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Effect of acrolein, a hazardous air pollutant in smoke, on human middle ear epithelial cells

Jae-Jun Song [a], Jong Dae Lee [b], Byung Don Lee [b], Sung Won Chae [c], Moo Kyun Park [b,*]

[a] Department of Otolaryngology-Head and Neck Surgery, Dongguk University Ilsan hospital, Ilsan, South Korea
[b] Department of Otolaryngology-Head and Neck Surgery, Soonchunhyang University College of Medicine, Seoul, South Korea
[c] Department of Otolaryngology-Head and Neck Surgery, Korea University College of Medicine, Seoul, South Korea

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ABSTRACT

Objective: Acrolein is a hazardous air pollutant. Tobacco smoke and indoor air pollution are the main causes of human exposure. Acrolein has been shown to cause cytotoxicity in the airways and induce inflammation and mucin production in pulmonary cells. We investigated whether acrolein caused cytotoxicity, induced inflammation or increased expression of mucin in immortalized human middle ear epithelial cell lines (HMEECs).

Methods: Cytotoxicity following acrolein treatment was investigated using the MTT assay, flow cytometry, and Hoechst 33342 staining of HMEECs. We measured expression of inflammatory cytokines tumor necrosis factor (TNF)-α and cyclo-oxygenase (COX)-2 and the mucin gene *MUC5AC* using semi-quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting.

Results: Exposure to >50 μg/mL acrolein caused a decrease in cell viability. Acrolein induced apoptosis and necrosis at 50 μg/mL. Acrolein at 5–50 μg/mL increased expression of TNF-α and COX-2, as shown by RT-PCR and Western blotting. Acrolein exposure at 5–50 μg/mL for 2–24 h increased MUC5AC expression, as determined by RT-PCR.

Conclusion: Acrolein decreased cell viability, induced an inflammatory response, and increased mucin gene expression in HMEECs. These findings support the hypothesis that acrolein, a hazardous air pollutant in tobacco smoke and ambient air, is a risk factor for otitis media.

1. Introduction

Otitis media is a worldwide pediatric healthcare problem. It affects hearing, speech, language development, and balance, and causes poor educational performance. It is the most common condition encountered in children and causes the most visits to a physician. At least 80% of children will have experienced one or more episode of otitis media by the age of 3 years [1,2].

Known risk factors of otitis media include infections, Eustachian tube dysfunction, allergy, immunology, biofilms, gastroesophageal reflux, and various environmental factors. Environmental factors include smoking, seasonality, obesity, daycare, and air pollution. Identification of preventable risk factors for otitis media, such as cigarette smoking, may have significant implications for health-care costs. Epidemiological studies have demonstrated that children frequently exposed to air pollutants have a higher incidence of otitis media. *In vitro* and *in vivo* studies have shown that air pollutants, such as tobacco smoke and diesel exhaust, can induce otitis media [3–6].

Acrolein is a respiratory irritant present in both the environment and tobacco smoke. It can be generated during cooking and endogenously at sites of injury. Acrolein is listed as a hazardous air pollutant by the US Environmental Protection Agency [7]. Acrolein has been shown to cause inflammation, cytotoxicity in the airways, and increased mucus production [8,9]. However, whether acrolein induces otitis media has not yet been determined.

To establish whether acrolein can induce otitis media, we investigated whether it affected viability of middle ear epithelial cells and induced inflammation in human middle ear epithelial cell lines (HMEECs), by measuring the expression levels of inflammatory cytokine and mucin genes.

2. Materials and methods

This study was approved by the Soonchunhyang University Bucheon Hospital Institutional Review Board.

* Corresponding author at: Department of Otorhinolaryngology-Head and Neck Surgery, Bucheon hospital, Soonchunyang University College of Medicine, 1174 Jung-dong, Wonmi-gu, Bucheon, Gyonggi-do, 420-767 South Korea.
Tel.: +82 31 621 5054; fax: +82 31 621 5440.
E-mail address: aseptic@schmc.ac.kr (M.K. Park).

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2.1. Cell culture

HMEECs were kindly provided by Dr. David J. Lim, House Ear Institutes, Los Angeles, USA [10]. HMEECs were maintained in a mixture of DMEM and BEBM (1:1) with various supplements, as described previously [6]. Cells were grown in a humidified atmosphere at 37 °C, containing 5% CO_2 and 95% air. Growth medium was changed every 3 days. After 7 days, the cells were stimulated with acrolein (National Institute of Standards and Technology, Gaithersburg, MD, USA). Acrolein was suspended in phosphate-buffered saline (PBS).

To determine the time course of acrolein stimulation, HMEECs were stimulated for 0, 1, 6, 12, and 24 h. To determine the dose-dependent manner of acrolein stimulation, HMEECs were stimulated with 0, 1, 5, 10, 20, and 50 μM acrolein. The control group was stimulated with PBS. Wells were sealed during the stimulation because acrolein is highly evaporative.

