INTRODUCTION

The bronchial epithelium has traditionally been recognized as a physical barrier protecting the host from its environment. However, epithelial cells play a central role in the regulation of airway immunity, affecting inflammation and host defenses in diseases of the airway. Epithelial cells release a wide range of proinflammatory mediators and multifunctional cytokines in response to exposure to inhaled environmental factors or microorganisms. The precise mechanisms are not fully understood, but epithelial cells are thought to play a major part in the regulation of host inflammatory status as well as airway structure and function.1,2

Lipid rafts are subdomains of the epithelial cell membrane that contain high concentrations of cholesterol and glycosphingolipids. They interact with one another and pack tightly together to form cell membrane structures. Thus, lipid rafts provide a platform for multiple signaling pathways and act as key modulators of certain disease pathways.4 Lipid entities render lipid rafts insoluble in nonionic detergents and cause them to separate from their surroundings. Cholesterol, the most abundant lipid component of animal cell membranes, regulates membrane fluidity and plays a crucial role in the formation and stabilization of membrane microdomains. It is also an important contributor to cell-cell adhesion, migration, and even endocytosis.4,5 However, despite increasing interest in the bronchial epithelium, the possible role of cholesterol in inflammation of the airway or the development of asthma has not been investigated.

Among the numerous cytokines and chemokines released from human airways, interleukin-8 (IL-8) is a representative marker of inflammation.6 The lipid entities of cell membranes are components of the immune system and important mediators of inflammation. Despite increasing interest in the function of epithelial cells in inflammation, the role of cholesterol in this process has not been described. Here, we investigated the effect of cholesterol depletion on the inflammatory process in airway epithelial cells via the expression of interleukin (IL)-8 as a marker of inflammation.

Effect of Cholesterol Depletion on Interleukin-8 Production in Human Respiratory Epithelial Cells

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Purpose: The lipid entities of cell membranes are components of the immune system and important mediators of inflammation. Despite increasing interest in the function of epithelial cells in inflammation, the role of cholesterol in this process has not been described. Here, we investigated the effect of cholesterol depletion on the inflammatory process in airway epithelial cells via the expression of interleukin (IL)-8 as a marker of inflammation.

Methods: A 549 cells were treated with 0.5% methyl-β-cyclodextrin as a selective cholesterol extractor. The IL-8 level was assessed by enzyme-linked immunosorbent assay and reassessed after cholesterol repletion. Mitogen-activated protein kinase (MAPK) inhibitors were used to determine the upstream signaling pathway for IL-8 production in cholesterol-depleted cells.

Results: We found a relationship between the amount of cholesterol in A 549 cells and inflammation of the airway. IL-8 production was increased in cholesterol-depleted A 549 cells and restored by cholesterol repletion. IL-8 production was decreased by pretreatment with the extracellular signal-regulated kinase (ERK) inhibitor U0126 but not with JNK inhibitor II or the p38 MAPK inhibitor SB202190.

Conclusions: Our findings suggest that inflammatory responses are increased in cholesterol-depleted epithelial cells via the MAPK signaling system, predominantly by the ERK pathway. We conclude that the lipid components of airwayepithelial cells may play a role in the inflammatory process.

Key Words: Cholesterol; epithelial cell; inflammation; interleukin-8; MAP kinase signaling system

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• Min Jung Kim and Jung Yeon Hong contributed equally to this work.
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MATERIALS AND METHODS

Cholesterol depletion and repletion

Methyl-β-cyclodextrin (MβCD; Sigma) binds specifically to cholesterol to disturb the association of proteins with lipid rafts.14 It is therefore presumed to change the structure and function of the cell membrane by disrupting lipid rafts.15,17 A stock solution of 10% MβCD in phosphate-buffered saline (PBS) was stored at 4°C. This solution was used at concentrations of 0.5, 1, and 2% (v/v). After serum starvation for 24 h, cells were incubated with the indicated concentrations of MβCD for 1 h at 37°C for cholesterol depletion. The culture medium was replaced with fresh serum-starved medium at the indicated times, and the cells were maintained at 37°C in an incubator with 5% CO2.

