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Effect of urban particles on human middle ear epithelial cells

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ABSTRACT

Objectives: The aim of this study was to examine the cytotoxic effect and inflammatory response of human middle ear epithelial cells (HMEECs) induced by urban particles (UP).
Materials and methods: Cell viability following UP exposure was assessed in HMEECs using the CCK 8 assay. The expression levels of the inflammation-related genes (COX-2 and MUC5AC) were analyzed using semi-quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and COX-2 production was analyzed using western blotting.
Results: Treatment with UP decreased cell viability in HMEECs in a dose- and time-dependent manner. UP exposure induced the significantly increased expression of COX-2 and MUC5AC. Western blotting showed dose dependently increased expression of COX-2 production.
Conclusions: UP decreased cell viability, increased the inflammatory response, and increased mucin gene production in HMEECs. These findings indicate that exposure to UP can contribute to the development of otitis media.

1. Introduction

Otitis media (OM) is an inflammatory disease of the middle ear cavity and one of the most common disease encountered in children [1]. At least 80% of children experience one or more episode of OM by the age of 3 years [2,3]. There are approximately 13.6 million pediatric office visits annually in the United States, and an estimated annual cost over USD 3 billion [4].

There are multiple factors that can give rise to OM, including a viral or bacterial infection, biofilm formation, congenital anomaly, Eustachian tube dysfunction, and environmental factors such as smoking and air pollution [5–7]. Previously, we have reported that environmental factors including air pollutants, chemicals, and micro-particles can induce an inflammatory response and contribute to the pathophysiology of OM [8–10]. In addition, epidemiologic studies have demonstrated that frequent exposure to air pollutants can cause OM in children [11–13].

Particulate matter (PM) consists of tiny droplets of solid or liquid matter suspended in the air in the form of atmospheric aerosol. PM is usually classified as suspended particulate matter, respirable suspended particle (RSP; particles with diameter of 10 micrometers or less), fine particles (diameter of 2.5 μm or less), ultrafine particles, and soot.

The sources of PM can be man-made or natural, and PM can adversely affect human health. Some particulates occur naturally, originating from volcanoes, dust storms, forest and grassland fires, living vegetation, and sea spray. Human activities, such as the burning of oil or coal, power plants, smoking, vehicles, and various industrial processes also generate significant amounts of PM. Oil and coal are the primary sources for heating homes and supplying energy, especially in developing countries. The establishment of large cities presents a threat to human health due to exposure to high concentrations of PM.

PM has been shown to induce inflammatory responses in airways and cause asthma in a mouse model [14]. PM increased the expression of tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), and interleukin-6 (IL-6) in the rat lung [15]. These findings using animal models support the possibility that PM is an important risk factor for the induction of OM. However, PM-induced inflammation has not been fully examined in human middle ear epithelial cells.

PM is classified according to particle size. PM that is less than 50 μm is called total suspended particles or suspended PM. PM that is less than 10 μm is called PM-10 or respirable suspended particles [16]. Inhalation of PM-10 is believed to have negative effects on human health, including asthma, lung cancer, cardio-vascular and respiratory diseases. PM that is less than 2.5 μm is

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called PM-2.5 or fine particles, and can have the worst effect on human health among PM of different sizes [17].

Urban particles (UP) are a type of suspended PM that consist of particles of various sizes with an average size of about 10 μm. UP contains many environmental materials including sodium, magnesium, aluminum, sulfur, chlorine, potassium, calcium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, arsenic, bromine, rubidium, strontium, cadmium, antimony, cerium, and lead. UP is atmospheric PM collected in an urban area and is intended primarily for use as a control material and in the evaluation of methods used in the inorganic analysis of atmospheric PM. Inhalation of UP may influence various human health conditions such as OM and respiratory disease [17,18].

The aim of this study was to examine the cytotoxic effect and inflammatory response of human middle ear epithelial cells (HMEECs) induced by UP.

2. Materials and methods

2.1. Preparation of urban particles and cell culture

Urban PM (SRM 1648) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). HMEECs (kindly provided by Dr. David J. Lim, House Ear Institutes, LA) were maintained in a mixture of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) and bronchial epithelial basal medium (Lonza, Walkersville, MD, USA) (1:1) [9,19,20]. The cells were grown to 60% confluence in six-well culture plates and kept at 37 °C in a carbon dioxide-enriched (95% air, 5% CO_2) humidified atmosphere. After starvation for 2 h, PM was added and the cells were incubated. The experimental group received a various concentrations of PM up to 300 μg/ml while the control group did not receive PM.

