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A nanocarrier system for the delivery of nucleic acids targeted to a pancreatic beta cell line

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ABSTRACT

Pancreatic β cells secrete insulin in response to glucose levels and thus are involved in controlling blood glucose levels. A line of pancreatic β cells "MIN6" has been used in studies related to the function of β cells and diabetes therapy. Regulating gene expression in MIN6 cells could accelerate these studies, but an efficient method for the transfection of nucleic acids targeted to MIN6 cells is required. We report here on a liposome-based carrier targeted to pancreatic β cells (Multifunctional envelope-type nano device for pancreatic β cells, β-MEND). We identified a lipid composition for use in preparing the β-MEND, which permits the particles to be efficiently internalized into MIN6, as evidenced by flow cytometry analyses. Intracellular observation by confocal laser scanning microscopy showed that the β-MEND efficiently delivered the oligo nucleic acids to the cytosol of MIN6 cells. Moreover, using a β-MEND encapsulating a 2′-O-Methyl RNA complementary to a microRNA that suppresses insulin secretion, the knockdown of the targeted microRNA and an up-regulation of insulin secretion were observed in MIN6. Thus, the β-MEND holds promise as an efficient system for delivering nucleic acids targeted to MIN6 and can contribute to research and therapy aimed at diabetes.

1. Introduction

Diabetes mellitus is a multi-factorial disorder characterized by high blood glucose levels, and appears to be caused, in part, by genetic and environmental factors [1,2]. Mutations of the gene related to pancreatic β cell function have been associated with some types of diabetes mellitus [3,4]. For example, maturity-onset diabetes of the young (MODY) are caused by mutations in any one of at least six different genes that encode the glycolytic enzyme glucokinase and five transcription factors [5]. These genes are associated with important pancreatic β cell functions, including the production and secretion of insulin which regulates blood glucose levels. Therefore, gene therapy targeted to pancreatic β cells and related studies could be useful for the treatment of many patients suffering from diabetes mellitus.

A line of pancreatic β cells "MIN6" has been established from mouse insulinomas, and has morphological characteristics that are similar to those of pancreatic β cells. In addition, MIN6 cells exhibit glucose-inducible insulin secretion comparable to that of cultured normal mouse islet cells [6]. Thus, the MIN6 cell line is useful in studies of the molecular mechanisms of pancreatic β cells, and has been used in research directed to the study of the pancreatic β cells function and diabetes therapy [7–9]. The regulation of gene expression in MIN6 cells could accelerate these studies, but an efficient method for the transfection of nucleic acids targeted to MIN6 cells is required. To date, while the transfection of nucleic acids into MIN6 cells using a viral vector [9] and electroporation [8] has been achieved, the transfection efficiency does not appear to be sufficient. Considering such a situation, a nanocarrier system capable of delivering nucleic acids targeted to MIN6 cells is essential for future research on the function of β cells and diabetes therapy.

In previous studies, we reported on the development of a multifunctional envelope-type nano device (MEND), which consists of a condensed plasmid DNA (pDNA) core and lipid envelopes [10,11], which showed transfection activities as high as that for a viral vector in dividing cells [12]. To date, we have been successful in efficiently packaging nucleic acids, including oligo DNA [13,14] and siRNA [15,16], and showed that the MEND system achieved

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efficient gene silencing and RNA knockdown [14–19]. These results prompted us to consider the possibility that a MEND system could be used to achieve the efficient cytosolic delivery of nucleic acids targeted to impregnable MIN6 cells.

