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Younger patients with COME are more likely to have mucoid middle ear fluid containing mucin MUC5B

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Abstract

Background—Chronic otitis media (COM) is one of the most common childhood diseases. Its pathophysiology is complex and multifactorial. The role of specific mucin glycoprotein subtypes in OM is only recently being elucidated.

Objective—To determine the relationship between middle ear fluid mucins and clinical variables of patients needing tympanostomy tubes (TT).

Methods—Middle ear effusions (MEE) from children receiving TT were collected over a 2-year period. Western blot characterization of mucins MUC5B and MUC5AC along with chart review of age, gender, effusion viscosity, hearing loss >30dB, history of allergies, and/or respiratory disease were performed.

Results—MEE samples from 58 patients were available for analysis. Overall, MUC5B was significantly more often detected in middle ear fluid relative to MUC5AC (90% vs. 51%,

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CONFLICT OF INTEREST STATEMENT

"The authors declare that they have no competing interests."

AUTHOR CONTRIBUTIONS: Dr. Preciado had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the report.

Study concept and design: Preciado, Val.

Acquisition of data, proteomic techniques: Duah, Poley, DeMason.

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p=0.005). MUC5B presence was statistically associated with mucoid effusions relative to serous effusions (100% vs. 57%, p=0.0064), MUC5AC presence was not significantly different in mucoid and serous fluid (55.1% vs. 37.5%, p=0.447). Patients younger than 48 months were more likely to present with mucoid effusion, compared to those older than 48 months of age (p=0.038). Finally, patients with effusions positive for MUC5B were younger than those with effusions negative for MUC5B (35.1 vs. 76 months, p=0.045). No other variables correlated to either effusion viscosity or specific mucin content.

Conclusion—Patients younger in age needing TT placement are more likely to present with mucoid effusions, predominantly containing MUC5B mucin. As such, we postulate a distinct pathophysiology for mucoid and serous effusions across ages in children with COM.

Keywords

Chronic Otitis Media with Effusion; Mucous; Serous; MUC5B; MUC5AC

INTRODUCTION

Chronic otitis media with effusion (COME) is defined as persistent middle ear fluid that may occur independently or after an acute infection from bacteria or virus¹. The condition is most prevalent in the first 2 years of life. Otitis media is the number one reason for antibiotic use and doctor visits with children^{2,3}. Furthermore, the most common surgical procedure among children, Tympanostomy Tubes (TT) placement, is the result of COME⁴. Effusions from the middle ear may be characterized as serous, mucoid or purulent. Purulent effusions are most prevalent in acute otitis media where the pathogen count is high, whereas serous or mucoid effusions typically have a low pathogen count by clinical culture, and are predominantly seen with COME⁵⁻⁷.

Mucins are a collection of large glycoproteins with several carbohydrate side chains.⁸⁻¹⁰ Many of the side chains are similar to glycolipids. This causes mucin to adhere to bacteria and activate host defense interactions that allow for the removal of the bacteria via the mucociliary transport system¹¹. Currently, the identified mucins that have the greatest association with airway mucus are the secreted MUC5AC and MUC5B proteins. Among other things, an increase or decrease in the mucins can alter the viscosity of the mucous.⁸ Using proteomics techniques, we have reported that mucin MUC5B is predominant in middle ear effusions¹².

The objective of this study was to determine the relationship between middle ear fluid characteristics and clinical variables of patients needing tympanostomy tubes. We hypothesized that MUC5B would be validated as the predominant mucin in mucoid middle ear fluid, and would be associated with mucoid effusion types.

METHODS

The study was approved by the Institutional Review Board of Children's National Medical Center in Washington DC. With written informed consent from parents or guardians, middle

ear effusions of children receiving TT placement were collected and analyzed over a 2-year period. In total 58 specimens were collected.

Patient charts were reviewed for age, race/ethnicity, gender, type of effusion, hearing loss >30db, history of allergies and respiratory diseases. Auditory function before the operation was determined from Pure Tone Audiometry (PTA) results documented by an Audiologist. PTA average results at 500, 1000, 2000, and 4000 Hz greater than 30 db were classified as 'significant' hearing loss. Effusions were broadly classified as mucoid vs. serous on the basis of presence or absence of gravitational flow of the effusion. Briefly, if the effusion did not flow upon flipping the tympanostomy trap vial upside down after ten seconds, it was considered as a mucoid effusion.

