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

Joseph E. Kerschner, MD; Tanya K. Meyer, MD; Amy Burrows, BS

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,1 causing an estimated 5 million annual episodes at a national cost of $3 billion to $6 billion.2 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.1

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.3,4 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,5-7 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.8-10 In addition, evidence exists that epithelial mucins interact with biofilms,11-13 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. Chinchillas were harvested from the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the National Institutes of Health, Guide for the Care and Use of Laboratory Animals. 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.

Confluent chinchilla middle ear epithelial cultures (Sigma-Aldrich Corp) were prepared for RNA harvest to assess mucin gene expression within the middle ear containing the middle ear epithelial layer. 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 used to grow the cell culture conditions. The temporal bone was wrapped in laboratory film (Parafilm M; 3M, St Paul, Minn). Labeled cells incubated only with full growth media containing 200 ng/mL of tumor necrosis factor alpha (TNF-α) 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.14-15 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 dithiothreitol (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 (Ecocube 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 × 10^5 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 these results. Uniform cell populations and culture conditions were ensured throughout and measurements were made against these results. Uniform cell populations and culture conditions were ensured throughout and measurements were made against these results.

After metabolic labeling, cell cultures were incubated with new growth medium containing a 1% combination of insulin, transferrin, and selenium, 50 ng/mL of hydrocortisone (Sigma-Aldrich Corp), and antibiotic/antimycotic solution (1000 U/mL 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; 3M, St Paul, Minn). Labeled cells incubated only with full growth media containing 200 ng/mL of tumor necrosis factor alpha (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.
with controls. Primer pairs used for the RT-PCR are listed in 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.

**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'-GTGCCCCCTAGCAGTACGG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GAAGTGGCCCTACTAAGTT-3'</td>
</tr>
<tr>
<td>CMUC2 (145)</td>
<td>Sense 5'-CGGTCTCTCTTACACTACCT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CGGTCTCTCTTACACTACCT-3'</td>
</tr>
<tr>
<td>CMUC4 (102)</td>
<td>Sense 5'-GCCCAAGCTACAGTGACTCA-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GCCCAAGCTACAGTGACTCA-3'</td>
</tr>
<tr>
<td>CMUC5AC (103)</td>
<td>Sense 5'-TCACCAATTAACGCAGCAA-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-TCACCAATTAACGCAGCAA-3'</td>
</tr>
</tbody>
</table>

Abbreviations: CMUC, chinchilla mucin gene; RT-PCR, reverse transcriptase–polymerase chain reaction.

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).

Chincilla 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).

**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.

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...
The expression of cells exposed to IL-1 and TNF-α is significant (P<.05) compared with the control culture. 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>Control</th>
<th>100 ± 3.68</th>
<th>100 ± 3.82</th>
<th>100 ± 4.07</th>
<th>100 ± 3.82</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CMUC2</td>
<td>100 ± 5.94</td>
<td>100 ± 3.68</td>
<td>100 ± 4.07</td>
<td>100 ± 3.82</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>CMUC2</td>
<td>133.1 ± 10.23</td>
<td>103.7 ± 6.54</td>
<td>99.2 ± 2.48</td>
<td>93.2 ± 8.59</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>CMUC2</td>
<td>127 ± 4.21</td>
<td>108.7 ± 5.64</td>
<td>101.8 ± 4.75</td>
<td>97.2 ± 11.00</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CMUC, chinchilla mucin gene; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α.

This differential regulation of 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 studies provided 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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