International Journal of Pharmaceutics 386 (2010) 122–130

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Intracellular fate of octaarginine-modified liposomes in polarized MDCK cells

Takahiro Fujiwara, Hidetaka Akita*, Hideyoshi Harashima

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

ARTICLE INFO

Article history:
Received 6 July 2009
Received in revised form 6 November 2009
Accepted 8 November 2009
Available online 13 November 2009

Keywords:
Golgi apparatus
Cationic peptide
Nanoparticles
Confocal microscopy

ABSTRACT

Octaarginine (R8)-modified liposomes have been used to deliver therapeutic substances into cells owing to the efficient cellular uptake via macropinocytosis. Recent analyses revealed that R8-modified liposomes are mainly taken up via macropinocytosis, and escape from endosomes efficiently to avoid lysosomal degradation in non-polarized NIH–3T3 cells. In the present study, we evaluated the intracellular fate of R8-modfied liposomes in polarized MDCK cells, comparing their trafficking with that of conventional cationic liposomes by confocal laser scanning microscopy (CLSM). In contrast to what occurs in NIH–3T3 cells, R8-modified liposomes are internalized by MDCK cells equally well via clathrin-mediated endocytosis and macropinocytosis. The most salient characteristic in subsequent intracellular trafficking in MDCK cells is that R8-modified liposomes become trapped in the endosomal compartment and subsequently, a portion of them colocalizes with the Golgi apparatus. Similar colocalization with the Golgi apparatus was observed for octalysine (K8)-modified liposomes. In contrast, cationic liposomes were found to colocalize predominantly with lysosomes stained with lysotracker. Collectively, in polarized MDCK cells, cationic peptide-modified liposomes may be subjected to a different sorting pathway from that used for liposomes composed of cationic lipids.

1. Introduction

Liposomes are promising devices for the delivery of encapsulated drugs and macromolecules to various organs and cells, protecting the molecular cargos from both clearance from the blood circulation and biological degradation (Weinstein and Leserman, 1984). For macromolecules such as oligonucleotides (Bennett et al., 1992; Capaccioli et al., 1993; Zelphati and Szoka, 1996; Lappalainen et al., 1997), plasmid DNA (pDNA) (Felgner et al., 1987; Tranchant et al., 2004) and short interfering RNA (siRNA) (Zhang et al., 2007), control of intracellular trafficking (i.e. cellular uptake, endosomal escape and nuclear delivery) is essential to maximize the functions of these nucleic acid cargos. Efforts to increase the cellular uptake of macromolecules include using liposomes composed of positively charged lipids, which bind to the plasma membrane efficiently (Stamatatos et al., 1988; Duzgunes et al., 1989; Leventis and Silvius, 1990; Wrobel and Collins, 1995), or liposomes modified with various ligands to promote targeting (Sapra and Allen, 2003). In particular, protein transduction domains (PTDs) are promising cellular uptake inducers which are applicable not only to liposomes (Torchilin et al., 2001; Khalil et al., 2006) but also to antibodies (Mie et al., 2003), proteins (Schwarze et al., 1999), and drugs (Rothbard et al., 2000; Kirschberg et al., 2003; Liang and Yang, 2005; Hayashi et al., 2007; Morishita et al., 2007). Well-known PTDs include HIV-1 (human immunodeficiency virus type-1) transactivator of transcription (TAT) (Kaplan et al., 2005) and penetratin (Terrone et al., 2003). Futaki and co-workers (Futaki et al., 2001; Suzuki et al., 2002; Khalil et al., 2004; Kosuge et al., 2008; Nakase et al., 2008) synthesized polypeptides composed solely of arginine, mimicking the sequences of PTDs, based on the concept that arginine residues are essential for efficient internalization of the TAT peptide. Optimization studies revealed octaarginine (R8) to be the most effective arginine oligopeptide for internalization (Futaki et al., 2001). The mechanism of cellular uptake of PTDs has been examined by using various cell types and conditions: PTDs or PTD-modified cargos are taken up via clathrin-mediated endocytosis (Khalil et al., 2006; Lundberg et al., 2003; Richard et al., 2003), caveolae-mediated endocytosis (Eguchi et al., 2001; Ferrari et al., 2003; Fittipaldi et al., 2003) and macropinocytosis (Kaplan et al., 2005; Nakase et al., 2004; Wadia et al., 2004). Our group reported that the surface density of R8 is one of the determinants for selection of the cellular uptake pathway: low-density R8-modified liposomes are taken up via clathrin-mediated endocytosis, whereas high-density R8-modified liposomes are taken up via macropinocytosis (Khalil et al., 2006). Moreover, it was revealed that the cellular uptake route greatly influences the liposome's subsequent intracellular fate. Liposomes taken up by macropinocytosis can avoid lysosomal degradation (Khalil et al., 2006, 2007), and are efficiently released from macropinosomes into the cytoplasm. In fact, pDNA encapsulated in high-density R8-modified liposomes

* Corresponding author. Tel.: +81 11 706 3735; fax: +81 11 706 4879.
E-mail address: akita@pharm.hokudai.ac.jp (H. Akita).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.11.005

exhibited high transfection activity, comparable to that of adenovirus (Khalil et al., 2006, 2007).

To date, intracellular trafficking of PTDs and PTD-modified cargos has been investigated mainly in non-polarized cells. Recently, insulin conjugated to or simultaneously administered with arginine-rich PTDs showed increased absorption in the intestine (Liang and Yang, 2005; Hayashi et al., 2007; Morishita et al., 2007). However, the mechanism of intracellular trafficking of PTDs in polarized epithelial cells remains to be clarified.

In the present study, we evaluated the intracellular trafficking of R8-modified liposomes in polarized MDCK cells in comparison to conventional cationic liposomes.

2. Materials and methods

2.1. Reagents

Egg phosphatidylcholine (EPC), cholesterol (Chol), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DOPE), and 1,2-dioleoyl-3-(trimetylammoium) propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). PKH67, phosphatidic acid (PA), cholesteryl hemisuccinate (CHEMS), amiloride, chlorpromazine, filipin, Triton X-100, Tween 20, bovine serum albumin (BSA) and Hoechst 33342 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lysotracker Green, Lipofectamine reagent, PLUS reagent, Mouse anti-ZO-1 antibody, and Alexa546-labeled Goat anti-mouse IgG antibody were purchased from Invitrogen (Groningen, The Netherlands). Mildform was purchased from Wako (Osaka, Japan).

2.2. Immunofluorescence staining

Confluent cells cultured on the base of a glass dish were fixed with Mildform for 15 min at room temperature, and then washed with PBS. Cells were treated with 0.25% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS, cells were incubated with 5% BSA and 0.1% Tween 20 in PBS and incubated with primary antibody overnight at 4 °C. Cells were then washed with 0.1% Tween 20 in PBS, and then incubated with the fluorescently labeled secondary antibody for 1 h at room temperature and stained with Hoechst 33342 for 5 min. Cells were washed with 0.1% Tween 20 in PBS and mounted in 90% glycerol in PBS. Samples were observed by using a fluorescence microscope, ECLIPSE TE2000-U (Nicon, Tokyo, Japan), and images were recorded with an EM-CCD C9100-13 camera system (HAMAMATSU Photonics, Hamamatsu, Japan).

