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Biosurfactants of MEL-A Increase Gene Transfection Mediated by Cationic Liposomes

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Many microorganisms growing on water-insoluble substrates have been known to produce surface-active compounds called biosurfactants. Although biosurfactants have received increasing attention due to their special properties, there has been no information available until now of a role for them with regard to gene transfection. Thus, we studied here the effects of biosurfactants on gene transfection by cationic liposomes with a cationic cholesterol derivative. Our results showed clearly that a biosurfactant of mannosylerythritol lipid A (MEL-A) increased dramatically the efficiency of gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. Among them, the liposomes with a cationic cholesterol derivative, cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine (I), were much more effective for gene transfection than the liposomes with DC-Chol (cholesteryl-3β-oxycarboxyamidoethylenedimethylamine) or liposomes without MEL-A in various cultured cells. This demonstrates that this new finding has great potential in the experiment of gene transfection and gene therapy mediated by nonviral vectors such as cationic liposomes. © 2001 Academic Press

Key Words: **biosurfactants; cationic liposomes; gene transfection; cationic cholesterol; NIH3T3 cells.**

Gene transfection across cell membranes is a fundamental technology for molecular and cell biology, and also for clinical gene therapy (1–4). Although the most efficient methods for gene transfection involve the use of viral vectors, there are still arguments about risks in regards to propagation and immunogenicity (5, 6). Recently, however, a variety of nonviral gene-delivery systems have been investigated and cationic liposomes have proven to be useful tools for delivery of plasmid DNA and RNA into cells (7–13). For the experiments of such kinds of cationic liposomes, cationic cholesterol derivatives were justified by their high transfection efficiency and low toxicity, although cationic cholesterol derivatives with dialkylamino head groups had been used there (7–9). Recently, however, we have synthesized a novel cationic cholesterol derivative containing a hydrophilic amino head group, cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine (I) shown in Fig. 1 and used it for gene transfection mediated by cationic liposomes (4). Although some success in getting DNA into cells has been achieved as mentioned above (4), gene delivery with nonviral vectors remains an inefficient process. More efficient DNA delivery systems are needed.

Then, biosurfactants have been receiving increasing attention due to their unique properties (low toxicity, biodegradability, and biological activities) compared to chemically synthesized counterparts, from an earth environmental point of view (14, 15). There have been many attempts to exploit and generate large-scale production of several type of biosurfactants (16). Diacylmannosylerythritol lipids (MELs) are abundantly produced by the yeast strain *Candida antarctica* T-34 up to 100 g/L (17, 18). These yeast glycolipids show not only excellent surface-activity (i.e., efficient decrease in surface tension) but also remarkable cell differentiation and growth inhibition activities against human leukemia, mouse melanoma and PC12 cells (19–22). On the other hand, glycolipids have received much attention as leading materials for drug-carrying microcapsules and artificial cells, owing to their stabilizing effect on liposomes (23, 24). Glycolipids also carry out vital functions in biomembrane, such as cell recognition, antigenicity, and cell–cell interaction.

In the present paper we showed that biosurfactants increased significantly the efficiency of the liposome-mediated gene transfection by cationic liposomes with a cationic cholesterol derivative. Although biosurfactants have been gotten attention due to their properties, there is no information for the effects on gene transfection mediated by the cationic liposomes. Thus, we have studied the effects of biosurfactants on the

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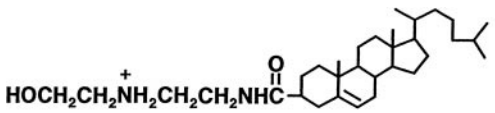

FIG. 1. Structure of cholesteryl-3β-carboxyamido ethylene-*N*-hydroxyethylamine (I). Cationic cholesterol derivative (I) used as a vector in this study has a hydroxy amino head group.

gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. Here, we used mannosylerythritol lipid A (MEL-A) from yeast. MEL-A showed excellent surface and interfacial tension lowering actions and antimicrobial activities particularly against gram-positive bacteria (19). These properties were equivalent to those of ordinary glycolipid-type synthetic surfactants. Our results showed clearly that MEL-A increased significantly the transfection efficiency of liposome-mediated gene transfer. This finding will break through in the experiments of gene transfection using nonviral vectors like cationic liposomes.