2.2. Cell viability assay

Cell viability was measured using the MTT (3-(4, 5-dimethylthiazoyl-2-yl) 2,5 diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) assay. HMEECs were seeded in plates and the culture medium was replaced after 24 h. Forty microliters of fresh MTT (5 g/L DW) were added. After the mixture was shaken at room temperature for 30 min, the optical density at 595 nm was measured using a microplate reader. Viability was expressed as a percentage of the values (corresponding to 100%) of untreated cells. Results were obtained from three repeated experiments.

2.3. Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometry

Annexin V–FITC/PI staining was used to determine the percentage of apoptotic cells. Cells were seeded in six-well culture plates after treatment with acrolein 10, 50 and 100 μM for 24 h. After collection, cells were washed with PBS and resuspended in 1× binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM CaCl]. Cells were transferred into a FACS tube, and stained using an annexin V–FITC apoptosis detection kit following the manufacturer's protocol (BD, San Diego, CA, USA). The cell cycle was assessed using a flow cytometer (Beckman Coulter, Fullerton, CA, USA). Results were obtained from three repeated experiments.

2.4. Hoechst 33342 staining

The nuclei of the HMEECs after 24 h treatment with acrolein 10, 50 and 100 μM were stained with chromatin dye Hoechst 33342. The cells were washed twice with PBS and fixed with 3.7% glutaraldehyde for 10 min at room temperature. After fixation, the cells were washed twice with PBS and incubated with 10 μg/mL Hoechst 33342 (Sigma–Aldrich, St. Louis, MO, USA) for 10 min at room temperature in the dark. After two washings, the cells were evaluated under an inverted fluorescence microscope (BX61; Olympus, Japan). Results were obtained from three repeated experiments.

2.5. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA from HMEECs was extracted using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. RNA was reverse-transcribed by incubation with 10 mM dNTP, 0.1 M dithiothreitol, 1 μL Oligo (dT) (500 μg/mL), and 1 μL SuperScript II (200 U/μL; Life Technologies, Grand Island, NY, USA) at 42 °C for 50 min, followed by heat inactivation at 70 °C for 15 min. After reverse transcription, aliquots of the cDNA were transferred to tubes containing primer pairs specific for the tumor necrosis factor (TNF)-α, cyclo-oxygenase (COX)-2, MUC5AC, and β-actin genes. The primer sequences of the genes used in this study are sense primer: 5′-AAGACCTTCTCCCCATTCTG-3′ and antisense primer" 5′-TTCCTCTGTGTGTGGATCTAG-3′ for TNF-α; sense primer: 5′-GTCAAAACCGAGGTGTATGT-3′ and antisense primer 5′-TGTGATCTGGATGTCAACAC-3′ for COX-2; sense primer: 5′-GGACTTCGAGCAAGAGATGG-3′ and antisense primer" 5′-AGCACTGTGTTGGCGTACAG-3′ for β-actin.

PCR amplification was performed in a thermocycler for 35 cycles (one cycle: 20 s at 94 °C, 10 s at 55 °C, and 30 s at 72 °C) with an initial denaturation at 94 °C for 2 min and a final extension at 72 °C for 5 min. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide stain, alongside a 100-bp DNA ladder (Bioneer, Daejon, Korea). Results were from three repeated experiments using triplicate samples.

To analyze the RT-PCR results semi-quantitatively, gel images were scanned and the intensity of the bands measured using a Scion imager (Scion, Frederick, MD, USA). Relative mRNA expression was calculated by determining the ratio between the individual mRNA and β-actin.

2.6. Western blotting

HMEECs were stimulated with 1, 5, 10, 20 and 50 μM acrolein for 1, 2, 6, 12, and 24 h. After treating the HMEECs with acrolein, the medium was removed and the cells washed twice in PBS (10 mM, pH 7.4). The cells were incubated in 0.4 mL ice-cold lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.50, 1 mmol/L EDTA, 0.1% Triton X-100] containing 0.5% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). The cells were centrifuged at 13,000 × *g* for 25 min at 4 °C, and the supernatant (total cell lysate) was collected, aliquoted, and stored at −70 °C. The protein concentration was determined using an RC DC Protein Assay kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Aliquots (25 μg protein) were mixed with sample buffer (Bio-Rad) containing 2% mercaptoethanol, boiled for 5 min, and separated by electrophoresis on 12% Tris–HCl gels. The contents were transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences), and the membranes were blocked with PBS, 0.1% Tween-20 containing 5% (w/v) dry milk, and 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were probed with TNF-α (1:1000; Cell Signaling, Inc. Beverly, MA, USA), COX-2 (1:200 Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Merck Biosciences, San Diego, CA, USA), or control goat IgG, followed by donkey anti-goat IgG coupled to horseradish peroxidase (1:10,000 dilution; Jackson Immuno Research Laboratories, West Grove, PA, USA). The membranes were developed using an ECL detection kit (Pierce, Rockford, IL, USA) and exposed to X-ray film (XAR5, Kodak, Rochester, NY, USA). Results were obtained from three repeated experiments.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of differences between controls and groups at each time point or dose. Scheffé'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. All data are expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS, version 11 (Chicago, IL, USA).