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Differential response of gel-forming mucins to pathogenic middle ear bacteria

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Abstract

Objective—To assess the differential response of the secretory gel forming mucins (GFM) to the most common bacterial pathogens causing otitis media, *Streptococcus pneumoniae* (SP), nontypeable *Haemophilus influenza* (NTHi), and *Moraxella catarrhalis* (Mcat), in a culture model of human middle ear epithelium (HMEEC).

Methods—*In vitro* cultured HMEEC was exposed to 5µg/ml of bacterial whole cell lysate (WCL). RNA was extracted to generate cDNA. The expression levels of each of the targeted mucin transcripts, *MUC2*, *MUC5AC*, *MUC5B* and *MUC19*, were detected by quantitative PCR.

Results—The submerged HMEEC exposed to NTHi-86028NP WCL demonstrated a significant increase of *MUC2*, *MUC5AC* and *MUC5B* as compared to the control non treated cells while *MUC19* transcript level remained unchanged. WCL of additional major OM pathogens significantly increase the transcription of these three mucin genes as well. A combination of NTHi and SP further synergistically induced *MUC2* and *MUC5AC* gene expression however, not all NTHi strains synergized with SP in the induction. Addition of Mcat WCL to the synergized combination of NTHi and SP did not participate in the synergistic response of mucins.

Conclusion—The specific pathogen combinations were important in determining the degree of synergistic effects to GFM expression. The current data are substantive in guiding future work to extend our understanding of OM pathogens and GFMs.

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Conflict of interest statement

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Keywords

otitis media; gel-forming mucin; bacteria

INTRODUCTION

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, knowledge deficits persist regarding OM pathogenesis. Particularly important is the interaction between various pathogens and the middle ear epithelium (MEE) as it relates to mucin gene expression and mucin production. It has been well-documented by our laboratory and others that pathogenic bacteria and viruses in the middle ear lead to inflammatory processes, cytokine activation and an increase in the MEE production of mucins [1–4]. These events leading to an increase in mucin production provide protection to the MEE from the invading pathogens and inflammatory events and assist in clearance of pathogens from the ME. However, mucins are also the primary components which persist in the ME following these infectious events, leading to thickened secretions and hearing loss associated with OM.

With this knowledge, a next logical step in understanding the pathogenesis involved in OM is a more thorough elucidation of the specific pathogen and mucin responses. The mucins which primarily lead to this hearing loss are the secretory, gel-forming mucins (GFM) found in the MEE which include the glycoproteins from mucin genes 2 (*MUC2*), 5A (*MUC5AC*), 5B (*MUC5B*) and 19 (*MUC19*). These mucins have been demonstrated in a number of studies, including from our laboratory, to be the primary gel-forming mucins of the MEE [5–8]. In addition, these gel-forming mucins are conserved in the MEE across multiple species including human, rat, mouse and chinchilla [9,10]. Each of these mucins have been shown to be up-regulated in response to non-specific cytokine inflammatory responses [11,12]. However, specific mucin gene and protein responses to common OM bacterial pathogens have not been investigated. The objective of this current investigation was to assess the differential response of these gel-forming mucin genes to the most common OM bacterial pathogens of *Streptococcus pneumoniae* (SP), nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (Mcat).

MATERIALS AND METHODS

Bacterial strains and growth

Bacteria utilized in this study were Nontypeable *Haemophilus influenzae* (strain 86028NP and 2019), *Streptococcus pneumoniae* (strain TIGR4 and D39), and *Moraxella catarrhalis* (strain 7169). All bacteria were grown on brain heart infusion (BHI) agar plates with exception of NTHi which requires the supplement of hemin and nicotinamide adenine dinucleotide (sBHI) at 37°C with 5% CO₂. The 12 hour bacterial culture was harvested. The pellet was suspended and sonicated in phosphate buffered saline as previously described [13]. The whole cell lysate (WCL) was collected and stored at –80°C. The bacterial WCL

was selected to simulate the clinical condition where most of OM pathogens undergo spontaneous or antibiotic-induced autolysis *in vivo* [14].

Cell culture

A culture model of human middle ear epithelium (HMEEC, provided by Dr. David J Lim) was an immortalized cell line whose primary characterization regarding transformation and growth properties have been published [15]. The culture was maintained in a humidified atmosphere at 37°C with 5% CO₂, in full growth media containing a 1:1 ratio mixture of Dulbecco's Modified Eagle Medium (Life Technologies) and Bronchial Epithelial Cell Basal Medium (Lonza), 10% Fetal Bovine Serum (FBS), 1% antibiotic/antimycotic, and supplemented with one BEGM Singlequots (Lonza) for every 500ml portion of the media. The cells were grown in the presence of 1µM retinoic acid (RA) for 5 days prior to treatment. The culture was serum starved overnight and subsequently treated with 5µg/ml of bacterial WCL, a minimal concentration that provided optimal response of mucin (data not shown), in basal media containing supplements and 1µM RA at 2, 4, and 6 hours. Cell lysate was harvested at the end of exposure time.

