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Synergistic effect of a biosurfactant and protamine on gene transfection efficiency

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ABSTRACT

Several barriers need to be overcome to ensure successful gene transfection, including passing of the foreign gene through the plasma membrane, escape of this material from lysosomal degradation, and its translocation into the nucleus. We previously showed that the biosurfactant mannosylerythritol lipid-A (MEL-A) enhanced the efficiency of gene transfection mediated by cationic liposomes by facilitating rapid delivery of foreign genes into target cells through membrane fusion between liposomes and the plasma membrane. Moreover, using MEL-A-containing cationic liposomes, the foreign gene was efficiently delivered into the nucleus because it was released directly into the cytosol and thus escaped lysosomal degradation. Here we investigated the effect of pre-condensation of plasmid DNA by a cationic polymer, protamine, on gene transfection. We found that the efficiency of pre-condensed DNA transfection mediated by MEL-A-containing OH liposomes was >10 times higher than that of non-condensed DNA transfection. In contrast, the efficiency of pre-condensed DNA transfection mediated by OH liposomes was only 1.5 times higher than that of non-condensed DNA transfection. MEL-A did not influence plasmid DNA encapsulation by cationic liposomes, but it greatly accelerated the nuclear delivery of pre-condensed plasmid DNA. Our findings indicate that MEL-A and protamine synergistically accelerate the nuclear delivery of foreign gene and consequently promote gene transfection efficiency.

1. Introduction

Gene transfection using non-viral vectors is a promising approach to ensure safe gene therapy. Cationic liposomes, polymers, and a mixture of cationic lipids and polymers have most often been used as non-viral gene delivery systems (Zhang et al., 2007; Karmali and Chaudhuri, 2007; Kudsiova et al., 2011; Nie et al., 2011). Of these non-viral vectors, cationic liposomes are considered to be a promising means of introducing foreign genes into target cells because of their high transfection efficiency, low toxicity, ease of preparation, and targeted application (Felgner et al., 1989; Farhood et al., 1992; Lasic, 1998; Nishiyama et al., 2005). However, the efficiency of transfection mediated by cationic liposomes has not yet reached levels comparable to mediated by viral vectors. The poor efficiency of transfection mediated by cationic liposomes is a result of the limiting barriers, including entry into target cells, endosomal/lysosomal escape, avoidance from degradation of exogenous DNA by nuclease, and transport into the nucleus.

Previous reports have shown that most cationic liposome–DNA complexes are taken up through endocytosis before exogenous DNA is transferred into the nuclei of target cells (Friend et al., 1996; Rejman et al., 2005). Therefore, efforts have been made to improve cellular uptake and enhance endosomal/lysosomal escape to ensure successful gene transfection (Maruyama et al., 1999; Kawaura et al., 2000; Kogure et al., 2007; Ko et al., 2009; Schäfer et al., 2010; Manjappa et al., 2011). We previously developed a new method for gene transfection by combining cationic liposomes with a biosurfactant, mannosylerythritol lipid-A (MEL-A), which is one of the glycolipid biosurfactants composed of both lipophilic and hydrophilic moieties (Desai and Banat, 1997). MEL-A, a major component of MELs produced by the yeast strain *Candida antarctica* T-34, has a surface active properties as well as cell differentiation activities against human leukemia, mouse melanoma, and PC12 cells (Kitamoto et al., 1993; Isoda et al., 1997; Wakamatsu et al., 2001). We found that MEL-A enhanced the efficiency of DNA transfection mediated by cationic liposomes (Inoh et al., 2001). The MEL-A–containing cationic liposomes could deliver larger amount of genetic material (DNA and siRNA) into the cytoplasm than cationic liposomes without MEL-A or than a commercially available liposomal agent (Inoh et al., 2004, 2010, 2011; Ueno et al., 2007). We further found that the improved efficiency of gene delivery into the cytoplasm was caused not by endocytosis but by membrane fusion between the liposomes and the plasma membrane.

Recently, much emphasis has been placed on gene transfection involving nuclear delivery systems for foreign genes. Although several studies have focused on promoting gene delivery into the nucleus (Ludtke et al., 1999; Zanta et al., 1999; Rebuffat et al., 2001;

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Mesika et al., 2005), gene transfection efficiency has not yet reached a sufficiently high level. These systems have the crucial disadvantage that DNA internalized into the cytoplasm is degraded by nucleases in the cytoplasm. Lechardeur et al. demonstrated that both single- and double-stranded circular plasmid DNA disappears from the cytoplasm of HeLa and COS cells with an apparent half-life of 50–90 min (Lechardeur et al., 1999). Given this background, it was projected that condensing DNA with polymers prior to being complexed with cationic liposomes would diminish the susceptibility of DNA to nucleases. Devising a system that can deliver a large amount of plasmid DNA into the nucleus without degradation by nucleases would constitute a breakthrough in gene delivery.

To establish an efficient nuclear delivery system for foreign genes using MEL-A–containing liposomes, we here prepared condensed plasmid DNA using protamine. Protamine is a cationic polypeptide that can assist in DNA condensation and can deliver DNA into the nucleus using the arginine-rich nuclear localizing signal (NLS) (Reynolds et al., 2005; Meistrich et al., 2003). Previous studies have shown that DNA pre-condensed by protamine could improve the efficiency of conventional cationic liposome-mediated transfection by protecting DNA from enzymatic degradation and by promoting nuclear delivery of DNA (Sorgi et al., 1997; Noguchi et al., 2002). In this study, we showed that a combination of protamine and MEL-A-containing cationic liposomes markedly promotes the nuclear delivery of plasmid DNA. Employing the synergistic effect of these agents may be a promising approach for successful gene transfection using non-viral methods.