2.3. Preparation of liposomes

Liposomes composed of DOTAP/DOPE (3/7), DOPE/PA (7/2), DOPE/CHEMS (9/2), and EPC/Chol (7/3) were prepared by a lipid hydration method. A lipid film was produced by evaporation of a chloroform solution of 135 nmol lipids. 10 mM Hepes solution (pH 7.4) was added and then incubated for 15 min to hydrate the lipid film. The glass tube was sonicated for approximately 1 min in a bath-type sonicator (Aiwa, Japan). For rhodamine labeling, 0.5% of rhodamine-DOPE was also incorporated into the lipid composition. For surface modification with R8 or K8, 10% (for DOPE/PA) or 5% (for other lipid compositions) of STR-R8 or STR-K8 was incorporated into the lipid composition.

2.4. Cell culture and transfection

The dog kidney epithelial cell line MDCK was obtained from Health Science Research Resources Bank (Osaka, Japan). Cells were maintained at 37 °C in Eagle's minimal essential medium with 10% fetal bovine serum and antibiotics under an atmosphere of 5% CO_2/95% air.

pAcGFP1-Golgi was purchased from Clontech (CA, USA). The Golgi apparatus was stained with AcGFP1-Golgi after transfection by using Lipofectamine PLUS following the manufacturer's instructions.

2.5. Flow cytometry

To investigate contributions of macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis, cellular uptake of liposomes was quantified by FACS with various inhibitors. Cells were pre-incubated in serum-free medium containing amiloride (final concentration, 2.5 mM) for 30 min, chlorpromazine (final concentration, 5 μg/mL) for 1 h, or filipin (final concentration, 3 μg/mL) for 1 h to block macropinocytosis, clathrin-mediated endocytosis or caveolae-mediated endocytosis, respectively. After pre-incubation, cells were incubated with rhodamine-labeled liposomes for 1 h in serum-free medium at a final concentration of 27.5 nmol lipids/mL. Then, the medium was removed, and cells were washed once with ice-cold PBS solution containing 0.1% sodium azide and 0.5% BSA, and with ice-cold PBS with heparin (40 units/mL) three times. No significant toxic effects were observed based on the morphology of the cells. The cells were then trypsinized and collected by centrifugation at 3000 rpm at 4 °C for 5 min. Cells were washed in ice-cold PBS two times by repeating the centrifugation and suspension steps. Finally, cells were suspended in 500 μL of ice-cold PBS solution containing 0.1% sodium azide and 0.5% BSA. The cell suspension was filtered through a nylon mesh (45 μm mesh size) to remove cell aggregates, and then analyzed by flow cytometry (BD Biosciences).

2.6. Confocal laser scanning microscopy

To investigate the intracellular trafficking of liposomes, cells were cultured on the base of a glass dish until confluence. For evaluation of endosomal escape, the plasma membrane was stained with PKH67, a green fluorescent cell linker following the manufacturer's protocol with some modifications. After washing, liposomes labeled with rhodamine were added to 1 mL of cell culture medium, followed by incubation for 6 h at 37 °C. The cells were washed with 1 mL of PBS solution containing heparin (40 units/mL) three times, and then the cell culture medium was replaced with 1 mL of Krebs–Ringer buffer (KCl 4.8 mM, KH_2PO_4 1 mM, $MgSO_4$ 1.2 mM, Hepes 12.5 mM, $CaCl_2$ 1.5 mM, NaCl 120 mM, $NaHCO_3$ 23.8 mM, glucose 5 mM, pH 7.3). Images were captured by confocal laser scanning microscopy using a Zeiss Axiovert 200 inverted fluorescence microscope with a 63× NA 1.4 planachromat objective lens (Carl Zeiss Co. Ltd., Jene, Germany).

To investigate the localization of liposomes in lysosomes, confluent cells were incubated with liposomes labeled with rhodamine, and then 6 h later the lysosome fraction was stained with Lysotracker Green following the manufacturer's instructions.

To investigate the localization of liposomes to the Golgi apparatus, cells transfected with pAcGFP1-Golgi were cultured on the base of glass dishes until confluence. Cells were washed with 1 mL of PBS for three times, and then incubated with 27.5 nmol total lipids of liposomes for 6 h at 37 °C. Then, the medium was removed and liposomes binding on the cellular surface were washed out in three changes of heparin solution (40 units/mL). Finally, the medium was replaced with Krebs–Ringer buffer and the cells were analyzed by confocal laser scanning microscopy.

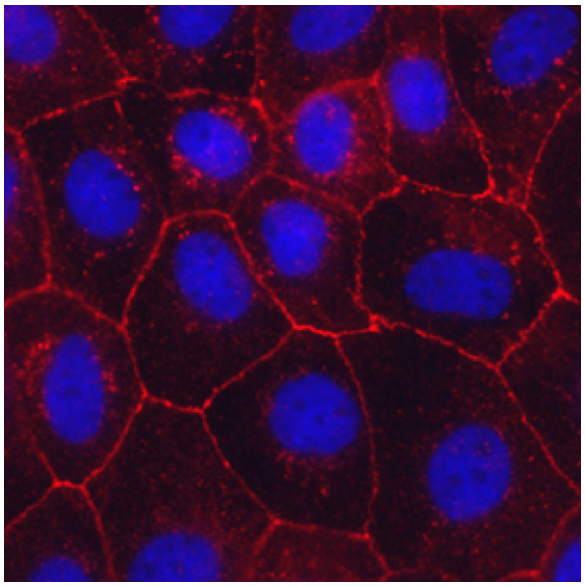

Fig. 1. Immunostaining of ZO-1, a tight-junction marker. MDCK cells were cultured until confluent, fixed and immunostained. Fluorescence images were captured by fluorescent microscopy. Blue and red signals represent the nucleus and ZO-1, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

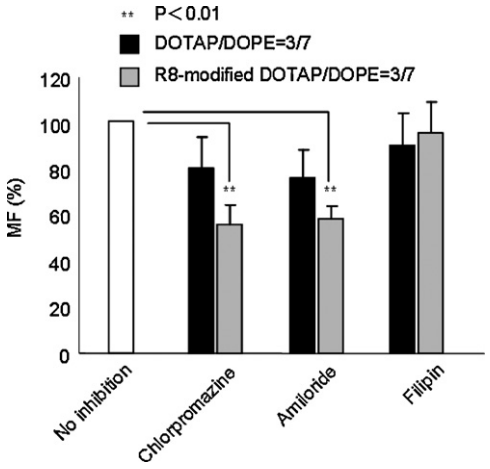

Fig. 2. Cellular uptake of liposome in the presence of inhibitors in MDCK cells. Mechanism of cellular uptake is different between types of cationic liposomes. MDCK cells were incubated for 1 h with liposomes composed of DOTAP/DOPE = 3/7 modified with R8 or unmodified in the absence or presence of the clathrin-mediated endocytosis inhibitor chlorpromazine (5 μg/mL), the macropinocytosis inhibitor amiloride (2.5 mM), or the caveolae-mediated endocytosis inhibitor filipin (3 μg/mL). The mean fluorescence (MF) of 10,000 cells was measured by flow cytometry and is expressed as the percentage of fluorescence measured in the absence of inhibitor. Error bars represent ± S.D. for three different experiments performed in triplicate.