Quantitative PCR

Total RNA from experimental cultures was prepared after the indicated treatments using TriZol reagent. cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies). Each cDNA reaction prepared for *MUC2*, *MUC5AC* and *MUC19* experiments was started with 400ng total RNA while a 1.5µg starting total RNA was required for a detection of *MUC5B*. Quantitative PCR (qPCR) was carried out on a ViiA7 by Life Technologies using their commercially available TaqMan primer-probes for *MUC2*, *MUC5AC*, *MUC5B*, *MUC19* and *HPRT1*. Samples were analyzed in triplicates and a no template control was included for each gene. The expression levels of each of the targeted mucin genes detected by qPCR were normalized to the mRNA level of the house keeping gene *HPRT1*. The relative fold change of mucin gene was calculated using 2^{-Ct} methodology [16].

Statistics

All experiments were performed in triplicate. The results were presented as mean ± standard error of means. Data of GFM response to single OM pathogen were analyzed by student's t-test. Differences in GFM synergistic response to multiple OM pathogens were determined by one-way ANOVA followed by Tukey's Post-hoc. All statistical analyses were performed using Prism software version 5.01 (GraphPad Software, Inc).

RESULTS

NTHi strain 86028NP whole cell lysate up regulated transcript expression of gel forming mucins in immortalized human middle ear epithelium cells (HMEEC)

To assess changes in gel forming mucin gene expression due to stimulation of bacterial whole cell lysate, HMEEC were exposed to 5 µg/ml NTHi-86028NP WCL at various time points (Figure 1). The submerged HMEEC exposed to NTHi WCL demonstrated 1.5–2 fold increase of *MUC2* as compared to the control non treated cells. The significant increase was

observed at every time point tested ($p < 0.05$). Increase of *MUC5AC* was also detected in similar a pattern at 5–7 fold increase over the level detected in non-treated cells ($p < 0.05$). The change in *MUC5B* mucin expression required a longer stimulation. The significant 2-fold increase of *MUC5B* mRNA level is observed after a 6 hour exposure ($p = 0.007$). *MUC19* transcript level remained unchanged in WCL treated HMEEC cells.

Whole cell lysate of additional major OM pathogens stimulated transcription of gel forming mucin genes

The assessment of the impact on GFM of an additional strain of NTHi (NTHi-2019) demonstrated increase of *MUC2*, *MUC5AC* and *MUC5B* with significant p value of 0.0014, 0.0001, and 0.0116, respectively (Figure 2). Similar induction was observed when cells were exposed to SP-D39 with the highest fold change of *MUC5AC* ($p < 0.0001$), about 3-fold change of *MUC5B* ($p < 0.0001$) and almost 1.5 fold change of *MUC2* ($p = 0.0028$). Exposure to SP-TIGR4 increased *MUC5AC* ($p < 0.0001$) and *MUC5B* ($p = 0.0042$) at comparable levels, but not *MUC2* ($p = 0.1989$). Mcat-7169 significantly induced *MUC2* ($p < 0.0001$) and *MUC5AC* ($p < 0.0001$) but not *MUC5B* ($p = 0.1243$). Both *MUC2* and *MUC5AC* demonstrated a more robust response to NTHi and Mcat than SP. A more robust response of *MUC5B* to SP was observed.

Synergistic effect between NTHi and SP further up-regulated GFM gene expression in in vitro HMEEC model

To assess impact of multiple microbial WCL on gel forming mucin expression, HMEEC were exposed to the combination of OM pathogens (Figure 3). Single bacterial strain WCL of NTHi and SP induced significant changes in levels of each mucin gene. A combination of NTHi and SP further synergistically induced *MUC2* and *MUC5AC* mRNA expression in HMEEC. A similar synergistic effect was not observed in the induction of *MUC5B* level. Note that not all NTHi strains (86028NP and 2019) synergized with SP (TIGR4 and D39) in the induction of expression. While all combinations of NTHi and SP (NTHi-86028NPD39; NTHi-86028NPTIGR4; NTHi-2019D39; and NTHi-2019TIGR4) further induced *MUC5AC* (p -value of < 0.0001 , 0.0004, 0.0005, and < 0.0001 , respectively), only a combined NTHi-2019 and D39 synergized the expression of *MUC2* ($p = 0.0002$). The addition of Mcat WCL to the synergized combination of NTHi-2019 and SP-D39 (Figure 4) did not participate in the synergistic response and demonstrated a reduction of *MUC2* response ($p = 0.0042$) to the same level as that of Mcat alone. A similar result of non-synergized Mcat was observed in *MUC5AC* response ($p = 0.9673$).

