MUC5AC Expression in Human Middle Ear Epithelium of Patients With Otitis Media
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Objective: To compare levels of middle ear (ME) MUC5AC expression in patients with otitis media (OM) with patients without OM. Mucin gene 5AC has been identified as a major secretory mucin in the ME and is fundamentally important in the development of ME mucoid effusions, hearing loss and also provides ME mucosal protection and bacterial clearance.

Design: Case control.

Setting: Tertiary, academic, pediatric otolaryngology practice.

Patients: Patients 9 months to 7 years old undergoing routine tympanostomy tube (TT) insertion for recurrent otitis media (RecOM) or chronic otitis media with effusion (COME) were compared with control patients without a history of OM undergoing cochlear implantation.

Methods: During routine TT placement or cochlear implantation, a 1-mm biopsy sample of the ME epithelium was obtained. RNA was extracted, and real-time reverse transcriptase–polymerase chain reaction was used to quantify levels of MUC5AC expression.

Results: Twenty-three patients with OM (12 with RecOM and with 11 COME) were evaluated using 5 controls. Mean age was not different between groups. In the RecOM group, mean expression of MUC5AC was 25.92 times greater than in controls. In the COME group, the mean expression was 155.40 times greater than in controls.

Conclusions: Levels of MUC5AC expression in the human ME are significantly increased in patients with RecOM and COME compared with controls. This study demonstrates MUC5AC gene changes in patients with OM and highlights the need for greater understanding of the molecular responses in OM; particularly that of mucin. A thorough exploration of these factors will provide opportunities to develop novel interventions for the extremely common problem of OM.


Although otitis media (OM) is a prevalent condition responsible for conductive hearing loss in the pediatric population and substantial health care costs totaling several billion dollars annually in the United States, little is still known about the molecular pathophysiologic characteristics of this condition. Increased production and secretion of mucins in middle ear epithelium (MEE), in response to the inflammatory events that occur in OM, provides both protection to the middle ear (ME) and contributes to ME disease that can subsequently occur. Mucins, such as mucin gene 5AC (MUC5AC), participate in protecting the underlying MEE from pathogen invasion and pathogen damage and assist with pathogen clearance. However, overproduction of these same mucins can lead to increased viscosity of ME fluids, limiting mucociliary clearance in the ME, and eventual accumulation within the ME, leading to hearing loss.1

It has been demonstrated that MUC5AC is an important component of the secretory mucins in the ME,1,2 however, very little data exist examining this mucin in in vivo studies, and to our knowledge no previous studies exist comparing the expression of this mucin in pediatric patients with a history of OM compared with those without this history. The current investigation was designed to explore the differences between these patients in MEE and to compare MUC5AC expression differences in patients with a history of recurrent OM (RecOM) and chronic OM with effusion (COME).
STUDY CRITERIA

Institutional review board approval was granted for this study through The Children's Hospital of Wisconsin (Milwaukee) human research review committee. Patients were invited to participate in this study after a decision for tympanostomy tube (TT) placement was made at the time of consultation for difficulties with OM (hereinafter, study group) or with a decision for cochlear implantation in patients without a history of OM (hereinafter, control group). Written informed consent was obtained from the parents or guardians of the children in both groups with a separate consent form for each group. Each patient fulfilling the study group criteria from a single tertiary referral pediatric otolaryngology practice (that of J.E.K.) was invited to participate during 2 separate periods of patient recruitment (March-June 2005 and July-September 2008). The participation rate was approximately 40% of patients invited. Formal analysis of decisions for or against participation was not conducted. Consecutive patients meeting criteria for inclusion in the control group from a single pediatric otolaryngology practice (that of B.C.P.) were invited to participate as well. Basic demographic information including age, sex, and race was collected. Inclusion criteria for the study group included age of 6 months to 12 years and meeting the clinical criteria for TT placement for either OME or RecOM. Otitis media with effusion was defined as the persistence of a ME effusion for longer than 3 months. Recurrent OM was defined as 3 or more acute OM presentations within a 6-month period in which clinical evidence of OM and ME effusion resolved between episodes. These were used as minimum criteria for consideration of TT placement. Inclusion criteria for the control group included patients in the same age range who met the clinical criteria for cochlear implantation and who had less than 1 episode of diagnosed OM per 12 months of life. Exclusion criteria included immunologic abnormality, either intrinsic or pharmacologic; anatomic or physiologic defect of the ear (except in the control group, who all had an anatomic or physiologic defect of the ear with resulting profound sensorineural hearing loss requiring cochlear implantation); a syndrome associated with OM (eg, Down syndrome or cleft palate); and chronic mastoiditis, cholesteatoma, or other OM complications except for conductive hearing loss. In addition, control patients were excluded if there was any evidence of ME disease at the time of cochlear implantation. Patients enrolled in this study are part of a larger, comprehensive study examining the pathophysiological characteristics of OM in children.