3. Results

3.1. ZO-1 localization in MDCK cells

To obtain evidence that cultured MDCK cells form a monolayer with tight junctions, cells were grown on the base of a glass dish until confluent and then stained for the tight-junction marker ZO-1. ZO-1 was clearly concentrated at tight junctions and a monolayer was evident (Fig. 1).

3.2. Preparation and characterization of liposomes

The size and ζ-potentials of liposomes used in the present study were measured by dynamic light scattering (Table 1). In the present study, we focused on positively charged liposomes (>30 mV), which are desirable for cell-surface binding and internalization. In addition, the size of the liposomes ranged from 100 nm to 300 nm, sufficiently small for cellular uptake via endocytosis. As reported previously, nanoparticles with a size of >500 nm are mainly taken up via caveolin whereas those less than 100 nm are taken up via clathrin pathway (Rejman et al., 2004). However, the size of liposomes described in the present study is a mean value, and a certain degree of dispersion in size (±200 nm) was observed. Therefore, we consider that 100 nm of difference in mean size has a minimal effect on the overall trends in cellular uptake and subsequent intracellular trafficking. In liposomes composed of DOPE/PA = 7/2, R8 was added at a density of 10% because 10% R8 is necessary to convert the ζ-potential from negative (−40 mV) to positive (+50 mV). A 5% R8 concentration was used in all other liposomes.

3.3. Investigation of the cellular uptake pathway

First, cellular uptake of liposomes was quantified in polarized MDCK cells by FACS analysis. R8-modified liposomes and conventional cationic liposomes (DOTAP/DOPE = 3/7) were incubated with cells pre-treated with an inhibitor of clathrin-mediated endocytosis (chlorpromazine), caveolae-mediated endocytosis (filipin), or macropinocytosis (amiloride). Uptake of R8-modified liposomes was inhibited by chlorpromazine and amiloride by approximately 40% ($p < 0.01$, t-test) (Fig. 2), indicating that R8-modified liposomes are taken up by MDCK cells via both clathrin-mediated endocytosis and macropinocytosis. In contrast, cellular uptake of liposomes composed of DOTAP/DOPE = 3/7 was not inhibited by any of the inhibitors (Fig. 2). It is postulated that liposomes composed of DOTAP/DOPE = 3/7 were taken up by cells via a pathway different from that used to internalize the R8-modified liposome (mechanism unknown).

3.4. Evaluation of endosomal escape

As shown in Fig. 3, significant amounts of liposomes were taken up by the cells after 6 h of incubation. However, only small numbers of liposomes were detected in cross-section passing through the center of the cells until 3 h incubation. Furthermore, the number of liposomes in the cells decreased prominently after 12 h compared with 6 h (data not shown).

Previous work has demonstrated that R8-modified liposomes or liposomes composed of fusogenic lipids can escape from endosomes in non-polarized cells (e.g. NIH–3T3 or HeLa cells). To confirm endosomal escape of liposomes in polarized MDCK cells,

Table 1
Characteristics of all liposomes used in this study.

Lipid composition	Modification 0%	R8-modified 5%	R8-modified 5%	R8-modified 10%	R8-modified 5%	K8-modified 5%
	DOTAP/DOPE = 3/7		DOPE/CHEMS = 7/2	DOPE/PA = 7/2	EPC/Chol = 7/3	
Diameter (nm)	169 ± 54.1	164 ± 55.9	275 ± 30.6	279 ± 66.7	112 ± 34.5	114 ± 28.3
ζ-Potential (mV)	50.2 ± 16.3	56.4 ± 0.495	53.4 ± 10.7	59.8 ± 10.2	40.8 ± 7.58	30.1 ± 9.16

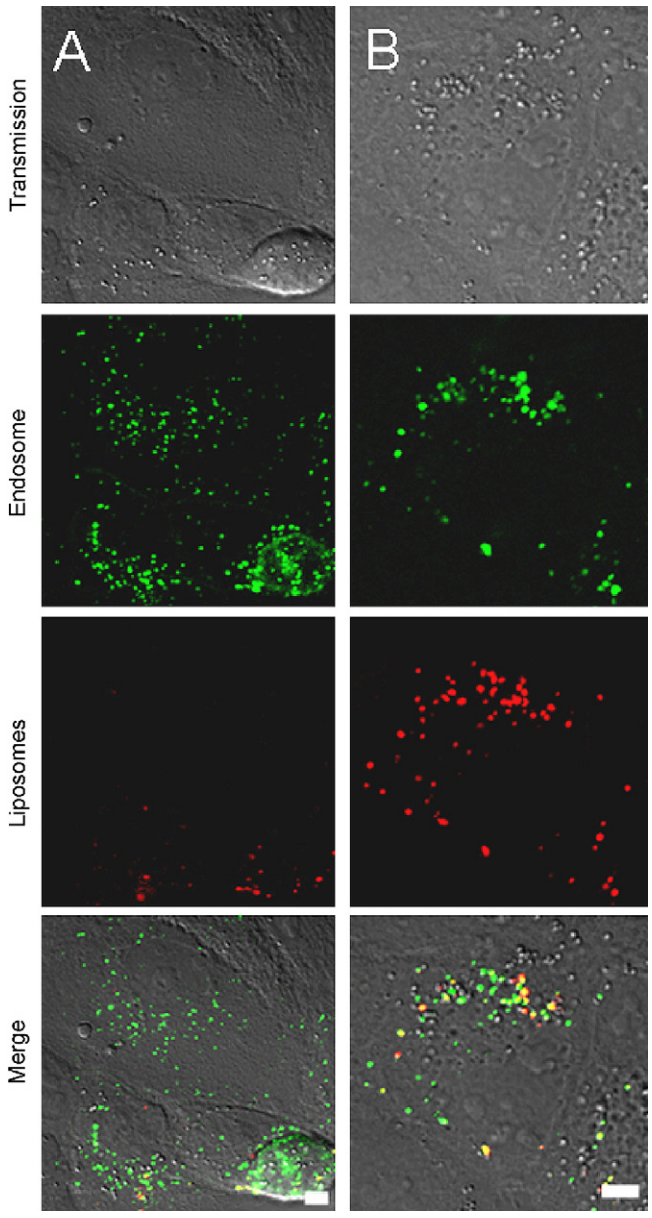

Fig. 3. Endosomal colocalization cationic liposomes to endosomes. MDCK cells were incubated with non-ligand-modified DOTAP/DOPE = 3/7 liposomes (A) and R8-modified ones (B) after staining the plasma membrane with PKH67. Fluorescence images were captured by confocal laser scanning microscopy. Green represents intracellular PKH67 signals (endosome). Bars = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

colocalization of liposomes and plasma membrane labeled with PKH67 was investigated.

Positively charged liposomes composed of DOTAP/DOPE = 3/7 (Fig. 3A) or R8-modified ones (Fig. 3B) were added to PKH67-labeled cells. At 6 h of transduction, PKH67 was detected in a punctuate pattern indicative of endocytosis. Although the liposomes were composed of fusogenic lipid (DOPE), most of the liposomes colocalized with the punctuate PKH67 signals (Fig. 3). Therefore, a major portion of the liposome population became trapped in the membrane compartment (endosome) after internalization. Because efficient endosomal escape of R8-modified liposomes was observed in NIH–3T3 cells when assessed by the same method (data not shown), inefficient endosomal escape is a specific phenomenon in MDCK cells.

