Chinchilla Middle Ear Epithelial Mucin Gene Expression in Response to Inflammatory Cytokines

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Objectives: To review the importance of mucin on a molecular level in the understanding of the pathophysiology of otitis media and to present new data demonstrating differential up-regulation of specific mucin genes during inflammation of middle ear epithelium.

Design: Primary chinchilla middle ear epithelial cultures (CMEECs) were established and exposed to tumor necrosis factor α and interleukin 1β. Expression of chinchilla mucin genes 1, 2, 4, and 5AC was analyzed by means of reverse transcriptase–polymerase chain reaction after this exposure and compared with that of controls. Mucin secretion was also characterized by means of exclusion chromatography and liquid scintillation.

Results: The CMEECs exposed to interleukin 1β and tumor necrosis factor α demonstrated significant up-regulation of mucin gene 2 (P = .005 and P = .007, respectively) and trends suggestive of up-regulation of mucin gene 5AC compared with the controls. These CMEECs also demonstrated significant increases in secretion of mucin compared with controls. Mucin genes 1 and 4 did not demonstrate up-regulation.

Conclusions: In CMEECs, the inflammatory mediators tumor necrosis factor α and interleukin 1β cause a differential up-regulation and expression of mucin genes. Elucidating the effect of specific cytokines on the regulation of mucin secretion in the middle ear is vital to understanding the pathophysiology of otitis media. A greater understanding of these mechanisms and variations between individuals and pathogens has the potential to significantly alter the approach and management of otitis media in children and lead to novel therapeutic interventions.

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Otitis media is the most common diagnosis in children who visit physicians for illness in the United States, causing an estimated 5 million annual episodes at a national cost of $3 billion to $6 billion. Approximately 5% to 10% of acute otitis media progresses to chronic otitis media with effusion, which is a leading cause of hearing loss in children. The most accepted treatment of chronic otitis media with effusion is tympanostomy tube insertion, which is now the most frequent pediatric surgical procedure requiring a general anesthetic.

Otitis media has significant potential for morbidity and presents increasing therapeutic challenges imposed by antimicrobial resistance. Despite this, much is still unknown about the cellular and molecular events in this disease process. Mucins are high-molecular-weight glycoproteins produced in a variety of conditions but are particularly important in respiratory epithelium such as that found in the nasal cavity, trachea, and middle ear. Variation in the quantity and character of middle ear secretions and specifically mucin secretion is known to be important in the pathophysiological mechanisms of otitis media. Mucins are the only component of middle ear effusions responsible for its rheological properties and are responsible for creating a high-viscosity fluid that can prevent normal mucociliary clearance, which, in turn, causes abnormalities such as chronic otitis media and hearing loss. However, mucins are also known to be important in normal host defenses through participating in mucociliary clearance of pathogens, providing protective barriers to underlying epithelium, and interacting with the host’s innate immune mechanisms. In addition, evidence exists that epithelial mucins interact with biofilms, which have recently been implicated as central to the pathogenesis of chronic otitis media, further supporting the importance of these glycoproteins in regulating middle ear epithelial physiology. The mounting evidence of the significance of mucin in middle ear...
pathophysiology prompted this investigation into the potential of differential up-regulation of middle ear mucin genes during periods of middle ear inflammation.

METHODS

ESTABLISHMENT OF CULTURES

All experiments conducted during this investigation were performed using in vitro cell culture techniques in the chinchilla that have been previously described. Briefly, cells used in all cultures were harvested from mature (aged 6-10 months) mixed-breed chinchillas weighing 400 to 600 g (Moulton Chinchilla Ranch, Rochester, Minn). The temporal bone, including the tympanic membrane and middle ear cavity, was removed bilaterally. Animals were treated in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act; the animal use protocol was approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee.