SPECIMEN COLLECTION

The MEE biopsy samples were collected as previously described. In brief, for the study group, a myringotomy incision was made in the antero-inferior tympanic membrane. A cup forceps was inserted through the myringotomy incision, and MEE specimens (<1 mm²) were obtained from the ME promontory in proximity to the Eustachian tube orifice. In the control group, a mastoidectomy was performed as routine approach for cochlear implantation. When the ME was exposed, an ME mucosa biopsy sample of approximately 1 mm² was taken with a cup forceps from a location similar to that in the study group. After the specimens were obtained in the operating room, they were treated in a similar manner in both the study and control groups. In brief, the MEE was immersed in RNAlater (Invitrogen, Carlsbad, California), placed on ice, and transferred directly to the laboratory. Biopsy specimens were kept at 4°C for up to 1 month. Specimens shipped to Milwaukee were refrigerated directly to the laboratory. Biopsy specimens were kept at 4°C for up to 1 month. Specimens shipped to Milwaukee were refrigerated directly to the laboratory.

RNA EXTRACTION

Total RNA was extracted from MEE using TriZol reagent (Invitrogen) following the manufacturer’s instructions. The yield and purity of total RNA was determined by spectrophotometry to ensure that all specimens analyzed were of high quality prior to experimental analysis. The RNA was stored at -80°C until further analysis.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

A total of 100 to 200 ng of RNA was reverse transcribed using Superscript III First Strand Synthesis System (Invitrogen). The random primed complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) (GeneAmp 2720, Perkin-Elmer, Waltham, Massachusetts) using specific MUC5AC primers as described previously. Accordingly, the MUC5AC primer pair (forward primer: 5'-GGGACCTTCTCTACAAATT-3' and reverse primer: 5'-TATATGTGGATCTTGAGGATAG-3') sequences were based on GenBank accession No. AJ001402, position, nucleotides 499 to 516 for the forward primer and 674 to 651 for the reverse primer. The 176 base pair (bp) amplified product was then digested with NlaIII enzyme. The 20 µL of reaction mixture for PCR contained 1.0 U of Platinum Taq DNA polymerase (Invitrogen), 0.2 µM each of deoxyribonucleotide triphosphates (Invitrogen), 0.2 µM of each primer, and 2 µL of cDNA template. One-tenth of the PCR reaction was run on 2% agarose gel at 100 V, and the product was visualized with GelStar (Lonza, Rockland, Maine). Reliability of the PCR product identified in the gels and generated by the selected primer pair was further assessed by restriction enzyme digestion and confirmed by comparison of the digested pattern to the previously published sequence of MUC5AC.

QUANTITATIVE PCR

Quantitative PCR (qPCR) was performed on an iCycler iQ (Bio-rad, Hercules, California) using TaqMan probe chemistry (Applied Biosystems, Foster City, California). TaqMan primer/probe sets for human MUC5AC and HPRT were obtained commercially (Applied Biosystems). Both primer/probe sets were designed across an exon junction and therefore specifically detect only transcribed gene product, unlikely to be affected by genomic DNA contamination. However, to ensure the absence of genomic amplification, the qPCR reaction contained the equivalent amount of each specific RNA template was included. The 20-µL reaction containing TaqMan PCR Master Mix, primer, probe, and template cDNA was performed at 50°C for 2 minutes, 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Sets of cDNAs from the control and study groups were run on the same plate. Each cDNA sample was analyzed in triplicate, and a no-template control reaction was included for each gene. The messenger RNA level of MUC5AC was normalized to an HPRT gene; HPRT was used for this purpose owing to its expression in MEE at a level similar to that of mucin genes and the lack of impact of the disease condition of OM in this gene’s expression. The relative fold change was then calculated using the 2^(-ΔΔCt) method.
PERFORMING ELISA