3.5. Intracellular trafficking of liposomes

Generally, liposomes or macromolecules which fail to escape from endosomes are subsequently subjected to lysosomal degradation or recycled back to the extracellular space. However, we observed that a significant fraction of R8-modified liposomes remained associated with membrane compartments separate from the lysosomal compartment stained with lysotracker (Figs. 3B and 4B), whereas ligand-unmodified liposomes almost always colocalized with lysosomes (Fig. 4A). Recently, it was reported that cholera toxin and Shiga toxin are retrogradely transported from endosomes to the Golgi apparatus via vesicular transport (Bonifacino and Rojas, 2006). Therefore, we examined whether liposomes are transported to the Golgi apparatus (Fig. 5). In the case of ligand-unmodified cationic DOTAP/DOPE liposomes,

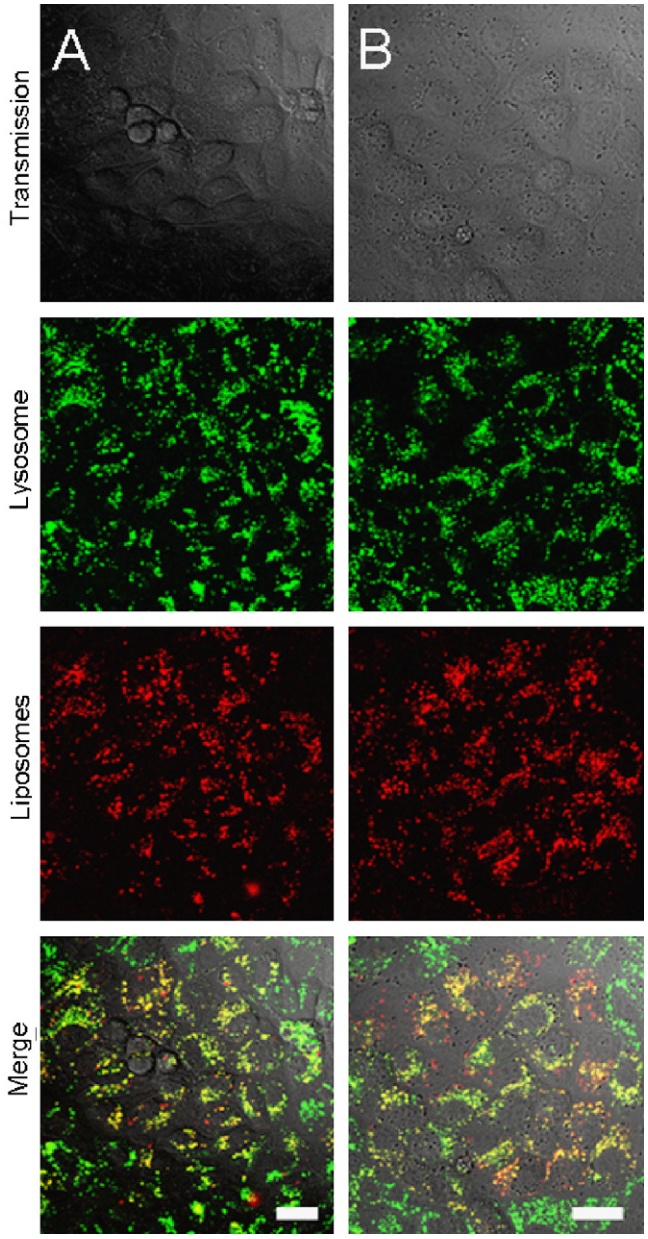

Fig. 4. Localization of liposomes and lysosomes. MDCK cells were incubated with non-ligand modified DOTAP/DOPE = 3/7 liposomes (A) or R8-modified liposomes (B) and cells were stained with lysotracker just before observation. Fluorescence images were captured by means of confocal laser scanning microscopy. Bars = 20 μm.

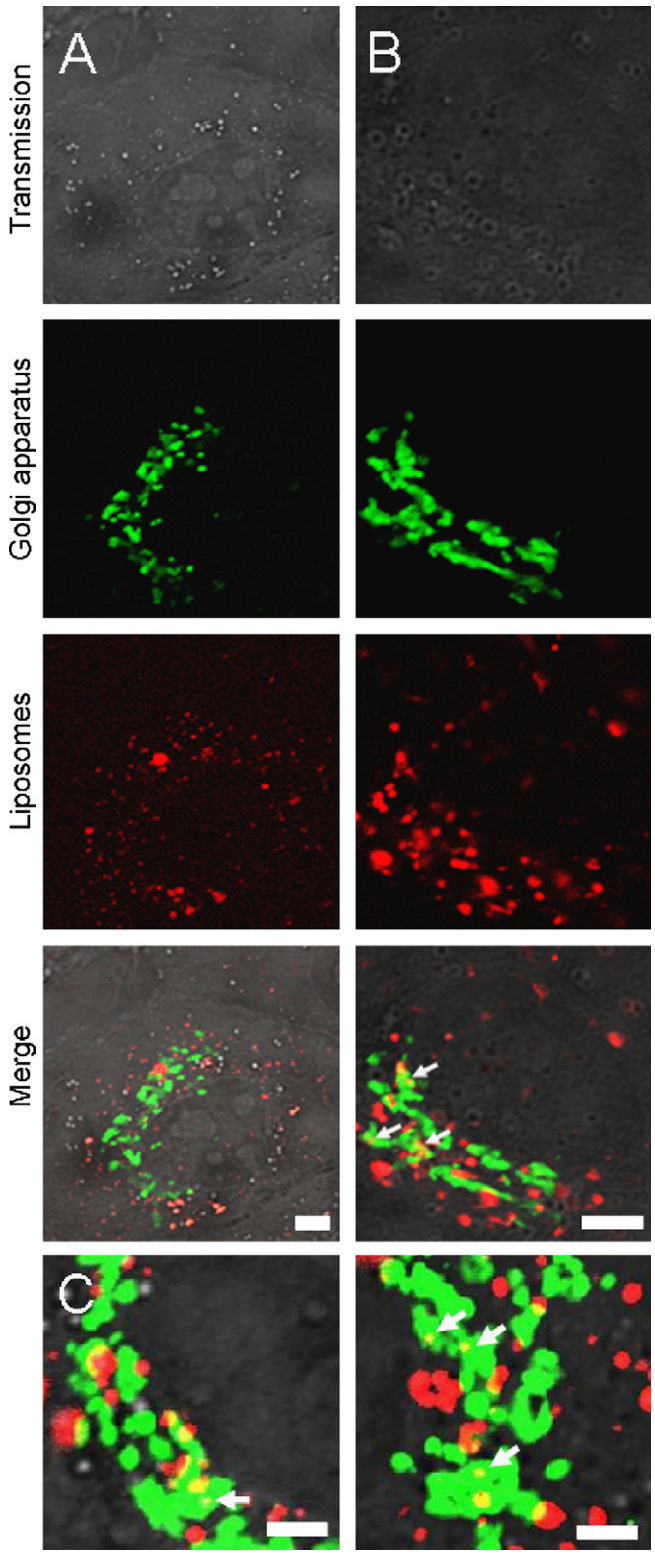

Fig. 5. Localization of a portion of R8-modified liposomes to the Golgi apparatus. MDCK cells, expressing GFP only in the Golgi apparatus were incubated with non-ligand-modified DOTAP/DOPE = 3/7 liposomes (A) or R8-modified liposomes (B). Panel C shows magnified views of the Golgi apparatus when R8-modified liposomes were internalized. Colocalization of liposomes and the Golgi apparatus is indicated by arrowheads. Fluorescence images were captured by means of confocal laser scanning microscopy. Bars = 5 μm.

they did not colocalize with the Golgi apparatus (Fig. 5A), which is consistent with their extensive colocalization with lysosomes stained with lysotracker (Fig. 4A). In contrast, some colocalization with the Golgi apparatus was observed for liposomes modified with R8 (arrowheads in Fig. 5B and C). Hence it is likely that a certain fraction of R8-modified liposomes was transported to the Golgi apparatus via a retrograde pathway after internalization.