2. Materials and methods

2.1. Materials

The synthesis of a cationic cholesterol derivative, cholesteryl-3β-carboxyamido ethylene-*N*-hydroxyethylamine (OH-Chol), has been described in our previous study (Okayama et al., 1997). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) was purchased from Sigma (St. Louis, MO, USA). Luciferase plasmid (pGL-3) was purchased from Promega (Madison, MI, USA). SYTO 13 and Lipofectamine™ 2000 were both purchased from Invitrogen (Carlsbad, CA, USA). The Label IT TM-rhodamine nucleic acid labeling kit was purchased from Mirus Bio Corporation (Madison, SO, USA).

The MEL-producing yeast strain *C. antarctica* T-34 was isolated from the exudate of a tree from Mt. Tsukuba, Japan. MEL-A (4-*O*-[(4′,6′-di-*O*-acetyl-2′,3′-di-*O*-alkanoyl)-β-D-mannopyranosyl] meso-erythritol) was produced by growing the yeast on a fermentation medium consisting of 8% (v/v) soybean oil, 0.2% $NaNO_3$, 0.02% KH_2PO_4, 0.02% $MgSO_4(7H_2O)$, 0.1% yeast extract, and tap water. MEL-A was extracted from the growth medium with ethyl acetate. Crude MEL-A was purified by silica gel column chromatography (Wako gel C-200) using a chloroform-acetate (9:1–5:5) mixture as solvent. NBD-conjugated MEL-A was synthesized as described in our previous study (Ueno et al., 2007).

2.2. Preparation of liposomes

Cationic liposomes containing MEL-A (MEL-A–containing OH liposomes) and without MEL-A (OH liposomes) were prepared by ultrasonication methods according to our previously described procedures (Inoh et al., 2001, 2004, 2010, 2011; Ueno et al., 2007). In brief, to prepare MEL-A-containing OH liposomes, the neutral lipid DOPE (20 nmol), OH-Chol (30 nmol) and MEL-A (10 nmol) were mixed in chloroform, while to prepare OH liposomes, DOPE (20 nmol) and OH-Chol (30 nmol) were mixed. These were dried with N_2 gas under reduced pressure to remove the chloroform solvent. The lipid film was hydrated with 400 μL of phosphate-buffered saline (PBS) for 1 h. Samples were sonicated in a bath-type sonicator (Branson model B 1200) to generate small unilamellar vesicles according to previously described procedures (Inoh et al., 2001, 2004, 2010, 2011; Ueno et al., 2007).

2.3. Preparation of pre-condensed DNA (protamine–plasmid DNA)

Pre-condensed plasmid DNA (protamine–plasmid DNA) was prepared by adding protamine (0.25–6.25 μg) to 5 μL of plasmid DNA (pGL-3) solution (1 mg/mL in Tris-EDTA buffer), and incubated for 30 min at a room temperature.

2.4. Preparation of cationic liposome–protamine–DNA complexes

To prepared cationic liposome–protamine–plasmid DNA complexes, we added 0–6.25 μg protamine to 5 μg plasmid DNA and then complexed this with MEL-A-containing OH liposomes and OH liposomes at a positive/negative ratio of 1.4 according to our previous report (Inoh et al., 2001, 2004).

2.5. Ethidium intercalation assay for determining the efficiency of plasmid DNA encapsulation by cationic liposomes

When added to a solution of DNA, ethidium ions intercalate between the base pairs of the DNA double helix and emit fluorescence under ultraviolet light. Ethidium bromide was added (final concentration, 0.5 μg/mL) to cationic liposome–DNA complexes, and the fluorescence of ethidium at 595 nm (after excitation at 520 nm) was continuously monitored using a spectrofluorometer (RF-5300PC; Shimadzu, Kyoto, Japan). The amount of DNA protected from ethidium intercalation was calculated from the intensity relative to the maximum intensity obtained when ethidium was added to free plasmid DNA in the absence of liposomes.

2.6. Cell culture and luciferase assay

NIH-3T3 and COS7 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Trace, Melbourne, Australia). The target cells were plated on a 60-mm culture dish at a density of 1×10^6 cells/dish. Plasmid pGL-3 DNA (5 μg) was complexed with the cationic liposomes (400 μL) in PBS for 15 min, and the complexes were then incubated with the target cells for 2 h at 37 °C in serum-free SFM101 medium (Nissui, Tokyo, Japan) or SFM101 medium in the presence of 10% serum (FBS). The cells were subsequently washed and cultured for 24 h in DMEM at 37 °C. Lipofectamine™ 2000 was employed as a positive control using the procedure recommended by the manufacturer.

A luciferase assay was performed using the Picagene luciferase assay kit (Toyo Ink, Tokyo, Japan). The transfected cells were washed three times with PBS and lysed in cell lysis buffer. The lysate was centrifuged at 12,000*g* at 4 °C for 3 min, and the supernatant was subjected to the luciferase assay. The relative light unit (RLU) of chemiluminescence was measured using a luminometer (TD-20/20; Turner Designs; Sunnyvale, CA, USA), and the luminescent RLU values were normalized to the amount of protein determined by the BCA assay. The value 1×10^8 RLU represents the activity of approximately 0.1 μM of the luciferase standard.

2.7. Bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated using a modified procedure (Inaba et al., 1992). Briefly, bone marrow was flushed from femurs and tibias of Balb/c mice. They were performed according to the Guiding