In performing ELISA, the effusion from the ME was homogenized in equal volume of radioimmunoprecipitation assay buffer containing 2mM of phenylmethylsulfonyl fluoride and protease inhibitor (Sigma, St. Louis, Missouri). The extract was centrifuged at 13 000 rpm at 4°C for 15 minutes. Clear supernatant was collected, and the total protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, Illinois). The total protein from sample extract was diluted in carbonate-bicarbonate buffer, pH 9.6, to coat microtiter plates at 4°C overnight. Nonspecific reactions were blocked with 2% bovine serum albumin fraction V in phosphate-buffered saline, and the plates were then incubated with monoclonal mouse anti-MUC5AC (clone 45M1; Fisher Scientific, Pittsburgh, Pennsylvania) at 1:200 for 1 hour. After washing, MUC5AC was detected using horseradish peroxidase–conjugated donkey anti-mouse IgG at 1:1500 (Jackson Laboratory, Sacramento, California). Color development was performed using tetramethylbenzidine (Sigma). To investigate the presence of MUC5AC protein, pooled ME effusion extract was used to determine the range of total protein concentration that provides linear response in a linear regression analysis. The optimal concentration, producing a midlinear response of 156 ng of total protein from each ME effusion extract, was assayed for the MUC5AC level.

RESULTS

A total of 28 MEE samples, 23 study group and 5 control group specimens, from children 9 months to 7 years old were collected for this study (Table). There were no significant differences in the mean ages of patients in the control group, the RecOM group or the COME group (P = .37; 1-way analysis of variance [ANOVA]). The sex of the control and study groups were also similar based on Fisher exact test and displayed no significant differences in comparing groups (control compared with RecOM group, P = .59; control compared with OME group, P > .99; RecOM group compared with OME group, P = .22). However, overall numbers of patients were small in each group. Age and sex were the primary determinants of selection in matching study patients to control patients. Given the difficulty in finding control patients without a history of OM, control for ethnicity was not possible, and there did exist some differences between the study and control groups, with the study population more heavily represented by white patients.

The primers used to identify the presence of MUC5AC in human ME tissue amplified a PCR product from MEE, which was an expected 176 bp in length, similar to one obtained from human MEE culture. The NlaIII restriction enzyme digestion resulted in the appropriate digested product of 130- and 46-bp fragments, confirming the correct amplicon was obtained from MEE samples (Figure 1). There were 23 samples from patients undergoing TT placement in the OM group, 12 with diagnosis of RecOM and 11 with diagnosis of COME.

The level of MUC5AC transcript in each sample was determined quantitatively using real-time PCR. The amount of RNA obtained from an MEE sample was minute; therefore, the control to detect possible genomic contamination for each sample was set up using the equivalent amount of RNA that would present in a qPCR reaction. There was no amplified signal from any

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<th>Table. Patient Demographic Information</th>
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Abbreviations: COME, chronic otitis media with effusion; RecOM, recurrent otitis media.

Figure 1. The MUC5AC gene was amplified from human middle ear mucosa biopsy. M indicates the standard DNA marker, size in base pairs (bp) as marked on the left; lane 1: A 176-bp MUC5AC polymerase chain reaction fragment amplified from recurrent otitis media-50 sample (50 refers to an internal tracking system used to deidentify but still track patients); lane 2: NlaIII restriction digestion yielded correct product of 130- and 46-bp fragments.
of these control reactions, indicating the absence of genomic DNA in all samples tested.

The mean level of MUC5AC in the RecOM group was 25.92 times higher than in the control group. In COME samples, the mean level of MUC5AC was 155.4-fold higher than that of the control patients (Figure 2). The expression data were not normally distributed among groups and were subjected to Kruskal-Wallis ANOVA on expression data were not normally distributed among

Figure 2. The MUC5AC gene transcript levels in middle ear mucosa from different study groups as measured by quantitative polymerase chain reaction. The error bar displayed is the standard error of the mean. COME indicates chronic otitis media; RecOM, recurrent otitis media.

ing epithelium, contributes to the normal physiologic characteristics of the MEE, and has the potential to contribute to pathophysiological disease in the ear. However, the precise mechanisms and interactions for the varied mucins have not been well characterized. An increase or decrease in production of any one mucin creates changes in the composition and function of the mucous blanket overlying the epithelium. These changes affect viscosity, mucociliary clearance, epithelial repair mechanisms, and bacterial pathogenesis in the ME.

The secreted or gel-forming mucins are of particular interest in ME pathogenesis because they are the primary mucins determining the viscoelastic properties of fluid overlying respiratory epithelium like that found in the ME. This is especially important in chronic disease states such as chronic bronchitis, asthma, and chronic OM. The gel-forming mucins that have received the most attention in the study of OM have been MUC2, MUC5AC, and MUC5B. Our in vitro work using both human and chinchilla cell cultures has demonstrated that MUC2 and MUC5AC are regulated by inflammatory cytokines in a differential manner. In addition, we have recently reported, in a study similar to this current work, that clinical specimens also demonstrate an upregulation of MUC2 in children with OM compared with controls. Previous investigations have also examined the expression of MUC5AC or the MUC5AC protein in the ME. However, none of these studies specifically compared ME mucosal tissue in children with and without a history of OM or allowed for a direct comparison in children with a diagnosis of OME and RecOM as designed in this current work.