3.6. Effect of lipid composition on intracellular fate

To examine whether lipid composition affects intracellular trafficking, R8 was added to the surface of liposomes composed of various kinds of lipids. Previous reports showed that lipid composition is an important determinant of endosomal escape. For example, we demonstrated that liposomes composed of DOPE/CHEMS = 9/2 and DOPE/PA = 7/2 (optimized for endosomal fusion) can efficiently escape from endosomes in NIH–3T3 cells via a pH-dependent fusogenic activity (Khalil et al., 2007; Farhood et al., 1995; Hafez and Cullis, 2000; El-Sayed et al., 2008; Akita et al., 2009).

As expected, non-fusogenic liposomes composed of EPC/Chol became trapped in the endosomal compartment of MDCK cells (Fig. 6B). However, endosomal escape was poor even in liposomes composed of DOPE/CHEMS = 9/2 (Fig. 6A). Similarly, liposomes composed of DOPE/PA = 7/2 showed low endosomal escape (Fig. 6C), whereas the escape activities of these liposomes were high in non-polarized cells (NIH–3T3 (El-Sayed et al., 2008), JAWS II (Akita et al., 2009), and HeLa cells; data not shown). Under the same conditions, a portion of R8-modified liposomes composed of EPC/Chol = 7/3 and DOPE/PA = 7/2 colocalized with the Golgi apparatus (Fig. 6E and F), whereas ones prepared with DOPE/CHEMS = 9/2 did not colocalize (Fig. 6D). These data suggest that lipid composition is one of the determining factors for intracellular transport of cationic peptide-modified liposomes.

3.7. Comparison of intracellular fate between R8- and K8-modified liposomes

To investigate whether specific transport in polarized cells of R8-modified liposomes is limited to arginine, liposomes were modified with another cationic peptide, K8. Liposomes modified with K8 showed almost the same behavior as R8-modified ones. Uptake of liposomes was inhibited by chlorpromazine and amiloride by 40% (Fig. 7). Furthermore, after internalization, neither liposomes escaped from the endosome (Fig. 8A), but both partially colocalized with the Golgi apparatus (Figs. 6E and 8B).

4. Discussion

In the present study, we analyzed the intracellular trafficking of cationic liposomes and its relationship to cell polarization in MDCK cells. Cationic liposomes containing DOTAP are commonly used for the delivery of pDNA or siRNA (Cardoso et al., 2007; Hatakeyama et al., 2007). These cationic liposomes can efficiently enter cells due to their high cellular binding activity. However, for liposomes to be effective carriers of therapeutic compounds, their degradation in the lysosome must be avoided. One promising strategy is to use fusogenic lipid (e.g. DOPE), which has a corn-type structure. These liposomes form hexagonal-II structures, which cause lipid-mixing between the endosomal membrane and the cationic liposome. As a result, encapsulated macromolecules are released to the cytosol. Another strategy is to exploit a cellular uptake pathway. R8-modified liposomes are taken up into cells via macropinocytosis, which circumvents lysosomal degradation and permits the liposomes to escape efficiently from the endosome to the cytosol (Khalil et al., 2006). It has been reported that surface modification

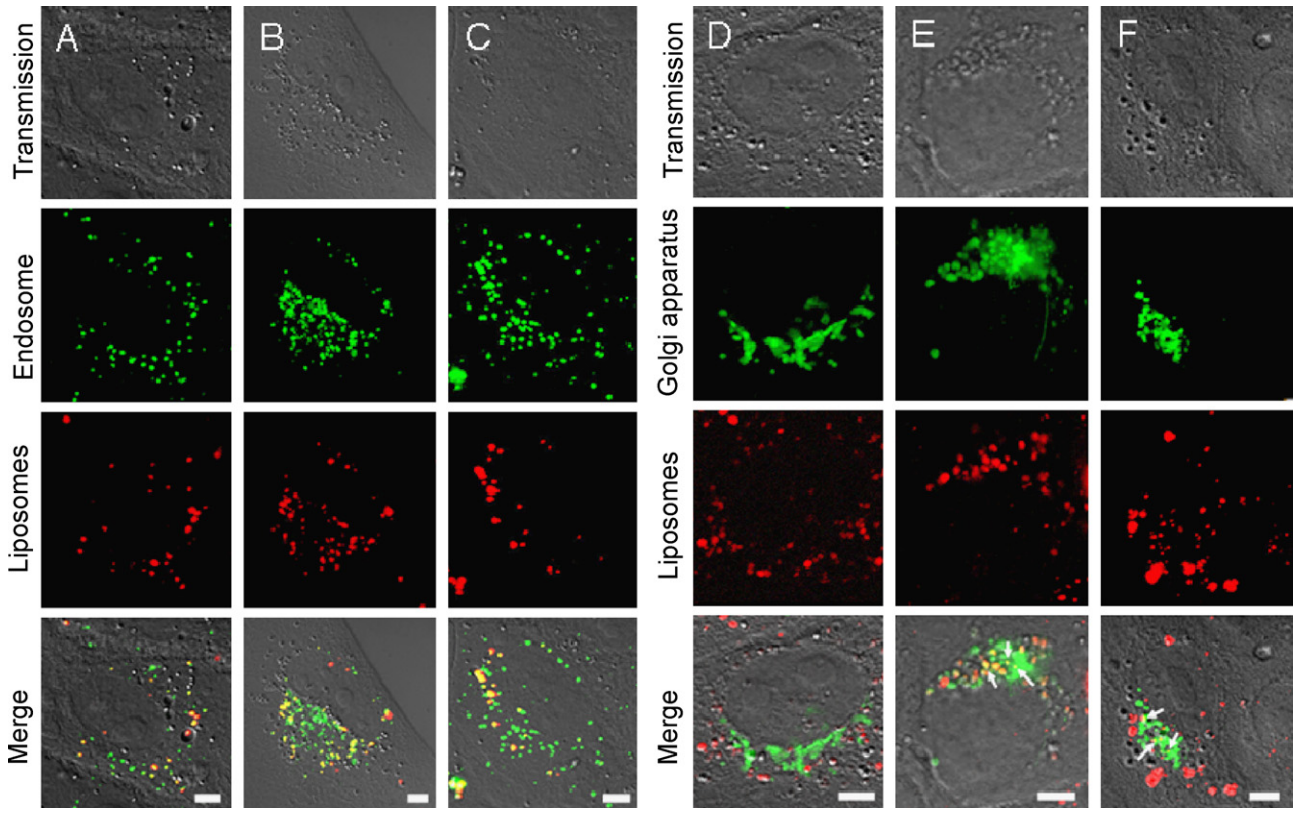

Fig. 6. Effect of lipid composition on intracellular fate of liposomes in MDCK cells. MDCK cells whose plasma membrane was stained (A–C) or which expressed GFP in only the Golgi apparatus (D–F) were incubated with R8-modified liposomes. (A) and (D) show intracellular dynamics of liposomes composed of DOPE/CHEMS = 9/2. (B) and (E) show transport dynamics of liposomes composed of EPC/Chol = 7/3. (C) and (F) show transport dynamics of liposomes composed of DOPE/PA = 7/2. Fluorescence images were captured by means of confocal laser scanning microscopy. Bars = 5 μm.

by R8 confers a high transfection activity of encapsulated pDNA through its high endosomal escape activity (Khalil et al., 2006; Kaplan et al., 2005; Zhang et al., 2006).

