed to BSA, and Mito-Tracker Red FM (Life Technologies, Inc.) in accordance with the manufacturer's instructions. HeLa cells (5.0×10^4 cells) were plated in 35-mm glass-bottom dishes coated with poly-*L*-lysine (Matsunami Glass Ind., Ltd., Osaka, Japan) in D-MEM containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for 48 h at 37 °C and under 5% CO_2, the cells were exposed to liposomes (final conc. 5 μg total lipids/mL) in culture medium. At predetermined times after the addition of liposomes containing NBD-labeled DOTAP, the cells were washed and held in PBS for imaging by confocal microscopy (Carl Zeiss LSM 780, Carl Zeiss Microscopy GmbH, Jena, Germany). Data were collected using dedicated software supplied by the manufacturers and exported as tagged image files.

4.6. siRNA transfer

Stealth RNAi oligonucleotides (25-mers) were obtained from Life Technologies, Inc. and their sequences shown in Table 2, with Stealth RNAi High GC Negative Control Duplex used as a negative control. Stealth RNAi oligonucleotides were transfected into cells using Lipofectamine RNAiMAX (Life Technologies, Inc.) according to the manufacturer's protocols. At 48 h before liposome addition, each siRNA (final conc. 10 nM) was added to the cells and incubated for 6 h, after which the culture medium was replenished and the cells were incubated for a further 42 h.

4.7. Statistical analyses

Results were presented as the mean + S.D. of six experiments. Analysis of variance (ANOVA) was utilized to test for statistical significance of differences among groups. Two-group comparisons were performed by Student's *t*-test, and multiple comparisons between control and test groups were performed by Dunnett's test.

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