In this study, we report on our attempts to develop β-MEND, which is a MEND that permits the efficient delivery of nucleic acids to pancreatic β cells. We conclude that the β-MEND reported on here, constitutes a breakthrough in research on the function of β cells and diabetes therapy. We first identified a lipid composition for the β-MEND that permits it to be efficiently internalized into MIN6 cells. This was achieved by varying the lipid composition of a panel of liposomes (LPs) labeled with fluorescent lipids and cellular uptake analysis by flow cytometry. We next constructed the β-MEND in which nucleic acids were encapsulated, and the fluorescent labeled nucleic acids that were internalized in MIN6 by the β-MEND were measured by flow cytometry. We also observed the intracellular trafficking of the nucleic acids using confocal laser scanning microscopy (CLSM). Moreover, when a β-MEND encapsulating a 2′-O-Methyl (2′-OMe) RNA which targets a microRNA (miRNA) that suppresses insulin secretion was used, the knockdown of the targeted miRNA and the up-regulation of insulin secretion were observed and evaluated in MIN6 cells.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dilawroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), egg yolk phosphatidyl choline (EPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) were obtained from Nippon Oil and Fats Co. (Tokyo, Japan). Cardiolipin (CL), cholesteryl hemisuccinate (CHEMS), phosphatidic acid (PA) and phosphatidyl grycerol (PG) were purchased from Sigma (St. Louis, MO, USA). Cholesterol (Chol), 3β-[N-(N′-,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), dimethyldiocatadecylammonium bromide (DDAB), 1, 2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (SOPE) and sphingomyelin (SM) were purchased from Avanti Polar lipids (Alabaster, AL, USA). Stearyl octaarginine (STR-R8) [20] was obtained from KURABO Industries Ltd (Osaka, Japan). The oligo DNA (5′-CTTCTCGTCCCCATGGATGACCCC-3′) and the oligo DNA labeled with fluorescent that Cy5 is conjugated to 5 terminal of the oligo DNA were obtained from Hokkaido System Science Co., Ltd (Sapporo, Japan). 2′-OMe RNA complementary to miR-375 (anti-miR-375) (5′-UGCAUCACGCGAGCCGAACGAACAAAUAAG) and antisense 2′-OMe RNA targeting green fluorescent protein (control RNA) (AAGGCAAGCUGACCCUGAAGU) were obtained from Greiner Japan (Tokyo, Japan). Protamine was purchased from CALBIO CHEM (Darmstadt, Germany). HeLa human cervix carcinoma cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). MIN6 cells, a mouse pancreatic beta cell line [6], were generously provided by Dr. J. Miyazaki (Osaka University, Japan). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Lipofectamine Plus (LFN Plus) and tetramethylrhodamine (TMRM) were purchased from Life Technologies Corporation (Carlsbad, CA, USA). All other chemicals used were commercially available reagent-grade products.

2.2. Cell culture and transfection studies

HeLa cells were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/mL), streptomycin (100 μg/mL) under an atmosphere of 5% CO_2 at 37 °C. MIN6 cells were cultured in DMEM containing 25 mM glucose and supplemented with 10% FBS and penicillin (100 U/mL), streptomycin (50 μg/mL) under an atmosphere of 5% CO_2 at 37 °C. MIN6 cells (4×10^5 cells/mL) or HeLa cells (1×10^5 cells/mL) were seeded with DMEM containing 10% FBS, on a 12 well plate (Corning, NY, USA; DMEM volume, 1 mL) for flow cytometry analysis and a 35 mm glass base dish (IWAKI, Tokyo, Japan; DMEM volume, 2 mL) for confocal laser scanning microscopy (CLSM). Moreover, MIN6 cells (4×10^5 cells/mL) were seeded with DMEM containing 10% FBS, on a 24 well plate (Corning; DMEM volume, 500 μL) for insulin secretion studies, a 12 well plate (Corning; DMEM volume, 1 mL) for cytotoxicity evaluation and reverse transcription and polymerase chain reaction (RT-PCR).

2.3. Preparation of NBD-labeled LPs

LPs were prepared by the lipid hydration method. A lipid film containing 0.5 mol % 7-nitrobenz-2-oxa-1, 3-diazole labeled DOPE (NBD-DOPE) (Avanti Polar lipids) was prepared on the bottom of a glass tube by evaporation of an ethanol solution containing 150 nmol lipids, as shown in Table 1. Next, 250 μL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) was applied to the lipid film, followed by incubation for 15 min at room temperature. The lipid film was then sonicated for 30 s in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan). R8-modified LP (R8-LP) was prepared by mixing the LP (DOPE/PA = 9:2, molar ratio) suspensions and an STR-R8 solution (10 mol% of lipid).

