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Cholesterol Enhances Cationic Liposome-Mediated DNA Transfection of Human Respiratory Epithelial Cells

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Cationic liposome transfection reagents are useful for transferring polynucleotides into cells, and have been proposed for human pulmonary gene therapy. The effect of adding cholesterol to cationic lipid preparations has been tested by first formulating the cationic lipid N-[1-(2,3-dioleoyloxy)propyl-N-[1-(2-hydroxy)ethyl]-N,N-dimethyl ammonium iodide (DORI) with varying amounts of dioleoylphosphatidylethanolamine (DOPE) and cholesterol. Cholesterol was found to enhance lipid-mediated transfection in both the respiratory epithelial cells and mouse fibroblasts. These findings will facilitate nucleic acid transfection of many cell types including differentiated epithelial cell monolayers, and therefore may be useful for examining gene regulation in various cell types and for developing pulmonary gene therapy.

KEY WORDS: cationic liposome; cholesterol; transfection; human respiratory epithelial cells.

ABBREVIATIONS: N-[1-(2,3-dioleoyloxy)propyl]-N-[1-(2-hydroxy)ethyl]-N,N-dimethyl ammonium iodide (DORI); dioleoylphosphatidylethanolamine (DOPE); N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethyl ammonium chloride (DOTMA); Eagle's modified essential medium (Mem); Dulbecco's Modified Eagle's Medium (DMEM).

INTRODUCTION

Methods for polynucleotide delivery are essential for the correlation of gene expression with phenotype, investigation of the regulation of gene expression, and gene therapy. Commonly used gene delivery methods include the use of

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recombinant viral vectors, physical methods (such as direct DNA injection), or pharmaceutical reagents. One pharmaceutical gene delivery technique involves the use of DNA packaged with lipids (liposomes). Originally, neutral or weakly charged lipids were utilized for liposomal gene delivery (1, 2, 3). The discovery that cationic liposomes spontaneously associate with DNA, fuse with cell membranes, and deliver the DNA into cytoplasm (4) has greatly advanced the utility of liposomal polynucleotide transfection.

Synthetic cationic transfection lipid preparations spontaneously interact with DNA in solution to form lipid-DNA complexes. When placed onto tissue culture cells, these lipid-DNA complexes interact with the plasma membrane (4). As a consequence of this process, a portion of the exogenous DNA becomes localized in the nucleus and is subsequently transcribed. There are two leading hypotheses defining the mechanism of cationic lipid-mediated transfection: 1) plasma membrane fusion and subsequent cytoplasmic delivery (4) or 2) a pathway involving endocytic uptake (5, 7, 8). These hypotheses are not mutually exclusive, and may be active to a greater or lesser extent in different cell types. Either transfection pathway may be facilitated by alterations in liposome formulation which effect the fluidity of lipid/polynucleotide/cell membrane complexes.

Sterols are commonly used for modulating the fluidity of both natural and artificial membranes (9). Cationic cholesterol derivatives have also been shown to mediate effective DNA transfection of cells and tissues when formulated into sonicated vesicles with DOPE (10). Therefore, these experiments have been designed to investigate the effect of cholesterol, the predominant mammalian sterol, on the efficacy of cationic liposome transfection formulations.

Since respiratory epithelial cells are a potential therapeutic target for cationic lipid-mediated gene therapy, experiments focusing on the transfection of the apical surface of polarized respiratory epithelial cells were performed. Human surface airway epithelial cells have been isolated and cultured *in vitro*, but even under optimal conditions such cells have a limited life span and senesce or terminally differentiate (11, 12). To circumvent this problem, cultures of polarized immortal human bronchial epithelial cells have recently been developed (13), and this cell line (16HBE14o-) was chosen as the principal target for the analysis of the effects of cholesterol on cationic lipid-mediated DNA transfection.

MATERIAL AND METHODS

Chemicals

Dioleoylphosphatidylethanolamine was purchased from Avanti Polar Lipids (Inc. (Birmingham, A1). Cholesterol was purchased from Sigma Chemical Company (St. Louis, MO). The cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N-[1-(2-hydroxy)ethyl]-N,N-dimethyl ammonium iodide (DORI) was prepared using a procedure developed in our laboratories (14).

Cell Culture

Human respiratory epithelial cells (16HBE14o-) were cultured by plating on 24 well tissue culture plastic ware coated with fibronectin, vitrogen (collagen), and bovine serum albumen as previously described (11, 12). Growth media consisted of Eagle's modified essential medium (MEM) supplemented with 10% fetal bovine serum, and cells were transfected as subconfluent monolayers.