Transbullar injection of 0.09% protease type XIV (Sigma-Aldrich Corp, St Louis, Mo) in Dulbecco Modified Eagle Medium/F-12 with 0.3% fetal bovine serum containing a 1% combination of insulin, transferrin, and selenium, 50 ng/mL of hydrocortisone (Sigma-Aldrich Corp), and antibiotic/antimycotic solution (1000 µL of penicillin G sulfate, 100 µg/mL of streptomycin sulfate, and 250 ng/mL of amphotericin B) was used to fill the middle ear cavity and bulla (the components added to growth media were obtained from Gibco Invitrogen Corporation [Carlsbad, Calif] unless otherwise indicated). The temporal bone was wrapped in laboratory film (Parafilm M, Chicago, Ill) and placed at a cell count of approximately 105 viable cells.

Suspended cells were pooled and centrifuged, and the pellet was then resuspended in 1 mL of Dulbecco Modified Eagle Medium/F-12 with 0.3% fetal bovine serum, 1% insulin-transferrin-selenium, 50 ng/mL of hydrocortisone, and the antibiotic/antimycotic solution. The cells were plated in 24-well, collagen type I–coated plates (Becton, Dickinson and Co, Bedford, Mass) at a cell count of approximately 1 × 10⁶/cm². The cells were grown in a humidified atmosphere at 37°C containing 95% air/5% carbon dioxide. All cells in these experiments were from primary cultures, and did not undergo passage.

Metabolic labeling with tritiated glucosamine (Becton, Dickinson and Co, Bedford, Mass) was performed using in vitro cell culture techniques in the chinchilla. Confluent chinchilla middle ear epithelial cultures (CMEECs) were incubated with 5 pCi (0.185 MBq) of tritiated glucosamine per 1 mL of full growth media at 37°C in 95% air/5% carbon dioxide for 24 hours before the assay. These media were then aspirated and replaced with new media containing the appropriate experimental culture conditions.

EXPERIMENTAL CONDITIONS

After metabolic labeling, cell cultures were incubated with new full-growth media containing 200 ng/mL of tumor necrosis α (TNF-α) or interleukin 1β (IL-1β) (R&D Systems, Inc, Minneapolis, Minn). Labeled cells incubated only with full growth media and without cytokine exposure served as control cells. After 16 hours, 900 µL of media was aspirated and reserved for mucin quantification. Aspirates were stored at −80°C for less than 2 weeks. After harvesting the aspirated media, the remaining cells were prepared for RNA harvest to assess mucin gene expression as described below. Experiments were repeated 4 times, with each experimental condition performed in duplicate or triplicate to give a total of 10 data points for each experimental condition, ie, cells exposed to TNF-α, cells exposed to IL-1β, or cells without cytokine exposure serving as controls. These repeated cell culture experiments provided the data for the mucin secretion and mucin gene expression experiments.

QUANTIFICATION OF MUCIN SECRETED

Mucin secretion from the epithelial cells was analyzed and quantified using procedures previously described in a number of laboratories. These investigations have demonstrated that secretions from respiratory and middle ear epithelia contain high-molecular-weight glycoconjugates. The glycoconjugates resistant to digestion by chondroitinase ABC and excluded after Sepharose CL-4B (Amersham Biosciences, Piscataway, NJ) column chromatography have been identified as mucin.

Stored aspirates were thawed and treated with 0.4 U/mL of testicular chondroitinase ABC (Sigma-Aldrich Corp) at 37°C for 5 hours to digest proteoglycans. After incubation, the digestion mixture was neutralized and applied to a Sepharose CL-4B column (0.7 × 50 cm) (Amersham Biosciences). Equilibrated with phosphate-buffered saline solution containing 0.02% (wt/vol) sodium azide (Sigma-Aldrich Corp). Columns were eluted with phosphate-buffered saline solution containing 0.02% sodium azide, 0.9% sodium chloride, and 5mM diithiothreitol (Sigma-Aldrich Corp) at a constant flow rate of 0.5 mL/min to collect 2-mL fractions. Void volume fractions were mixed with 8 mL of scintillation solution (Ecoscint A; National Diagnostics, Atlanta, Ga), and we counted the radioactivity of fractions with a liquid scintillation system (Tri-Carb 4530; Perkin Elmer, Inc, Downers Grove, Ill). Radioactivity at peak fractions was defined as the amount of mucin in the supernatant. The radioactivity of each sample was divided by its own viable cell number, and the average individual value of radioactivity was calculated. The calculated radioactivity was standardized by expressing the values as radioactivity per 5 × 10⁶ viable cells.