2.4. Preparation of MEND encapsulating Cy5-labeled oligo DNA

Negatively charged nanoparticles of oligo DNA (final oligo DNA concentration, 61.5 μg/mL) were prepared as follows; a solution of oligo DNA was added to the protamine solution under vortexing at a nitrogen/phosphate (N/P) ratio of 1.0 for β-MEND (DC-Chol/EPC/SM = 3:4:3, molar ratio). While a solution of oligo DNA was added to the STR-R8 solution under vortexing at an N/P ratio of 3.0 in the case of the preparation of positively charged nanoparticles for the R8-MEND (DOPE/PA/STR-R8 = 9:2:1, molar ratio). A lipid film was prepared by the evaporation of a chloroform solution of 825 nmol lipid [(DC-Chol/EPC/SM = 3:4:3, molar ratio) or (DOPE/PA = 9:2, molar ratio)] on the bottom of a glass tube, followed by hydration with 1.5 mL of 10 mM HEPES buffer (pH 7.4) for 15 min at room temperature. The glass tube was sonicated in a bath-type sonicator (AU-25C; Aiwa Co.), followed by sonication for 10 min in ice-cold water with a probe-type sonicator (Digital Sonifier 250; Branson Ultrasonics Co., Danbury, CT, USA) to produce small unilamellar vesicle (SUV). β-MEND was constructed by mixing negatively charged oligo DNA nanoparticles and with twice the volume of positively charged SUV (DC-Chol/EPC/SM = 3:4:3, molar ratio) suspension. For the preparation of R8-modified MEND (R8-MEND), positively charged oligo DNA nanoparticles were mixed with twice the volume of negatively charged SUV (DOPE/PA = 9:2, molar ratio) suspension, followed by incubation with STR-R8 (10 mol% of total lipids) for 30 min at room temperature.

2.5. Preparation of β-MEND encapsulating 2′-OMe RNA

A solution of 2′-OMe RNA was added to the protamine solution under vortexing at an N/P ratio of 1.0. A lipid film was prepared on the bottom of a glass tube by evaporation of an ethanol solution containing 150 nmol lipids (DC-Chol/EPC/SM = 3:4:3, molar ratio). 250 μL of a solution of 2′-OMe RNA nanoparticles was applied to the lipid film, followed by incubation for 15 min at room temperature. The lipid film was then sonicated for 30 s in a bath-type sonicator (85 W, Aiwa Co.).

Table 1
Characteristics of LPs. PG, phosphatidyl grycerol; CHEMS, choresteryl hemisuccinate; PA, phosphatidic acid; CL, cardiolipin; Chol, cholesterol; SM, sphingomyelin; DDAB, dimethyldiocatadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA, 1,2-di-O-octadecenyl-3-trimethylammonium propane; DC-Chol, 3β-[N-(N′-,N′-dimethylaminoethane)-carbamoyl] cholesterol; EPC, egg yolk phosphatidyl choline; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilawroyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; SOPE, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine. Data are represented as the mean ± S.D. ($n = 3–5$).

Liposome type	Lipid composition	Lipid X	Diameter (nm)	ζ potential (mV)
A-type LP	DOTAP/EPC/Lipid X (3:4:3, molar ratio)	PG	412 ± 127	−19 ± 13
		CHEMS	113 ± 11	−28 ± 0
		PA	142 ± 34	−41 ± 9
		CL	108 ± 24	29 ± 10
		Chol	109 ± 17	39 ± 8
		SM	92 ± 3	43 ± 7
B-type LP	Lipid X/EPC/SM (3:4:3, molar ratio)	DDAB	122 ± 6	50 ± 4
		DOTAP	92 ± 3	43 ± 7
		DOTMA	119 ± 17	70 ± 9
		DC-Chol	102 ± 6	57 ± 6
C-type LP	DC-Chol/Lipid X/SM (3:4:3, molar ratio)	EPC	102 ± 6	57 ± 6
		DOPC	109 ± 11	48 ± 7
		DLPC	107 ± 17	51 ± 5
		DMPC	95 ± 10	51 ± 11
		DPPC	121 ± 21	55 ± 3
		POPC	104 ± 6	53 ± 5
		SOPC	110 ± 10	21 ± 17
		DOPE	117 ± 2	43 ± 4
		SOPE	106 ± 7	58 ± 5

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