As a control for cell specific effects, NIH 3T3 cells were also tested for DNA transfection using the various liposome formulations. NIH 3T3 were obtained from ATCC (CRL 1658). 3T3 cells were cultured on standard 24 well tissue culture plates using Dulbecco's Modified Eagle's Medium and 10% calf serum.

Liposome Formulation

An appropriate mass of the cationic lipid DORI and a neutral lipid (DOPE and/or cholesterol), as solutions in chloroform, were added to 1.9 ml sample vials to yield a 50:50 mole ratio of DORI:neutral lipid. The chloroform was removed via rotary evaporation at 37°C. The resulting thin lipid films were placed under high vacuum overnight to insure that all traces of solvent have been removed. The lipid mixture was resuspended with vortex mixing using 1 ml sterile water for injection (American Reagent Laboratories Inc.), resulting in a total lipid concentration of 2 μmole lipid/1 ml water. This solution was sonicated until clear using a Branson sonifer 450 sonicator equipped with a cup horn (35°C, 15 minutes).

Plasmid DNA

The plasmid pCMVL consists of the *P. pyralis* luciferase cDNA subcloned into the plasmid pRc/CMV (Invitrogen). This plasmid was transformed into competent E. coli DH5-α cells, amplified in terrific broth, and prepared by alkaline lysis with the isolation of covalently closed circular plasmid DNA using two rounds of CsCl-EtBr gradient ultracentrifugation. The plasmid DNA was subsequently treated with DNAse-free RNAse, phenol/chloroform extracted, and purified by precipitation from an ethanol/sodium acetate solution. DNA purity was determined by agarose gel electrophoresis and optical density (OD 260/280 greater than or equal to 1.8).

Transfection of Cultured Cells

Tissue culture well plates (24 well) were plated 24 hours prior to transfection at approximately 80% confluency with either NIH 3T3 cells or 16HBE14o- cells. The growth media was removed via aspiration and the cells were washed once with 0.5 ml PBS/well. The liposome/DNA complexes were formed through sequential addition of an appropriate amounts of DMEM (serum-free), plasmid pCMVL (4 micrograms), and liposome formulation into a 2 ml Eppendorf tube

gave a total volume of 800 ml. The addition of these substances was followed by thorough vortex mixing and incubation for 15 minutes at room temperature. A 200 ml aliquot of the resultant transfection complex was added to each well and the cells were incubated for 4 hours at 37°C. At this time, 500 microliters of the appropriate growth media + 10% calf serum/well was added and the cells were incubated for an additional 48 hours.

Luciferase Assay

Relative luciferase activity was determined by using the Enhanced Luciferase Assay Kit and Monolight 2010 luminometer (both from Analytical Luminescence Laboratories, San Diego, CA). This was accomplished by directly applying 233.3 ml of concentrated luciferase lysis buffer to each well and placing the cells on ice for 15 minutes. Removal of growth media was not necessary prior to the application of the lysis buffer. This technique proved to be more efficient and avoids the possibility of cell loss during media removal. An analogous experiment where the growth media was removed afforded similar results. Luciferase light emissions from 31.1 microliters of the lysate were measured over a 10 second period, and results expressed as a function of an assumed total lysate volume of 933.3 μl.

RESULTS

To investigate the possibility that added cholesterol could enhance liposomal transfection ability, liposomes containing mixtures of the cationic lipid DORI and a neutral lipid component, DOPE and/or cholesterol, were formulated and screened for their ability to transfect pCMVL DNA into human airway epithelial cells and NIH 3T3 murine fibroblasts. Optimal experimental conditions were obtained by varying the ratio of DOPE to cholesterol while keeping the amount of DORI (50:50 mole ratio of DORI:neutral lipid mixture) and the DORI:DNA phosphate charge ratio (2:1) constant. These experimental conditions were specifically selected to allow direct comparison between the tri-component liposomes and 50:50 DOPE:DORI liposomes. Previous work by ourselves and others (6) has shown that a 50:50 DOPE:DORI formulation is optimal for bi-component transfection preparations composed of DOPE and DORI.

The addition of cholesterol to the cationic lipid formulations enhances the transfection of differentiated human respiratory epithelial cells (Figure 1). This observation was consistent for all formulations, including those composed of only DORI and cholesterol as well as the tri-component liposomes. Furthermore, variable transfection activity was observed among the tri-component formulations. The most active DNA transfection preparation was formulated using a molar ratio of 30:50:20 DOPE:DORI:cholesterol.