MUCIN GENE EXPRESSION WITH REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTIONS

Standardized conditions were maintained through all experiments to allow for quantification of the reverse transcriptase–polymerase chain reaction (RT-PCR) results and comparison of these results. Uniform cell populations and culture conditions were ensured throughout and measurements were made against standardized curves. Cell culture morphology and colony purity were maintained and assessed as previously described.

Total RNA was harvested from the cells in culture using the RNeasy Mini Kit (Qiagen Inc, Valencia, Calif). Deoxyribonuclease digestion was performed using RQ1 ribonuclease-free deoxyribonuclease (Promega, Madison, Wis). Yield and purity were determined by means of spectrophotometry. Purified RNA was stored at −70°C until RT-PCR analysis. Complementary DNA was obtained using Superscript III RNase H−RT (Invitrogen Corp, Carlsbad, Calif). Each RT reaction used 3 µg of the previously purified RNA. The complementary DNA was amplified by means of PCR (GeneAmp 2400; PerkinElmer, Inc). The 30-µL reaction mixture for PCR contained 1.0 U of platinum Taq DNA polymerase (Invitrogen Corp), 5mM of each deoxyribonucleotide triphosphate (Invitrogen Corp), 0.1µM of each primer, and 10 µL of complementary DNA template. A negative control that contained every component except the RT was used for each reaction. We used glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene to determine the quantity of complementary DNA in each sample. The PCR were run on 2% agarose gel at 100 V. The PCR product was visualized with fluorescent nucleic acid stain (GelStar; Cambrex Bio Science Rockland, Inc, Rockland, Me) and quantitated using the Kodak 1D Image Analysis Software Version 3.6.

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The CMEECs incubated with 200 ng/mL of IL-1β exhibited statistically significant increases in mucin secretion compared with controls as measured by means of fractionation with a Sepharose CL-4B column and liquid scintillation of appropriate fractions (P = .005). Similarly, CMEECs incubated with 200-ng/mL TNF-α exhibited statistically significant increases in mucin secretion compared with controls (P = .04) (Figure 2).

Chinchilla mucin gene expression in CMEECs after 16 hours of exposure to 2000-ng/mL IL-1β demonstrated differential up-regulation of mucin genes. The CMUC expression was assessed in the same cell colonies that had exhibited a significant increase in mucin secretion as described above. Compared with controls, CMUC2 demonstrated statistically significant up-regulation when compared with controls (P = .005). Similarly, CMUC2 demonstrated statistically significant up-regulation after 16 hours of CMEEC exposure to 200 ng/mL of TNF-α (P = .007) (Figure 3 and Table 2).

After the exposure to IL-1β and TNF-α, CMUC5AC demonstrated trends suggestive of up-regulation that did not reach statistical significance, and CMUC4 and CMUC1 did not demonstrate any propensity toward up-regulation. Trends suggested that the inflammatory cytokines, particularly TNF-α, might cause some down-regulation of CMUC4, although these results did not reach statistical significance (Figure 3 and Table 2).

Despite the prevalence of otitis media, its potential for morbidity, and the enormous health care expenditures resulting from its treatment, much is still unknown about

**RESULTS**

**STATISTICAL ANALYSIS**

Experimental conditions were repeated 4 times, with each experiment containing replicate wells to provide 10 total data points for each experimental condition. We calculated means with the standard error of the mean for statistical analysis. We analyzed data with paired t tests to assess the difference in mucin gene expression between cells exposed to inflammatory cytokines compared with controls. Similarly, we used t test analysis to compare the mucin secretion in cells exposed to inflammatory cytokines and controls.