Cell viability

A 549 cell viability at various concentrations of MβCD was measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The day before the experiment, 100 μL cells were seeded into 96-well microplates at a density of 1 × 104 cells per well. After 24 h of incubation, 10 μL cells per well were treated with various concentrations of MβCD for 1 h, followed by incubation with an additional 10 μL Cell Counting Kit-8 solution for 1 h. The absorbance was then measured at 450 nm with an enzyme-linked immunosorbent assay (ELISA) reader; the compensated absorbance was 590 nm.

Lipid extraction and cholesterol assay

A 549 cells pretreated with MβCD to deplete cholesterol were washed in cold PBS buffer and extracted with Folch solution containing chloroform and methanol at a 2:1 ratio. The cholesterol released by the Folch method was solubilized in the organic phase, and the amount of cholesterol was measured with a cholesterol probe following vaporization of the organic phase. The organic phase was dried under a speed vacuum (Savant Instruments Inc., New York, NY, USA) for 30 min, then solubilized with 2-propanol and 10% Triton X-100. Membrane cholesterol was assayed using a Cholesterol/Cholesteryl Ester Quantitation Kit (Calbiochem-Novabiochem, La Jolla, CA, USA), according to the manufacturer’s instructions. Samples and standards were loaded and reacted by the addition of a reaction mixture containing cholesterol buffer, probe, and enzyme without light for 1 h. The absorbance at 570 nm was measured with an ELISA reader; the compensated absorbance was 590 nm. A BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to quantify the extracted proteins, which were collected with Mammalian Protein Extraction Reagent (Pierce) and normalized to the volume.

Cell culture

The human epithelial-like lung carcinoma cell line A 549 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in F12K medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum containing 100 U/mL penicillin and streptomycin (GibcoBRL, Grand Island, NY, USA). At all stages of culture, the cells were maintained in an incubator at 37°C with 5% CO2.

Measurement of human IL-8 and MAP kinase (MAPK) assay

Reverse transcription-PCR (RT-PCR) was used to examine the mRNA expression of IL-6, IL-8, and TNF-α. Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) prior to cDNA synthesis. PCR amplification was carried out using specific primer pairs. The primer sequences used were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank). Fold changes were calculated using the comparative ΔΔCt method. The gene encoding glyceraldehyde-3-phosphate dehydrogenase (GADPH) was analyzed as an internal control in each sample.

Western blot analysis

MβCD-treated cells were lysed with lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA; 10% glycerol; and 0.5% NP-
Protein samples were mixed with 5× SDS-PAGE buffer containing β-mercaptoethanol and heated at 95°C for 5 min. Whole-cell lysates (30 μg) were separated by 10% SDS-PAGE, then electrotransferred to nitrocellulose membranes (GE Healthcare, Stockholm, Sweden). The membranes were blocked for 1 h with 5% skim milk in TBST buffer at room temperature. The blots were incubated overnight with specific antibodies (1:1,000), followed by incubation with secondary antibodies (1:2,000), and then washed three times with TBST buffer at 4°C. After incubation with secondary antibodies (1:2,000), the signal was detected using the ECL Plus (GE Healthcare) and CL-XPosure™ film (Thermo Scientific, Waltham, MA, USA) or an ImageQuant™ LAS 4,000 Mini Biomolecular Imager (GE Healthcare).

Statistical analysis

All data are expressed as the mean ± SEM of at least 3 individual experiments. A statistical analysis comparing the treatment and control groups was carried out using Student’s t-test. *P < 0.05 was considered significant.