This current study is interesting in that MUC5AC was identified in all specimens from the ME of both controls and patients with OM. This differs from the previous study by Lin et al in which MUC5AC was identified only in the Eustachian tube and not the uninfamed ME of their control patients. However, this current study used live patients and children compared with cadaveric tissue and

Figure 3. Middle ear fluid (MEF) samples from patients with otitis media were examined for MUC5AC protein. An equal amount of total protein from each sample was assayed by indirect enzyme-linked immunosorbent assay as described in the “Methods” section. Protein extraction from gastric stomach (“stomach”) served as a positive control. All MEF samples tested positive for MUC5AC. COME indicates chronic otitis media with effusion; OD, optical density; RecOM, recurrent otitis media. The sample numbers refer to an internal tracking system used to deidentify but still track patients.
adult patients in the control population in the study by Lin et al. Also, the current investigation consistently demonstrated the presence of MUC5AC in MEF compared with a much lower rate of identification in a previous study. However, similar to results identified in previous investigations, the current work also demonstrated a significant upregulation in MUC5AC in patients with OM compared with controls. An additional novel finding in this investigation was the demonstration that not only is MUC5AC expression in MEE directly associated with OM but that there is a progression of upregulation. Patients with RecOM have higher levels of expression compared with patients without a history of OM, and patients with OME have significantly higher levels than both the control and RecOM groups. With respect to the events surrounding the pathophysiologic characteristics of OM, a number of investigators have endeavored to elucidate important mediators of mucin regulation. Inflammatory cytokines have been identified as important modulators in OM clinically. Our laboratory has demonstrated that the inflammatory cytokines tumor necrosis factor (TNF) and interleukin 1β (IL-1β) regulate mucin production and produce a differential regulation of mucin gene expression in MEE in a dose- and time-dependent fashion. Corroborating this work, others have shown the affect of inflammatory cytokines on mucin protein production. Smirnova et al demonstrated TNF stimulation of MUC5AC mucin secretion in vitro and that mucin secretion response associated with TNF is higher in earlier stages of cell growth, suggesting that specific inflammatory cytokines have a temporal relationship in the progression of OM. Furthermore, TNF affected early mucin expression, and IL-6 demonstrated a later and more sustained effect on expression. Both TNF and IL-6 were also specific to the type of mucin expressed, with a greater effect seen in MUC5AC expression in comparison with MUC5B expression.

In addition to molecular studies examining the affect of cytokines on MEE mucin regulation, studies have determined the role of specific pathogens in this process. Pathogens important in OM, nontypeable Haemophilus influenzae (NTHi) and Streptococcus pneumoniae (SP) each have been shown to induce mucin overproduction in vitro experimentation with a synergistic effect in mixed infections. Both SP and NTHi upregulate MUC5AC expression in human epithelial cells through specific mitogen-activated protein kinase pathways, with the ability to suppress the NTHi-induced MUC5AC expression through the use of glucocorticoids. It is important to note that the similarities between the MEE and other areas of disease in the respiratory system also provide for overlap in studies that uncover the mechanisms of mucin regulation. In lung disease states, such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis, the mucin profile is altered in a manner similar to that of OM states, with changes in inflammatory activation of mucin gene expression of MUC5AC and MUC2. Both mucins have several common active cis-elements in their promoters, suggesting that they may have common regulatory pathways. Because commonalities exist between the 2 disease states and their mucin expression, studying modulation in one may help to gain insight into the other. Although there exists a need for further specificity in understanding the regulation of mucin production in the ME, the connection between bacterial influences, inflammatory cytokines, and agents such as glucocorticoids provide potential novel therapeutics for treating this disease. The data presented in these experiments provide a bridge between bench research and the clinical pathologic characteristics of OM and underscore the need for a more thorough understanding of ME mucin biology to achieve these novel interventions.

In conclusion, analysis of MEE samples demonstrates a significant upregulation in expression of the mucin MUC5AC in patients with OM compared with controls. In those with OM there exists a progressive upregulation, significantly higher in those with COME than in those with RecOM. These in vivo data corroborate previous in vitro data, demonstrating the importance of this mucin in inflammatory states of the ME and suggest possible novel strategies for intervention in OM.

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**Announcement**

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