**COMMENT**


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Figure 1. Reverse transcriptase–polymerase chain reaction mucin gene products demonstrating up-regulation of chinchilla mucin gene 2 (CMUC2) after exposure of chinchilla middle ear epithelial cultures to tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). Bands labeled as control are middle ear cells that were not exposed to inflammatory cytokines and demonstrate significantly less CMUC2 expression (less bright) than bands exposed to TNF-α and IL-1β.

Figure 2. Total mucin secretion from chinchilla middle ear epithelial cultures compared with controls after inflammatory cytokine (tumor necrosis factor α [TNF-α] and interleukin 1β [IL-1β]) exposure for 16 hours. Control cells were not exposed to inflammatory cytokines. Bars indicate mean values; limit lines, SEM; and asterisk, significant (P < .05) compared with the control culture.

Table 1. Primer Pairs Used in RT-PCRs

<table>
<thead>
<tr>
<th>Gene (Base Pair)</th>
<th>Primer</th>
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<tbody>
<tr>
<td>CMUC1 (123)</td>
<td>Sense: 5'-GGCCTGCTAGCTTACACAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CCCGAGCTTCGCGACCTACGAG-3'</td>
</tr>
<tr>
<td>CMUC2 (145)</td>
<td>Sense: 5'-CCGTCCTCTCCACATCATCTACGAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CTCTCTCCAGCGATGTAAGTGTACGAG-3'</td>
</tr>
<tr>
<td>CMUC4 (102)</td>
<td>Sense: 5'-GCCCAAGCTACGTGAGGACAGTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-ATGAGCTGCTGTGGTTATGTTGTAAGTGA-3'</td>
</tr>
<tr>
<td>CMUC5AC (103)</td>
<td>Sense: 5'-CTCACATATACCACCGCACAGAAGTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-TGACCAGACCTACGTGTCAGACG-3'</td>
</tr>
</tbody>
</table>

Abbreviations: CMUC, chinchilla mucin gene; RT-PCR, reverse transcriptase–polymerase chain reaction.
of IL-1 and TNF-α of control cells. Data are given as mean ± SEM. Figure 3. Expression of chinchilla mucin genes (CMUCs) 2, 5AC, 1, and 4 expression in chinchilla middle ear epithelial cultures (CMECs) after exposure to tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) for 16 hours compared with control cells not exposed to inflammatory cytokines. Bars indicate mean values; limit lines, SEM; and asterisk, significant (P<.05) compared with the control culture.

Table 2. Mucin Gene Expression of Control Cells

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Gene</th>
<th>CMUC2</th>
<th>CMUC5AC</th>
<th>CMUC1</th>
<th>CMUC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100 ± 5.94</td>
<td>100 ± 3.68</td>
<td>100 ± 4.07</td>
<td>100 ± 3.82</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td>133.1 ± 10.233</td>
<td>103.7 ± 6.54</td>
<td>99.2 ± 2.48</td>
<td>93.2 ± 8.59</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>127 ± 4.217</td>
<td>108.7 ± 5.64</td>
<td>101.8 ± 4.75</td>
<td>97.2 ± 11.00</td>
</tr>
</tbody>
</table>

Abbreviations: CMUC, chinchilla mucin gene; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α. *Control cells were not exposed to inflammatory cytokines. Mucin gene expression of cells exposed to IL-1β and TNF-α are presented as percentages of control cells. Data are given as mean ± SEM. †Indicates statistically significant at P<.05.

Mucins are a family of glycoproteins, and at present at least 18 unique human mucin genes have been identified and shown to be expressed in tissues such as lung, nose, salivary glands, and gastrointestinal tract. Mucins can be either membrane bound or secretory, can protect and shield the underlying epithelium, can maintain mucociliary clearance, and can interact with pathogens to affect adherence and host invasion. Variation in the quantity and character of middle ear secretions, specifically mucin, is known to be important in the pathophysiology of otitis media. Mucins are responsible for the high viscosity of middle ear effusions that prevent normal mucociliary clearance, which predisposes to the development of chronic otitis media and subsequent hearing loss.