To ascertain whether the enhancement of transfection which was observed with added cholesterol was limited to respiratory epithelial cells, NIH 3T3 cells

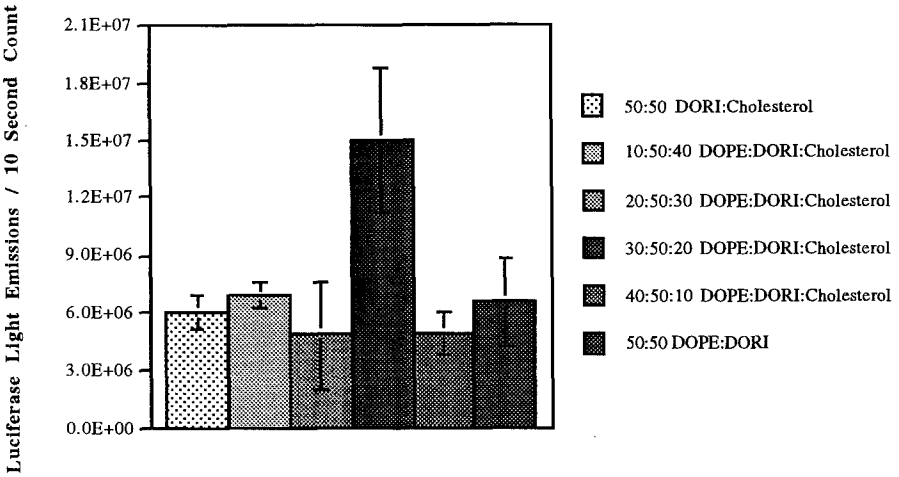

Fig. 1. Formulations containing 50 mole % of the cationic lipid DORI and varying amounts of DOPE and cholesterol were screened for their ability to functionally deliver the plasmid pCMVL (1 μg) into human respiratory epithelial cells. Cell lysates obtained 48 hours after transfection were analyzed for luciferase specific activity. Each data point reflects the mean value of total light units derived from four transfections and the standard deviation from this mean.

were transfected using the same formulations as were used for respiratory epithelial cell transfection (Figure 2). Again, it was determined that the tri-component formulation consisting of 30:50:20 DOPE:DORI:cholesterol was optimal for transfection of NIH 3T3 cells with DNA.

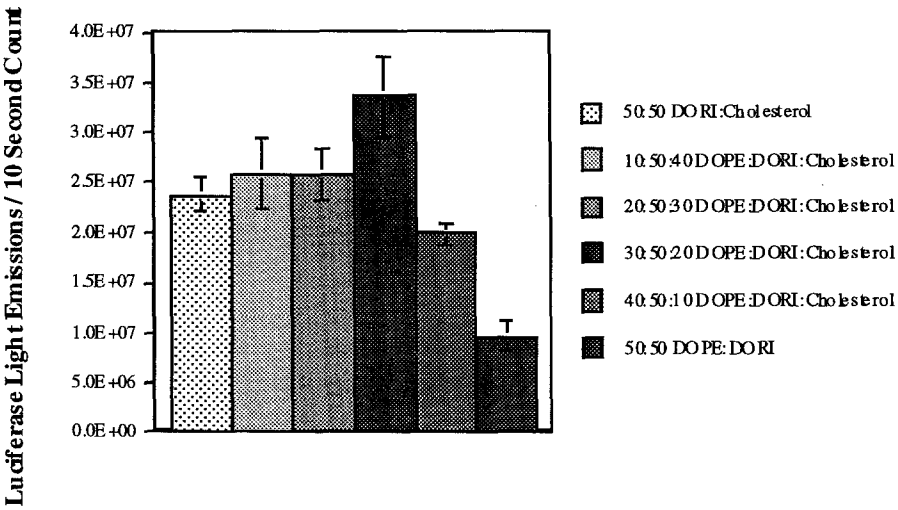

Fig. 2. Optimization of NIH 3T3 fibroblast transfection mediated by liposomes containing the cationic lipid DORI (50 mole %) and variable amounts of DOPE and cholesterol. Analysis for luciferase specific activity 48 hours after transfection indicates that liposomes comprised of a 30:50:20 mole ratio of DOPE:DORI:cholesterol are the most effective in mediating pCMVL delivery. These results compliment those obtained with the human respiratory epithelial cells.

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