RESULTS

Induction of cholesterol depletion by MβCD in A 549 cells

We estimated the amount of cholesterol in the growth media of control and MβCD-treated cultures. After treatment with 0.5, 1, or 2% MβCD for 1 h at 37°C, the cells showed a decreased amount of cholesterol (3.15 ± 0.52, 4.73 ± 0.75, and 4.17 ± 0.68 μg/mL, respectively) compared to untreated cells (8.22 ± 0.11 μg/mL). However, there was no significant difference in the amount of cholesterol among the MβCD-treated groups. In addition, the amount of cholesterol in the cell was at its lowest following treatment with 0.5% MβCD, showing an approximately 60% decrease compared to the control (Fig. 1). Subsequent experiments were thus carried out using epithelial cells treated with 0.5% MβCD.

Alteration of cell viability by MβCD

To determine whether cholesterol depletion by MβCD had any effect on cell viability, we compared the untreated cells to the A 549 cell cultures treated with 0.5, 1, or 2% of MβCD for 1 h. There were no dramatic changes in overall cell viability compared to the control (96.8, 95.3, and 94.2%, respectively, Fig. 2) but statistical differences showed in 1 and 2% of MβCD-treated cells. We concluded that 0.5% MβCD should be used in our subsequent experiments.

Effect of cholesterol depletion on human IL-8 production

The effect of cholesterol depletion on IL-8 production was examined in airway epithelial cells treated with 0.5% MβCD. Cholesterol-depleted cells produced more IL-8 than control cells at different time points (2, 5, 10, and 24 h). IL-8 production increased in a time-dependent manner, with maximum production at 24 h. Cells treated with 0.5% MβCD produced 300.4 ± 7.9 pg/mL at 2 h, 477.7 ± 1.5 pg/mL at 5 h, 816.7 ± 55.2 pg/mL at 10 h, and 867.6 ± 38.9 pg/mL at 24 h compared to 40.8 ± 2.3 pg/mL at 2 h, 80.4 ± 0.2 pg/mL at 5 h, 158.2 ± 8.3 pg/mL at 10 h, and 244.6 ± 6.0 pg/mL at 24 h for control cells. We verified that the IL-8 level decreased after treatment with both 0.5% MβCD and 70 μg/mL cholesterol, similar to in the control cells (Fig. 3A). When tested by RT-PCR with GAPDH as an internal control, the A 549 cells depleted of cholesterol with 0.5% MβCD showed greater IL-8 mRNA expression. IL-8 expression peaked at 2 h and returned to baseline 24 h after MβCD stimulation (Fig. 3B).

Role of MAPK signaling in IL-8 production

To investigate the link between IL-8 production in cholesterol-depleted cells and the MAPK signaling pathway, which is in-
involved in airway inflammatory diseases, we examined the effect of MAPK inhibitors on IL-8 production. IL-8 production was decreased by pretreatment with the ERK inhibitor U0126 (50 μM), but JNK inhibitor II (100 μM) and the p38 MAPK inhibitor SB202190 (50 μM) did not significantly reduce IL-8 production (Fig. 4A). The level of IL-8 was decreased by almost 80% by U0126 but by only 10.9 and 31.4% by JNK inhibitor II and SB202190, respectively, compared to MβCD treatment alone.

To demonstrate the intracellular activation of MAPK signaling, we examined the phosphorylation of ERK, p38 MAPK, and JNK in MβCD-treated cells from 0 to 60 min. As shown in Fig. 4B, we detected small amounts of phosphorylated ERK 1/2 MAPK in untreated A 549 cells, whereas treatment with MβCD induced ERK 1/2 MAPK phosphorylation. ERK 1/2 MAPK activation was maximized at 30 min, but neither p38 MAPK nor JNK activation was detected.

Effect of cholesterol depletion on the production of other cytokines

To identify the effect of cholesterol depletion on inflammatory responses in the airway, we examined the production of other cytokines released from A 549 cells. When tested by RT-PCR with GAPDH as an internal control, cholesterol depletion by MβCD resulted in elevated IL-6 and TNF-α mRNA expression in the A 549 cells. IL-6 and TNF-α expression peaked 2 h after MβCD stimulation (Fig. 5A and B).