Western blot analysis was performed for each specimen to determine the presence of the mucin proteins by using established protocols.¹² For mucin protein blotting, samples containing 40 µg total protein were separated by electrophoresis in 1.0% agarose gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The positive control for MUC5B was human saliva to which protease inhibitor cocktail (Sigma, St Louis, MO) was added at a 1:100 dilution on collection and for MUC5AC was Calu-3 or human nasal epithelial cell secretion. For MUC5AC immunostaining, the membrane was probed using LUM5-1 (41), a rabbit polyclonal anti-MUC5AC antibody generously provided to us by Dr. Mehmet Kesimer, University of North Carolina at Chapel Hill at a 1:4,000 dilution in a 2.5% milk solution in phosphate-buffered saline for 1 h at room temperature followed by incubation at 4 °C overnight. The antibody (LUM5-1 epitope) was raised against the sequence RNQDQQGPFKMC in the cysteine rich nontandem repeat domains of MUC5AC. MUC5B expression was detected with a rabbit polyclonal α-MUC5B H-300 (Santa Cruz Biotechnology, Dallas, TX) at 1:300 dilutions.

Univariate analysis for numerical variables was performed by Mann-Whitney tests, while categorical variables were examined by Chi-square tests. Multivariate regression analysis was also conducted with the independent variables considered being age, gender, significant hearing loss presence, effusion viscosity (serous/mucous), presence allergy history, history of previous tubes, snoring, and asthma. Dependent variables analyzed were presence of MUC5B and MUC5AC. P values less than 0.05 were considered statistically significant. The intercept and overall pvalues are described in the multivariate regression results. In statistical terms the intercept signifies the value at which the regression line would cross the y axis if all of the predictors (independent variables) were 0.

RESULTS

Effusions from a total of 58 patients were included in the study (Table 1). Out of the 58 patients, 19 were female (32.76%) and 39 were male (67.24%). Ethnicities varied with 9 Hispanic/Latino (15.52%), 24 African-American (41.37%), 11 Caucasian (18.97%), and 14 other (24.14%). The average age of all patients was 40 months (+/-4.5). Forty-six patients (79%) contained mucous type effusions, while 12 (21%) had serous effusions. Ten patients (17%) had a history of seasonal allergies and 42 (72%) had significant hearing loss. Thirty-

four (59%) of the patients had only an underlying respiratory disease or either a respiratory disease combined with another disease process.

Western blots determined that MUC5B was significantly more detected in middle ear fluid relative to MUC5AC (90% vs 51%, $p=0.005$) (Figures 1 and 2). MUC5B was always present in mucoid effusions compared to serous effusions (100% vs 57%, $p=0.0064$). However, MUC5AC was not significant in either effusion type (55.1% vs 37.5%, $p=0.447$). Mucoid effusions were also correlated with age. Patients under 48 months were more likely to have mucoid effusions relative to patients over 48 months ($p=0.038$). Patients positive for MUC5B were younger than those negative for MUC5B (35 mon. vs 76 mon., $p=0.045$). Multivariate analysis revealed no significance difference for MUC5AC presence and any of the clinical variables (Table 2). MUC5B presence, however, was found to significantly correlate on multivariate analysis with effusion viscosity, while controlling for other variables ($p=0.000486$) (Table 3).

DISCUSSION

The goal of this study was to explore the hypothesis of MUC5B being the predominant mucin found in mucoid middle ear effusions and also to view the relationship of middle ear fluid with a variety of clinical variables.

In line with our proteomics report, we found that MUC5B was more frequently present in MEE than MUC5AC.¹² This result was also seen in the study conducted by Elsheikh et al. Their results also showed that the expression of MUC5AC mRNA in the MEE was fairly low when compared to MUC5B.¹³ Analysis of the results determined that there was no statistical correlation between MUC5AC mucin protein and any of the clinical variables. MUC5B however, was statistically associated with mucoid effusions, which were also found to be related to patients younger than 48 months. Along those lines, patients that presented with MUC5B were younger in age than patients negative for MUC5B.

Mucins are vital components of the middle ear. Their structure allows for the trapping of pathogens that may enter via the Eustachian tube. Mucoid type effusions with MUC5B may be present more often in younger children with COME because this molecule may serve as a critical component of the airway innate immune defense mechanism¹⁴, particularly with the developing innate immune system of infants. MUC5AC on the other hand has been postulated to be important in inflammatory allergic response.¹⁵ As such, it may play a role in otitis media due to allergic etiologies, which may not be as mature in younger infants. Importantly, although mucins are considered critical in terms of protecting epithelium from pathogen invasion, they may contribute to effusion non-clearance due to the increased viscosity associated with their abundance. Moreover, it has been postulated that these molecules, particularly MUC5B, may become associated with neutrophil extracellular traps, which are commonly found within middle ear effusion. Presumably, these complexes further contribute to the marked viscosity of the effusions¹⁶. Finally, some have posited that MUC5AC is secreted earlier upon infectious/inflammatory stimuli, in a more robust fashion, relative to MUC5B which not only responds in a comparatively mutated fashion, but at much later time points after an initial infectious insult¹⁷. One can perhaps extrapolate that