MATERIALS AND METHODS

Materials. The synthesis of a cationic cholesterol derivative, cholesteryl-3β-carboxyamido ethylene-*N*-hydroxyethylamine was described in our previous paper (4). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) was obtained from Sigma (St. Louis, MO). Luciferase plasmid (pGL3) was obtained from Promega (Madison, WI).

Biosurfactants. As for biosurfactants we used here mannosylerythritol lipid (MEL-A). The complete structural characterization of MEL-A was described in our previous paper (17, 18). The structure of MEL-A is shown in Fig. 2. MEL-A were synthesized 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. Synthesis was performed in 500-ml Erlenmeyer flasks containing 50 ml of the fermentation medium by incubation at 30°C for 7 days on a rotary shaker (220 rpm). MEL-A was extracted with ethyl acetate from the reaction medium. The ethyl acetate layer gave an yellow syrup as crude MEL-A after removal of the solvent. The crude MEL-A was purified by silica-gel

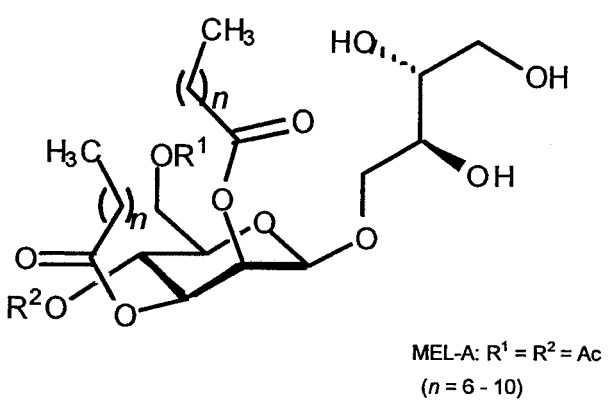

FIG. 2. Structure of biosurfactant MEL-A. MEL-A consists of 4-*O*-[β-D-mannopyranosyl]*meso*-erythritol esterified two fatty acids and two acetic acids.

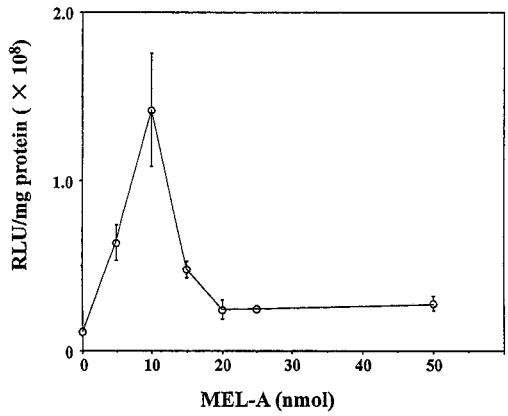

FIG. 3. Dose dependence of transfection efficiency by cationic liposomes containing MEL-A. NIH3T3 cells were transfected with MEL-A containing cationic liposomes with derivative (I). Transfection efficiency was estimated by luciferase assay and plotted against the amount of MEL-A in cationic liposomes.

(Wako-gel C-200) column chromatography with chloroform–acetate (9:1 to 5:5) mixture as solvent systems. MEL-A showed $[\alpha]_D^{22}$ −46.6°. The components of the fatty acids were octanoate (17.5%), decanote (71.3%), dodecanote (10.1%), and tetradecanoate (1.1%).

Preparation of liposomes. DOPE was combined with one of cationic derivatives of cholesterol in chloroform and dried with N_2 gas to remove chloroform solvent. Molar ratios of DOPE/cholesterol derivatives were 2:3 and 3:2 for derivative (I) and DC-Chol, respectively (9, 11). MEL-A containing liposomes were prepared by 5∼50 nmol of MEL-A in the liposomes (DOPE + cholesterol). Mixtures were dried under reduced pressure to remove chloroform solvent. The dried film was vacuum desiccated for at least 30 min and suspended by vortexing and the samples were sonicated in a bath type sonicator (Branson Model B1200) to generate small unilamellar vesicles (SUVs) following our previous procedures (4, 9, 11). The diameter of the cationic liposomes was measured using a multiangle light-scattering instrument (Otsuka Electronics).

Target cells. NIH3T3 cells and COS-7 cells were cultured in DMEM from Gibco (Grand Island, NY) supplemented with 10% FBS (TRACE). HeLa cells were cultured in MEM from Nissui (Tokyo, Japan) supplemented with 10% FCS (Boehringer Mannheim). Plasmid pGL3 DNAs were complexed to the cationic liposomes in SFM101 (Nissui) at room temperature for 15 min and then the complex was incubated with target cells for 4 h at 37°C. Then the cells were washed and cultured for another 40 h in growth medium at 37°C before luciferase assay (11).