DISCUSSION

The epithelium, which represents the first contact surface with the external environment, forms a tight and impermeable barrier for the maintenance of tissue homeostasis under normal circumstances. The structural integrity of the epithelium is also
Cholesterol, an essential component of the plasma membrane, has been implicated in the regulation of membrane fluidity, permeability, and cell surface receptor function.4–6 Because cholesterol contributes to the establishment of a cornified barrier, it plays a crucial role in keratinocytes. Atopic dermatitis has even been shown to occur in parallel with keratinocytes disrupted by lipid rafts.17 We hypothesize that inflammatory diseases of the airway are influenced by airway epithelial cells with disrupted lipid rafts, similar to keratinocytes in atopic dermatitis.

To clarify the relationship between cholesterol and inflammation of the airway, we used MβCD to damage lipid rafts in airway epithelial cells. We identified a concentration of MβCD capable of inducing significant changes in cholesterol levels in the cell. We then demonstrated that the amount of cholesterol in the cell was significantly decreased after incubation with 0.5% MβCD, without effect on cell viability.

IL-8 is a major chemokine of the CXC chemokine subfamily that acts as a chemoattractant for neutrophils released from the bronchial epithelium.8 IL-8 plays a key role in triggering and perpetuating inflammation in patients with severe asthma. In addition, the expression and release of IL-8 increase in response to other stimuli such as allergens and infectious particles.10–13 Hence, we used IL-8 as an inflammatory marker in this study, and IL-8 production rose in cholesterol-depleted epithelial cells. Based on these results, we recognize that IL-8 production in human respiratory epithelial cells could be affected by the cholesterol content in the membrane through the depletion of cholesterol in the cell in the absence of any other allergen.

MAPK regulates IL-8 expression and release in response to various allergens and viruses in airway epithelial cells, resulting in an influence on cell signaling pathways in diseases of the airway. Among ERK, JNK, and p38 MAPK, ERK-regulated IL-8 production has been suggested as a distinct signaling process in chronic diseases of the airway.10–13 We investigated the effect of MAPK inhibitors on IL-8 production using U0126, JNK inhib-
itor II, and SB202190 and sought to determine whether IL-8 production in cholesterol-depleted cells was affected by the MAPK signaling pathway. Our results suggest that activation of the ERK pathway is a relatively strong contributor to IL-8 production in cholesterol-depleted cells, in agreement with previous studies of allergen-induced inflammation of the airway.10,13

In addition, we investigated the expression of other cytokines generated by airway epithelial cells. IL-6 can stimulate T cells and contribute to the development of Th2-mediated inflammation. It was previously demonstrated that epithelial IL-6 expression increased after exposure to fungi, house dust mites, or common respiratory viruses.1 In addition, airway epithelial cells produce pleiotropic cytokines, including TNF-α, which plays an important role in severe refractory asthma. Targeting TNF-α has been shown to reduce pleiotropic cytokines, including TNF-α.17 What is interesting is that patients with genetic differences in their keratinocytes from patients with atopic dermatitis.18 Genome expression profiling has been performed in cholesterol-depleted keratinocytes from patients with atopic dermatitis.17 What is interesting is that patients with genetic differences tend to be susceptible to both this condition and other related allergic diseases.24,25 Such large-scale genome profiling and genetic experiments have confirmed that the disruption of lipid rafts can lead to inflammatory disease under certain circumstances.

In conclusion, our findings suggest that the disruption of lipid rafts plays an important role in inflammation of the airway. Bronchial epithelial cells with cholesterol depletion released a large amount of IL-8, and IL-8 production was regulated by the MAPK signaling pathway, predominantly through ERK. In addition, cholesterol depletion in airway epithelial cells induced the elevation of other proinflammatory cytokines generated by the airway epithelium. Considering that the bronchial epithelium may be a novel target for new therapies for inflammatory diseases of the airway, additional studies of cholesterol and lipid rafts in human airway epithelial cells are needed.

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