In the present study, the trafficking observed in polarized cells was strikingly different from that in non-polarized cells. The most prominent difference was the poor endosomal escape efficiency in polarized cells regardless of the liposome's lipid composition. In NIH/3T3 cells, liposomes taken up via clathrin-mediated endocytosis remained in endosomal compartments, and were degraded in the lysosome (Khalil et al., 2006). Unexpectedly, a major portion of the R8-liposomes was trapped in the endosomes at 6 h of incubation. Therefore, it is possible that the transfection activity of liposomes in MDCK cells may be low, at least for short incubation times.

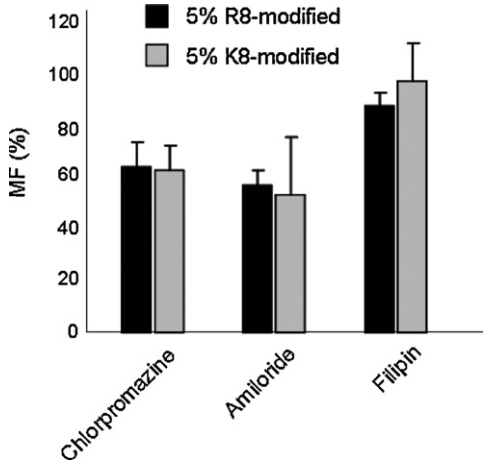

Fig. 7. Cellular uptake of R8- and K8-modified liposomes in MDCK cells in the presence of inhibitors. Mechanism of cellular uptake is different among different types of cationic liposomes. MDCK cells were incubated for 1 h with liposomes composed of EPC/Chol = 7/3 modified with R8 or K8 in the absence or presence of the clathrin-mediated endocytosis inhibitor chlorpromazine (5 μg/mL), the macropinocytosis inhibitor amiloride (2.5 mM) or the caveolae-mediated endocytosis inhibitor filipin (3 μg/mL). The mean fluorescence (MF) of 10,000 cells was measured by flow cytometry and is expressed as the percentage of the fluorescence measured in the absence of inhibitor. Error bars represent ± S.D. for three different experiments performed in triplicate.

In recent reports, exogenous toxins (i.e. Shiga toxin and cholera toxin) were shown to be transported by retrograde transport, which is specific transportation from endosomes to the Golgi apparatus without endosomal escape (Bonifacino and Rojas, 2006; Tarrago-Trani and Storrie, 2007). The cellular uptake pathway and/or membrane-associated proteins involved in sorting in the retrograde pathway have recently been investigated (Lauvrak et al., 2004; Saint-Pol et al., 2004; Derby et al., 2007). However, the molecular mechanism remains to be clarified. In the present study, a portion of liposomes modified with R8 or K8 was detected at the Golgi apparatus (Figs. 5B and 8B) without escaping from the endosome (Figs. 3B and 8A). These results indicate that some liposomes may be subjected to retrograde transport. Because conventional liposomes composed of cationic lipids (DOTAP) did not colocalize with the Golgi apparatus at all (Fig. 5A), but colocalized with lysosomes (Fig. 4A), the surface display of sequential cationic peptides was presumably recognized as a sorting signal for retrograde transport. In fact, a portion of DOTAP-based liposomes surface-modified with R8 localized to the Golgi apparatus (Figs. 3B and 5B).

It was previously demonstrated that the type of cellular uptake pathway can strongly influence subsequent intracellular trafficking events (Khalil et al., 2006). As shown in Fig. 7, R8- or K8-modified liposomes were internalized via both clathrin-mediated