RESULTS

MEL-A containing cationic liposomes with a cholesterol derivative (I) or DC-Chol were complexed with plasmids for luciferase (pGL3) and were studied by luciferase activity after 4 h-incubation with the target cells (NIH3T3 cells). Some of the results are shown in Fig. 3. The MEL-A containing cationic liposomes increased clearly the transfection efficiency compared with the control cationic liposomes (without MEL-A) shown in Fig. 3. The MEL-A containing liposomes (10 nmol) were the most effective for the luciferase activity in our present experimental conditions. Thus, in the

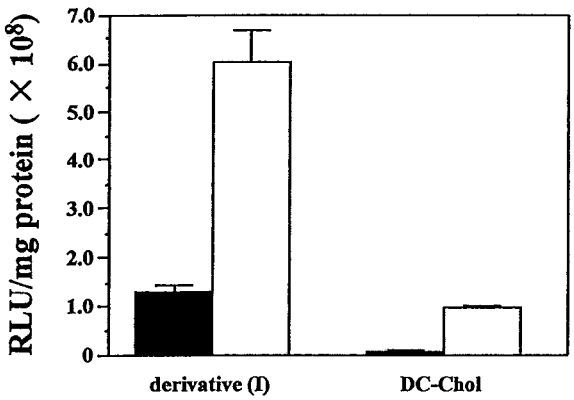

FIG. 4. Effects of MEL-A on the transfection efficiency mediated by cationic liposomes. NIH3T3 cells were transfected with luciferase gene (pGL3) using two different cationic cholesterol derivatives: derivative (I) and DC-Chol. Transfection efficiency was estimated by luciferase assay and values were normalized to the amount of total protein. In both cases, transfection efficiency with cationic liposomes containing MEL-A (open columns) was higher than that with cationic liposomes lacking MEL-A (filled columns).

present experiments we used the MEL-A containing liposomes (10 nmol) to study the effect of MEL-A on gene transfection of cationic liposomes. We used two kinds of the MEL-A containing cationic liposomes with a cationic cholesterol derivative: one was a derivative of a hydrophobic amino head group (I) and the other was DC-Chol. The results showed that both MEL-A containing cationic liposomes increased the luciferase activity in NIH3T3 cells as shown in Fig. 4. We did similar experiments using other target cells such as COS-7 and HeLa cells. The results are shown in Fig. 5A and Fig. 5B. All of the results showed that the liposomes with MEL-A increased more significantly transfection efficiency of pGL3 gene into the target cells than the liposomes without MEL-A. In addition, the liposome of the derivative (I) had greater transfection efficiency than the liposomes of the DC-Chol.

The average diameters of the MEL-A containing liposomes with derivative (I) and DC-Chol were 200–300 nm, while those of liposomes without MEL-A were 400–500 nm as shown in Table 1. This suggests that MEL-A enhanced the transfection efficiency partly because of minimizing DNA-induced aggregation and reducing particle sizes of MEL-A containing liposomes.

As we had reported previously that the cationic cholesterol with a hydroxyethylamino head group (I) promoted more significantly liposome-mediated gene transfection than those by the cationic liposomes containing DC-Chol (4), it was reasonable that the MEL-A containing cationic liposomes with the derivative (I) gave a much higher transfection efficiency than those with the DC-Chol. However, it seemed that the effects by the MEL-A itself were almost similar in the derivative (I) and DC-Chol. In addition, we found that transfection efficiency by the cationic liposomes with a cationic cholesterol derivative (I) was about 10 times as high as that by commercially available cationic liposomes, such as Lipofectin. The results suggested that the MEL-A containing cationic liposomes promoted further the transfection efficiency of plasmid DNAs into target mammalian cells depending on the efficiency of cationic liposomes.