Each mucin gene product has somewhat different characteristics. Although several studies have examined which of these mucins or their products are expressed in the middle ear, the profile of mucin genes expressed in the middle ear is still not yet clear. Potential variations in this profile or mucin gene expression during periods of inflammation are even less well understood. However, the results of this study clearly demonstrate the ability of differential mucin gene regulation during periods of inflammation and cytokine exposure in middle ear epithelium.

This differential regulation of mucin gene expression may also be important in host-pathogen interactions as otitis media develops into a chronic disease state. Chronic otitis media has recently been described as a biofilm disease. As a biofilm disease, sessile bacterial colonies at a relatively low metabolic rate maintain their viability in part through escaping the host's normal immune surveillance and pathogen clearance mechanisms. Alterations in the mucociliary clearance abilities of the middle ear epithelium are involved in this process, and specific alterations in mucin composition may also be required for these events to take place. Several investigations have demonstrated the importance of mucin-pathogen interactions for the establishment and maintenance of biofilms in other organ systems.

Undoubtedly, differences exist in the middle ear mucin gene expression across populations and between individuals elicited in response to external stimuli such as pathogens, allergens, or other environmental irritants. These pathogen responses also likely vary depending on the type of pathogen involved, the host-pathogen interactions that result, and the type of inflammatory cascade initiated. Some preliminary work in animal models has identified that pneumococcal infection of the middle ear leads to differential up-regulation of mucin genes. Defining the mechanisms involved in these responses on a
molecular and cellular level and characterizing the differences among individuals and pathogens are crucial steps to broadening our understanding of the pathophysiology of otitis media and providing avenues for novel treatment strategies. These avenues may include cytokine and mucin modulation through cytokine inhibitors, modulation of mucin secretion through a more thorough understanding of the signaling pathways leading to mucin gene up-regulation, or reduction in bacterial biofilm formation through greater understanding of middle ear variables required for their formation.

In many respects, these investigations continue a paradigm shift in thinking about otitis media. This change in thinking recognizes the importance of anatomic factors such as eustachian tube dysfunction but that, in addition, molecular and genetic responses to pathogens and mucin gene expression likely play a central role in determining individual susceptibility to otitis media and especially the development of chronic otitis media.

The prevalence, cost, and morbidity of otitis media coupled with the declining efficacy of current treatment methods mandate a better understanding of the pathophysiology of otitis media and subsequent generation of novel treatment strategies.

The study provides evidence that the inflammatory mediators TNF-α and IL-1β cause a differential up-regulation and expression of mucin genes from middle ear epithelium. Elucidating the effect of specific cytokines on the regulation of mucin secretion in the middle ear is vital to understanding the pathophysiology of otitis media. Additional experiments examining the molecular pathways associated with cytokine modulation of mucin gene expression and protein secretion from middle ear epithelium are needed and are ongoing in our laboratory.

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This study was supported by grant NIDCD DC0192 from the National Institutes of Health, Bethesda, Md (Dr Kerschner [primary investigator]).

This study was presented at the 19th Annual Meeting of the American Society of Pediatric Otolaryngology; May 2, 2004; Phoenix, Ariz.

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## CONCLUSIONS

The prevalence, cost, and morbidity of otitis media coupled with the declining efficacy of current treatment methods mandate a better understanding of the pathophysiology of otitis media and subsequent generation of novel treatment strategies.

The study provides evidence that the inflammatory mediators TNF-α and IL-1β cause a differential up-regulation and expression of mucin genes from middle ear epithelium. Elucidating the effect of specific cytokines on the regulation of mucin secretion in the middle ear is vital to understanding the pathophysiology of otitis media. Additional experiments examining the molecular pathways associated with cytokine modulation of mucin gene expression and protein secretion from middle ear epithelium are needed and are ongoing in our laboratory.

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