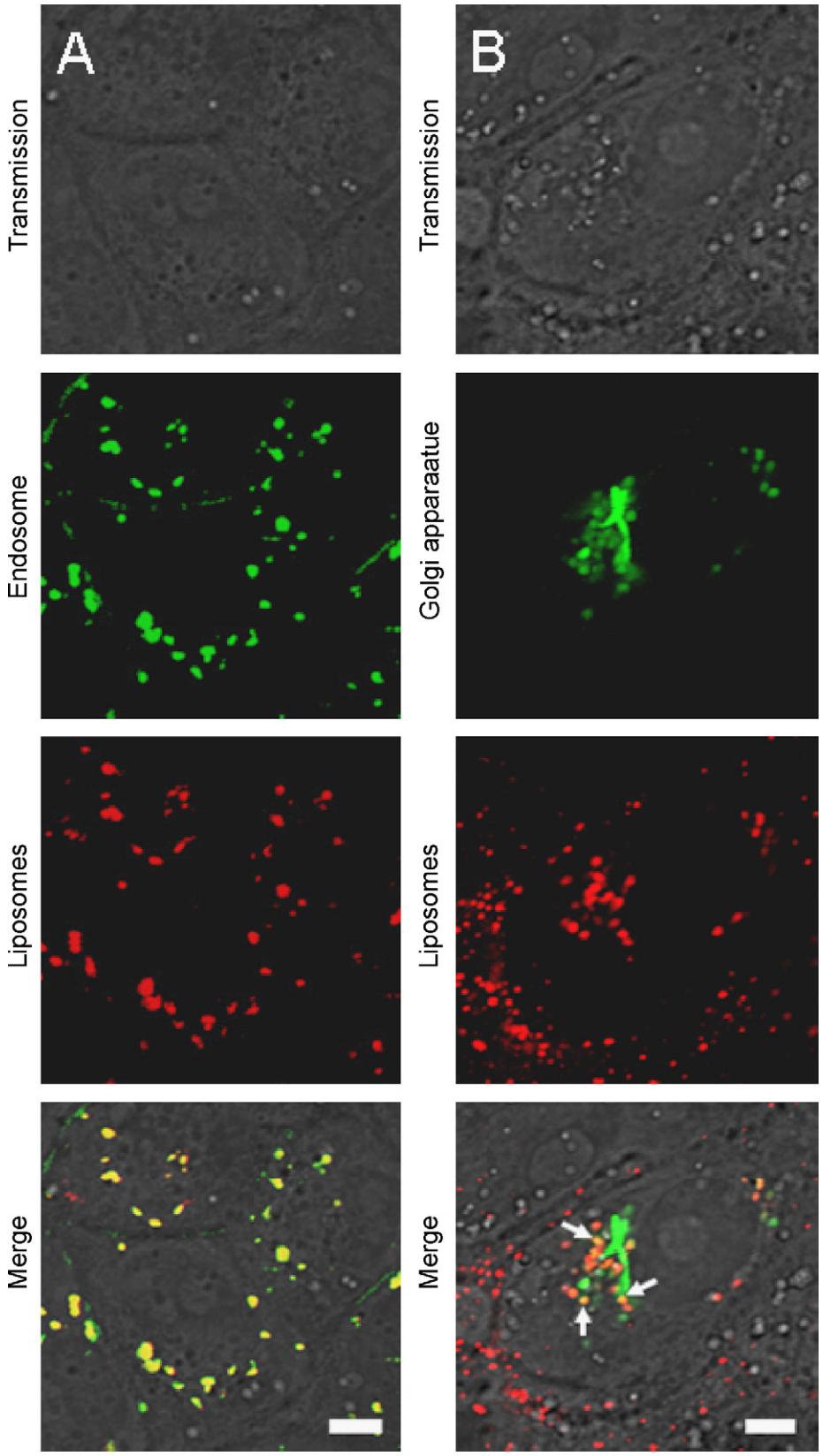

Fig. 8. Intracellular trafficking of R8- and K8-modified liposomes in MDCK cells. MDCK cells whose plasma membranes were stained (A) or that expressed GFP only in the Golgi apparatus (B) were incubated with K8-modified EPC/Chol = 7/3 liposomes. Fluorescence images were captured by confocal laser scanning microscopy. Bars = 5 μm.

endocytosis and macropinocytosis, suggesting that either or both of these pathways may be responsible for sorting to the Golgi apparatus. In non-polarized NIH–3T3 cells, it was reported that R8- or K8-modified liposomes are predominantly taken up via macropinocytosis (Khalil et al., 2006; El-Sayed et al., 2008), and endosomal escape of R8-modified liposomes was higher than that of K8-modified liposomes whereas both liposomes remained in endosomes in MDCK cells. These differences may be explained by differences in the binding mechanism of cationic peptides to non-polarized cells (NIH–3T3) versus polarized (MDCK) cells. It is known that TAT, one of the PTDs, is internalized via macropinocytosis in non-polarized cells (Kaplan et al., 2005) through its interaction with heparin sulfate proteoglycan (HSPG) (Tyagi et al., 2001) or lipid rafts on the surface of the cell membrane (Wadia et al., 2004). Similarly, it was demonstrated that internalization of R8 via macropinocytosis is highly dependent on binding with proteoglycan (Fuchs and Raines, 2004; Payne et al., 2007). In contrast, in polarized MDCK cells, cellular expression of HSPG on the apical membrane is low due to its low level of biosynthesis (Tveit et al., 2005). Additionally, it was shown that TAT does not mediate entry into epithelial cells via proteoglycan (Violini et al., 2002). Therefore, it is possible that another classical pathway (i.e. clathrin-mediated endocytosis) may contribute to cellular uptake in parallel with macropinocytosis. Unexpectedly, the uptake of ligand-unmodified liposome was not inhibited by treatment with chlorpromazine, amiloride, or filipin. Therefore, such liposomes may be taken up by an unknown pathway. A recent report showed that cellular uptake of Lipofectamine (LFN), a lipoplex formed with pDNA and cationic liposomes was not inhibited by chlorpromazine and filipin in polarized (BS-C-1) cells, but that Lipofectamine localizes in vesicles containing flotillin-1 (Payne et al., 2007). Therefore, ligand-unmodified cationic liposome may also be taken up via a non-classical pathway.

Finally, we obtained evidence that indicates that lipid composition also affects intracellular trafficking in polarized cells. In the present study, liposomes composed of DOPE/CHEMS = 9/2 did not colocalize with the Golgi apparatus. Because both DOPE and CHEMS are artificial lipids, they are recognized as xenobiotics and subject to sorting to the lysosomal compartment.

Collectively, our results indicate that liposomes modified with peptides composed of contiguous cationic amino acids (R8 or K8) are taken up into polarized cells via clathrin-mediated endocytosis and macropinocytosis, and partially transported to the Golgi apparatus, whereas conventional cationic liposomes (DOTAP/DOPE) are taken up by an unknown pathway and predominantly transported to the lysosome. Although the molecular mechanism remains to be elucidated, these cationic peptides-modified liposomes are subject to a different sorting mechanism from those of liposomes prepared with cationic lipid composition.

Acknowledgments

This work was supported in part by Grants-in-Aid for Young Scientists (A) and by Grants-in-Aid for Scientific Research on Priority Areas from the Japan Society for the Promotion of Science. We thank Dr. Shiroh Futaki (Kyoto University, Japan) for providing STR-R8 and STR-K8. The authors thank Dr. Daryl Henderson for his helpful advice in writing the English manuscript.

References

Akita, H., Kudo, A., Minoura, A., Yamaguti, M., Khalil, I.A., Moriguchi, R., Masuda, T., Danev, R., Nagayama, K., Kogure, K., Harashima, H., 2009. Multi-layered nanoparticles for penetrating the endosome and nuclear membrane via a step-wise membrane fusion process. Biomaterials.

Bennett, C.F., Chiang, M.Y., Chan, H., Shoemaker, J.E., Mirabelli, C.K., 1992. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. Mol. Pharmacol. 41, 1023–1033.

Bonifacino, J.S., Rojas, R., 2006. Retrograde transport from endosomes to the trans-Golgi network. Nat. Rev. Mol. Cell Biol. 7, 568–579.

Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T., Quattrone, A., 1993. Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. Biochem. Biophys. Res. Commun. 197, 818–825.

Cardoso, A.L., Simoes, S., de Almeida, L.P., Pelisek, J., Culmsee, C., Wagner, E., Pedroso de Lima, M.C., 2007. siRNA delivery by a transferrin-associated lipid-based vector: a non-viral strategy to mediate gene silencing. J. Gene Med. 9, 170–183.

Derby, M.C., Lieu, Z.Z., Brown, D., Stow, J.L., Goud, B., Gleeson, P.A., 2007. The trans-Golgi network golgin, GCC185, is required for endosome-to-Golgi transport and maintenance of Golgi structure. Traffic 8, 758–773.

Duzgunes, N., Goldstein, J.A., Friend, D.S., Felgner, P.L., 1989. Fusion of liposomes containing a novel cationic lipid, N-[2,3-(dioleyloxy)propyl]-N,N,N-trimethylammonium: induction by multivalent anions and asymmetric fusion with acidic phospholipid vesicles. Biochemistry 28, 9179–9184.

Eguchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M., Nakanishi, M., 2001. Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells. J. Biol. Chem. 276, 26204–26210.

El-Sayed, A., Khalil, I.A., Kogure, K., Futaki, S., Harashima, H., 2008. Octaarginine- and octalysine-modified nanoparticles have different modes of endosomal escape. J. Biol. Chem. 283, 23450–23461.

Farhood, H., Serbina, N., Huang, L., 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta 1235, 289–295.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84, 7413–7417.

Ferrari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M., Beltram, F., 2003. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Mol. Ther. 8, 284–294.

Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F., Giacca, M., 2003. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. J. Biol. Chem. 278, 34141–34149.

Fuchs, S.M., Raines, R.T., 2004. Pathway for polyarginine entry into mammalian cells. Biochemistry 43, 2438–2444.

Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., Sugiura, Y., 2001. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J. Biol. Chem. 276, 5836–5840.

Hafez, I.M., Cullis, P.R., 2000. Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. Biochim. Biophys. Acta 1463, 107–114.