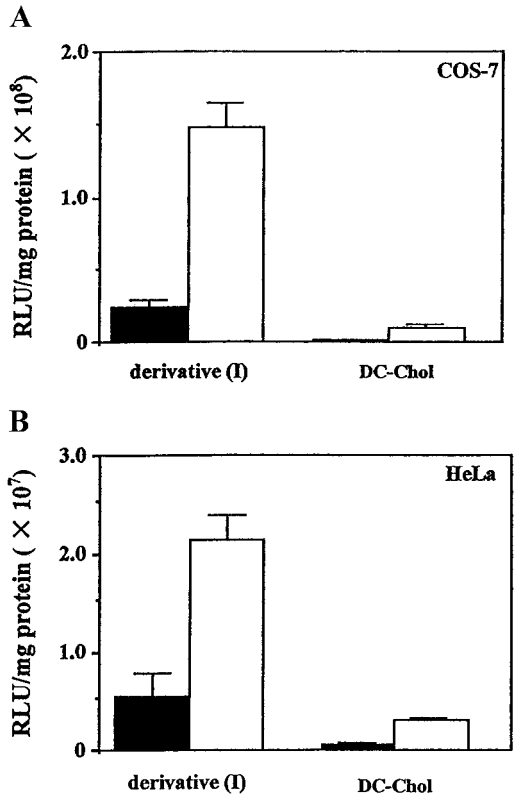

FIG. 5. Comparative study of the effects of MEL-A on the transfection efficiency mediated by cationic liposomes in various target cells. The effects of MEL-A on transfection efficiency were compared in COS-7 (A) and HeLa (B) cells. Enhanced efficiency due to the addition of MEL-A was observed in both cell lines.

DISCUSSION

The microbial extracellular glycolipid known as MEL is a biosurfactant composed of both lipophilic and hydrophilic moieties. MEL is produced in large

TABLE 1

Mean Diameter of Liposomes Estimated by Light Scattering

Liposome	Diameter (nm)
DC-Chol	479 ± 14
DC-Chol + MEL-A	221 ± 87
Derivative (I)	448 ± 76
Derivative (I) + MEL-A	275 ± 88

amounts by the yeast *C. antarctica* T-34 when this microorganism is grown on vegetable oils as the sole carbon source. The hydrophilic moiety of MEL-A was identified as 4-*O*-*β*-D-mannopyranosyl-*meso*-erythritol (18). Recently, we reported that MEL induces the differentiation in granulocytes of HL-60 promyelocytic leukemia cells (20).

Gangliosides and GSLs (glycosphingolipids), which are ubiquitous constituents of the plasma membranes of mammalian cells, were recently found to be active in the modulation of cell proliferation in oncogenesis and differentiation (25, 26). Cellular differentiation and oncogenic transformation are accompanied by dramatic changes in absolute and relative levels of GSLs (27). Moreover, ceramide, the product of the hydrolysis of sphingomyelin in the sphingolipid cycle, was recently reported to induce apoptosis in human neuroepithelioma cells (28). The accumulation of ceramide also seems to be associated with several antiproliferative responses, which include the differentiation of cells, apoptosis, and cell cycle arrest (29, 30). Mammalian and microbial glycolipids differ in the details of specific residues, but their backbones are similar. Thus, we postulated that MEL might have biological effects and most likely has antiproliferative effects on tumor cells in addition to inducing cell differentiation.

In the present study, we found that MEL-A containing cationic liposomes with a cationic cholesterol derivative of a hydrophobic amino head group (I) and DC-Chol promoted the efficiency of gene transfection into mammalian cultured cells. The efficiency of MEL-A containing liposomes was about five to seven times greater than that by the control liposomes without MEL-A in our experimental conditions. MEL-A has 4-*O*-[(4′,6′-di-*O*-acetyl-2′,3′-di-*O*-alkanoyl)-*β*-D-mannopyranosyl]*meso*-erythritol residues on the terminus of fatty acids. We checked MEL-A itself did not increase transfection efficiency into the target cells. The value was almost similar to that by naked DNA. This indicated that the enhancement of transfection efficiency by MEL-A containing liposomes was caused by collaboration of MEL-A and liposomes.

We have shown in the present paper that, in addition to cationic liposomes, other types (biosurfactants) of lipid formulation can be used to facilitate gene transfer into target cells. It seemed that this new delivery system has advantages over the currently used liposome formulations. As for gene transfer, Liu *et al.* reported that the cationic lipid formulation using a synthetic surfactant, Tween 80, had more favorable biological activities than traditional cationic liposomes as a carrier for gene delivery (31). However, there have been no reports for cationic lipid formulations using biosurfactants described in the present paper. Thus, the present finding has great potential in the experiment of gene transfection and gene therapy because biosurfactants have many excellent properties (low toxicity, biodegradability, environmental protection, etc.) compared to synthetic surfactants.

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