2.2. Cell viability assay

Cell viability was measured using a cell counting kit (CCK08; Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. HMEECs were seeded in 96-well plates, each well containing 1×10^4 cells in serum-free media for 24 h. Then, fresh media containing final PM concentrations of 0, 25, 50, 100, 150, 200, 250, or 300 μg/ml was added to each of 6 wells and cells were incubated for 24 and 48 h. At the end of the treatment, CCK-8 solution was added to each well and incubated for 150 min at 37 °C. Then, the plates were put on a shaker to mix at room temperature for 5 min and the optical density was measured at 450 nm using a microplate reader (SpectraMax plus 384, Molecular devices, Sunnyvale, USA).

2.3. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis

Primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed using a LightCycler 480 Real-time RT-PCR System (Roche, Basel, Switzerland). Each reaction mixture contained 10 μl of LightCycler 480 SYBR Green I Master (Roche), 4 pmol of sense and antisense primers each, and 0.4 μl of cDNA in a final volume of 20 μl. Reaction mixtures were incubated at 95 °C for 5 min to activate FastStart Taq DNA Polymerase, followed by amplification for 50 cycles. Data were analyzed using LightCycler 480 software 1.5 (Roche). Target mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, and calculated using the comparative Ct method. Six cell pellets were used for each experimental condition. Primers were constructed for COX-2 (forward; TTGCTGGCAGGGTTGCTGGT, reverse; TTGCTGGCAGGGTTGCTGGT), MUC5AC (forward; CAGCACAACCCCTGTTTCAAA, reverse; GCGCACAGAGGATGACAGT) and GAPDH (forward; GAGTCCACTGGCGTCTTCAC, reverse; TTCACACCCATGACGAACAT).

2.4. Western blot analysis

HMEECs were seeded in 96-well plates, each of the 6 wells containing 2.2×10^5 cells for experimental condition. The cells were then stimulated with PM 0, 100, 200, or 300 μg/ml for 24 h. After treating the HMEECs with PM, the medium was removed and the cells were washed twice in phosphate-buffered saline (PBS; 10 mM, pH 7.4). Then, 0.2 ml of lysis buffer (PRO-PREP; Intron, KOREA) was added and after a 1-h incubation at −20 °C, the cells were centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant that contained the total cell lysate was collected and stored at −20 °C. Protein concentration of the lysates was measured by Quick start Bradford 1X Dye Reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were mixed with sample buffer (62.5 mM Tris–HCl [pH 6.8], 1% sodium dodecyl sulfate, 2.5% glycerol, 0.5% 2-β-mercaptoethanol, bromophenol blue), boiled for 5 min, and separated by electrophoresis on 10% Tris–HCl gels. The protein content of the gels was transferred to a PVDF membrane (Hybond P; Amersham Biosciences Corp.), and the membranes were blocked with TBS-T (20 mM Tris, 500 nM NaCl, with 0.1% Tween-20) containing 5% (w/v) skim milk for 1 h at room temperature. Membranes were probed with antibodies against COX-2 (1:200, Santa Cruz Biotechnology, Dallas, Texas, USA) and β-actin (1:3000, Sigma–Aldrich, St. Louis, MO, USA) followed by peroxidase-conjugated anti-mouse IgG (1:5000, Vector laboratories, Burlingame, CA, USA). The membranes were developed using an ECL detection kit (Pierce, Rockford, IL, USA) and the signal was captured on an image reader (LAS3000; Fuji Photo Film, Tokyo, Japan). Results were obtained from three independent experiments.

2.5. Statistical analysis

All data are expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used to determine statistical significance differences between control and groups at each time or dose point. Scheffe's *F*-test was used to correct for multiple comparisons when statistically significant differences were identified in the ANOVA. $P < 0.05$ for the null hypothesis was accepted as indicating a statistically significant difference. Statistical analyses were performed using SPSS for Windows (Ver. 12.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cell viability assay

Six cell pellets were used for each experimental condition. UP decreased cell viability in a dose- and time-dependent manner. At low concentrations of UP, the decrease in cell viability was minimal. However, at higher concentrations (>200 μg/ml), the decrease was significant at both time points (Fig. 1). Microscopic evaluation showed a unified, single layer of epithelial cells in the control group (Fig. 2A). In the UP-treated group (300 μg/ml), particles were attached to the cells and the number of viable cells markedly decreased (Fig. 2B).

3.2. Real-time RT-PCR analysis

The COX-2 and MUC5AC gene expression was evaluated using real-time RT-PCR. COX-2 mRNA expression was significantly