DISCUSSION

Pathogen-specific influences on mucin production and particularly the development of chronic OME remains an area of significant knowledge deficit. *Streptococcus pneumoniae*, non-typeable *Haemophilus influenza* and *Moraxella catarrhalis* are recognized as primary pathogens identified in the middle ear of children with OME [17–18]. Studies have demonstrated that chronic OM is often a polymicrobial disease, with two or more of these most common OM pathogens participating in a middle ear infectious process at the same time [1,17,19].

Experimental evidence has accumulated revealing that these concurrent pathogens have symbiotic relationships enhancing their pathogenicity. This includes sharing of genetic information to acquire antimicrobial resistance and collaborative molecular process to improve persistence in the middle ear [20–23]. However, there exists little molecular data elucidating which of these pathogens is most likely to predispose patients to the development of pathology such as middle ear epithelial (MEE) changes and up-regulation of gel forming mucins (GFM), or data examining potential synergistic effects on mucin production by common OM pathogens. The data which does exist are conflicting and hampered, in many cases, by a study design that does not account for the inadequacy of traditional culture techniques for identification of viable pathogens or an understanding of the frequency of polymicrobial infections in OM [1,24–26]. Given these areas of knowledge deficit this current data set was constructed to address the compelling question of what is the differential impact of common OM bacterial pathogens on middle ear epithelial GFM production. This work assessed this aspect of GFM response between bacterial species and within species in the controlled environment of the cell culture.

The bacterial isolates utilized in this study, NTHi-86028NP, NTHi2019, SP-D39, SPTIGR4 and Mcat-7126, were selected based upon availability of genomic sequence and co-infection data published previously [27–28]. Initial testing was completed with NTHi-86028NP and demonstrated up-regulation of transcript expression for each of the GFMs tested, *MUC2*, *MUC5AC* and *MUC5B* except *MUC19*. These experiments also showed that there was a differential response of these GFM to this pathogen with *MUC5AC* demonstrating a 6 to 7-fold increase compared to the doubling of expression for *MUC2* and *MUC5B*. Understanding these differential expressions is critical in deciphering the pathophysiology of OM as each of these mucins not only plays a protective role in the underlying epithelium, but with hyper-secretion can lead to mucous-stasis and resulting hearing loss. Understanding these specific pathophysiologic events is also critical in attempting to design strategies to ameliorate the impact of increased GFM on hearing in children following inflammatory events. Similarly, the temporal relationships to bacterial exposure also demonstrate that there are difference in the regulation of these genes with *MUC2* and *MUC5AC* demonstrating up-regulation more quickly than *MUC5B*. The data is also substantive in that we have identified a more robust MEE up-regulation of GFMs *MUC2*, *MUC5AC* and *MUC5B* compared with *MUC19* using inflammatory cytokines in a previous publication [11]. Despite its large size, this relatively passive response by *MUC19* to inflammatory cytokines in our previous studies, and pathogens in this current work, would suggest that other mechanistic and pathway investigations are needed to elucidate the function and interaction this GFM plays in middle ear physiology and pathophysiology.

An important objective of this current investigation was to assess the differential response of MEE GFMs to a variety of pathogens within families and between species. Our previous investigations had primarily examined the root inflammatory cytokines which are ubiquitous in infection and inflammation (TNF- α , IL-1 β , IL-6, and IL-8) rather than specific pathogens [11–12, 29–32]. It is well-known that pathogens have differential infectious virulence. There have been a variety of mechanisms proposed for this virulence including ability to stimulate inflammatory pathways or escape immunologic eradication [33–34]. However, specific