relative to the acute responder MUC5AC, MUC5B is more critical for long-term baseline homeostasis than MUC5AC, as has been postulated by others^{18,19}. It is possible that those patients with MUC5AC in our study had experienced a more recent bacterial infection in the middle ear, and that this was the reason for the MUC5AC presence. However, due to the retrospective nature of our report, we were unable to deem when the most recent infection occurred for each patient prior to the tympanostomy tube placement procedure. This remains a limitation of this study. Another important limitation includes the inability to directly quantify specific mucins (i.e. MUC5AC and MUC5B). Antibody based techniques typically used for protein quantification, such as enzyme-linked immunosorbent assays (ELISA), have often proven erroneous at distinguishing MUC5B from MUC5AC mucin due to lack of the assay's specificity, presumably because the extensive mucin O-glycosylation masks amino acid epitopes under the non-denaturing conditions used in ELISA, which predictably blocks accessibility of antibodies⁹. In our hands, the best way of distinguishing these two mucins remains Western blot analysis using polyclonal antibodies raised against the non-glycosylated domains of each mucin. For all samples we loaded the maximum amount of protein possible onto the agarose gels. As such, the primary goal was not to quantify the mucins per se, but to detect whether they were present (yes/no) in each sample. Finally, another limitation of the study was the fact that viscosity for each sample was not quantified, but rather broadly qualified as serous or mucoid on the basis of gravitational flow. Indeed, a direct measure of viscosity would add validity to our findings.

CONCLUSION

In conclusion patients younger than 48 months needing TT placement were more likely to present with mucoid effusions, predominately containing MUC5B. MUC5AC was not statistically associated with either effusion type or clinical variable.

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FIGURE 1. Representative western blots for MUC5AC

The blots represent blotting for 13 different samples (1 sample per lane). The positive controls (far left and far right) are secretions from Calu-3 cells (known to contain large amounts of MUC5AC). S=serous sample. M=mucoid sample. Please note that the MUC5AC signal from Calu-3 cell secretions often appears weaker than that from biospecimens (such as middle ear effusions), as the protein amount tends to be more dilute.

FIGURE 2. Representative western blots for MUC5B

The blots represent blotting for 17 different samples (1 sample per lane). The positive control (far left) is human saliva (known to contain large amounts of MUC5B). S=serous sample. M=mucoid sample.

TABLE 1

Demographics of patients

Total Patients	58
Age	
Mean (months, +/- STD)	40.2 (+/-4.5)
# pts > 48 months old	17
# pts ≤ 48 months old	41
Effusion Quality	
Serous	12 (21%)
Mucoid	46 (79%)
Gender	
Male	39 (67%)
Female	19 (33%)
History of Seasonal Allergies	10 (17%)
Significant Hearing Loss > 30dB	42 (72%)

TABLE 2

Multivariate analysis for MUC5AC presence

	Correlation Coefficients	Standard Error	t Stat	P-value
Intercept	0.32597829	0.337815324	0.96496	0.34228
Significant Hearing Loss	0.04780952	0.217800995	0.21951	0.827739
Allergy	0.04739226	0.254547621	0.186182	0.853555
Age	-0.0009455	0.003527153	-0.26805	0.790496
Gender	-0.0467204	0.203809488	-0.22924	0.820241
Previous Tube Insertion	0.23497268	0.33695674	0.697338	0.490961
Sleep Disturbed Breathing	0.136115	0.204130687	0.666803	0.509994
Asthma/Bronchitis	-0.1749042	0.218069775	-0.80206	0.428829
Serous/Mucous	0.21010342	0.226320164	0.928346	0.360638

TABLE 3

Multivariate analysis for MUC5B presence

	Correlation Coefficients	Standard Error	t Stat	P-value
Intercept	0.69913339	0.166715888	4.193562	0.000301
Significant Hearing Loss	0.06226862	0.115322613	0.539952	0.594008
Allergy	-0.0051148	0.118485842	-0.04317	0.965911
Age	-0.0031039	0.001738008	-1.78589	0.086253
Gender	-0.1157458	0.101091618	-1.14496	0.263069
Previous Tube Insertion	0.10665921	0.180575535	0.590663	0.560049
Sleep Disturbed Breathing	0.02278488	0.099271241	0.229522	0.820331
Asthma/Bronchitis	-0.0533884	0.115362481	-0.46279	0.647519
Serous/Mucous	0.41623984	0.103856363	4.007841	0.000486