Hatakeyama, H., Akita, H., Kogure, K., Oishi, M., Nagasaki, Y., Kihira, Y., Ueno, M., Kobayashi, H., Kikuchi, H., Harashima, H., 2007. Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. Gene Ther. 14, 68–77.

Hayashi, Y., Takayama, K., Suehisa, Y., Fujita, T., Nguyen, J.T., Futaki, S., Yamamoto, A., Kiso, Y., 2007. Development of oligoarginine–drug conjugates linked to new peptidic self-cleavable spacers toward effective intestinal absorption. Bioorg. Med. Chem. Lett. 17, 5129–5132.

Kaplan, I.M., Wadia, J.S., Dowdy, S.F., 2005. Cationic TAT peptide transduction domain enters cells by macropinocytosis. J. Control. Release 102, 247–253.

Khalil, I.A., Futaki, S., Niwa, M., Baba, Y., Kaji, N., Kamiya, H., Harashima, H., 2004. Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. Gene Ther. 11, 636–644.

Khalil, I.A., Kogure, K., Futaki, S., Harashima, H., 2006. High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. J. Biol. Chem. 281, 3544–3551.

Khalil, I.A., Kogure, K., Futaki, S., Hama, S., Akita, H., Ueno, M., Kishida, H., Kudoh, M., Mishina, Y., Kataoka, K., Yamada, M., Harashima, H., 2007. Octaarginine-modified multifunctional envelope-type nanoparticles for gene delivery. Gene Ther. 14, 682–689.

Kirschberg, T.A., VanDeusen, C.L., Rothbard, J.B., Yang, M., Wender, P.A., 2003. Arginine-based molecular transporters: the synthesis and chemical evaluation of releasable taxol-transporter conjugates. Org. Lett. 5, 3459–3462.

Kosuge, M., Takeuchi, T., Nakase, I., Jones, A.T., Futaki, S., 2008. Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjug. Chem. 19, 656–664.

Lappalainen, K., Miettinen, R., Kellokoski, J., Jaaskelainen, I., Syrjanen, S., 1997. Intracellular distribution of oligonucleotides delivered by cationic liposomes: light and electron microscopic study. J. Histochem. Cytochem. 45, 265–274.

Lauvrak, S.U., Torgersen, M.L., Sandvig, K., 2004. Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. J. Cell Sci. 117, 2321–2331.

Leventis, R., Silvius, J.R., 1990. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. Biochim. Biophys. Acta 1023, 124–132.

Liang, J.F., Yang, V.C., 2005. Insulin-cell penetrating peptide hybrids with improved intestinal absorption efficiency. Biochem. Biophys. Res. Commun. 335, 734–738.

Lundberg, M., Wikstrom, S., Johansson, M., 2003. Cell surface adherence and endocytosis of protein transduction domains. Mol. Ther. 8, 143–150.

Mie, M., Takahashi, F., Funabashi, H., Yanagida, Y., Aizawa, M., Kobatake, E., 2003. Intracellular delivery of antibodies using TAT fusion protein A. Biochem. Biophys. Res. Commun. 310, 730–734.
Morishita, M., Kamei, N., Ehara, J., Isowa, K., Takayama, K., 2007. A novel approach using functional peptides for efficient intestinal absorption of insulin. J. Control. Release 118, 177–184.
Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J.C., Jones, A.T., Sugiura, Y., Futaki, S., 2004. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol. Ther. 10, 1011–1022.
Nakase, I., Takeuchi, T., Tanaka, G., Futaki, S., 2008. Methodological and cellular aspects that govern the internalization mechanisms of arginine-rich cell-penetrating peptides. Adv. Drug Deliv. Rev. 60, 598–607.
Payne, C.K., Jones, S.A., Chen, C., Zhuang, X., 2007. Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands. Traffic 8, 389–401.
Rejman, J., Oberle, V., Zuhorn, I.S., Hoekstra, D., 2004. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem. J. 377, 159–169.
Richard, J.P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M.J., Chernomordik, L.V., Lebleu, B., 2003. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J. Biol. Chem. 278, 585–590.
Rothbard, J.B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., McGrane, P.L., Wender, P.A., Khavari, P.A., 2000. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. Nat. Med. 6, 1253–1257.
Saint-Pol, A., Yelamos, B., Amessou, M., Mills, I.G., Dugast, M., Tenza, D., Schu, P., Antony, C., McMahon, H.T., Lamaze, C., Johannes, L., 2004. Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. Dev. Cell 6, 525–538.
Sapra, P., Allen, T.M., 2003. Ligand-targeted liposomal anticancer drugs. Prog. Lipid Res. 42, 439–462.
Schwarze, S.R., Ho, A., Vocero-Akbani, A., Dowdy, S.F., 1999. In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285, 1569–1572.
Stamatatos, L., Leventis, R., Zuckermann, M.J., Silvius, J.R., 1988. Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. Biochemistry 27, 3917–3925.
Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K., Sugiura, Y., 2002. Possible existence of common internalization mechanisms among arginine-rich peptides. J. Biol. Chem. 277, 2437–2443.
Tarrago-Trani, M.T., Storrie, B., 2007. Alternate routes for drug delivery to the cell interior: pathways to the Golgi apparatus and endoplasmic reticulum. Adv. Drug Deliv. Rev. 59, 782–797.
Terrone, D., Sang, S.L., Roudaia, L., Silvius, J.R., 2003. Penetratin and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. Biochemistry 42, 13787–13799.
Torchilin, V.P., Rammohan, R., Weissig, V., Levchenko, T.S., 2001. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc. Natl. Acad. Sci. U.S.A. 98, 8786–8791.
Tranchant, I., Thompson, B., Nicolazzi, C., Mignet, N., Scherman, D., 2004. Physicochemical optimisation of plasmid delivery by cationic lipids. J. Gene Med. 6, S24–S35.
Tveit, H., Dick, G., Skibeli, V., Prydz, K., 2005. A proteoglycan undergoes different modifications en route to the apical and basolateral surfaces of Madin–Darby canine kidney cells. J. Biol. Chem. 280, 29596–29603.
Tyagi, M., Rusnati, M., Presta, M., Giacca, M., 2001. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. J. Biol. Chem. 276, 3254–3261.
Violini, S., Sharma, V., Prior, J.L., Dyszlewski, M., Piwnica-Worms, D., 2002. Evidence for a plasma membrane-mediated permeability barrier to Tat basic domain in well-differentiated epithelial cells: lack of correlation with heparan sulfate. Biochemistry 41, 12652–12661.
Wadia, J.S., Stan, R.V., Dowdy, S.F., 2004. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat. Med. 10, 310–315.
Weinstein, J.N., Leserman, L.D., 1984. Liposomes as drug carriers in cancer chemotherapy. Pharmacol. Ther. 24, 207–233.
Wrobel, I., Collins, D., 1995. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. Biochim. Biophys. Acta 1235, 296–304.
Zelphati, O., Szoka Jr., F.C., 1996. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. Pharm. Res. 13, 1367–1372.
Zhang, C., Tang, N., Liu, X., Liang, W., Xu, W., Torchilin, V.P., 2006. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J. Control. Release 112, 229–239.
Zhang, S., Zhao, B., Jiang, H., Wang, B., Ma, B., 2007. Cationic lipids and polymers mediated vectors for delivery of siRNA. J. Control. Release 123, 1–10.