examinations of these pathogenic responses to mucin response have not been investigated. Given the role of GFM in protecting underlying mucosa the experiments performed in this investigation are critical to better understand the pathogenesis of OM. The results demonstrated that of the two strains of NTHi investigated, there was a similar *MUC5AC* response in comparison to *MUC2* and *MUC5B* with *MUC5AC* demonstrating a far greater up-regulation. And *MUC5AC* also demonstrated the most robust response across the spectrum of all pathogens tested. However, between families of pathogens differences were also noted with SP having a less robust up-regulation of *MUC5AC* and *MUC2* than either NTHi or Mcat but having a more robust up-regulation of *MUC5B* than either NTHi or Mcat. An additional important finding was that within a family, “virulence”, at least as it pertains to mucin up-regulation, was similar in the NTHi organisms tested with NTHi-86028NP consistently having a more robust response than NTHi-2019 for *MUC2* and *MUC5AC*. As for SP, SP-D39 demonstrated a more robust response than SP-TIGR4 for all of the mucin genes tested. These data represent a critical early understanding of the specific pathogen-host responses which exist in mucin pathophysiology. With the significant strain and serotype diversity among organism it is apparent that a single set of assumptions may not hold across a family and this may have important implications when designing therapeutic interventions. Examining across all of the data sets it was also evident that among the three bacterial species, NTHi and Mcat had the most significant impact on *MUC5AC* and *MUC2* whereas SP demonstrated the most significant effect on *MUC5B* response. Given the variety of responses from various strains/serotypes a sweeping generalization cannot be made from these data. However, clinically, it has been observed that infections with Mcat have demonstrated an increased incidence of OME following infection and there is some evidence in this data set that Mcat may indeed play an important role in greater mucin gene up-regulation with potential downstream implications on hearing [35]. The data in this current investigation is the first to provide some controlled evidence experimentally to support these previous clinical observations.

We also explored the synergistic effect of polymicrobial exposure on GFM expression when combining different NTHi and SP pathogens. There has been some study of the impact of multiple bacteria showing an up-regulation of *MUC5AC*. This investigation demonstrated *MUC2* also displayed increased gene expression due to synergistic interactions among multiple pathogens. However, it is critical to note that the specific pathogen combinations were important in determining the degree of synergistic effects. Again, these results are substantive in guiding future work which will require not only an assessment of potential pathways, alternative pathway signaling systems and feedback mechanisms within MEE but also pathogen-specific investigations related to mucin regulation and virulence factor characterization specifically aimed at investigating the mucin affect. Similar to the single pathogen experiments, there existed differential up-regulation of GFM with exposure to multiple pathogens and *MUC5AC* demonstrating the most significant synergistic up-regulation with all pathogen combinations tested. Although Mcat demonstrated robust up-regulation of both *MUC2* and *MUC5AC* compared with other pathogens tested, it did not participate in the synergistic impact noted for NTHi and SP. Further studies to elucidate additional synergistic interactions and mechanisms involved are ongoing.

The current data, generated in an *in vitro* environment, provides a critical environment to rapidly and inexpensively test our hypotheses regarding mucin gene regulation and bacterial pathogens, using multiple different pathogens and combinations. However, this model lacks a well- developed submucosal or goblet cell formation and certainly lacks the immunologic components which are critical in directing pathogens and setting up pathogen-host interactions. Further *in vivo* animal experiments are currently underway utilizing the data obtained in these experiments to extend our understanding of pathogens and GFMs.

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Figure 1. NTHi strain 86028NP whole cell lysate (WCL) up-regulated transcript expression of gel forming mucins in immortalized human middle ear epithelium cells (HMEEC)
 NTHi treatment of submerged HMEEC significantly increased the mRNA level of *MUC2* and *MUC5AC* at all time-points tested. *MUC5B* required a longer stimulation showing a significant change at 6 hour after exposure. *MUC19* remained unchanged at all time-points tested. The x-axis shows the hours (hr) during which the cells were exposed to 5µg/ml of NTHi-86028NP WCL. The y-axis represents the relative fold induction obtained from qPCR on total RNA extracted from the cells. Asterisk (*) designated significant difference from control group (pvalue < 0.05).

Figure 2. Stimulation of gel forming mucin genes by major OM pathogens (NTHi, SP and Mcat)
 The relative fold increase of mRNA level of *MUC2* and *MUC5AC* were detected at 4 hours after exposure to single strain bacterial WCL. There was significant increase of *MUC2* in cells exposed to all but TIGR4. *MUC5AC* also demonstrated a significant increase after exposure to all bacterial WCL tested. At 6 hour exposure, *MUC5B* demonstrated the significant increase in all but Mcat. Asterisk (*) designated significant difference from control group (pvalue < 0.05).

Figure 3. Synergistic effect between NTHi and SP induce the expression of gel forming mucin gene in HMEEC

Single bacterial strain WCL of NTHi and SP induced significant changes in levels of each mucin gene. Combination of NTHi and SP further synergistically induced *MUC2* and *MUC5AC* mRNA expression but not *MUC5B*. Note that not all NTHi strains (86028NP and 2019) synergized with SP (TIGR4 and D39) in the induction of expression. Asterisk (*) indicated the significant synergistic effect between NTHi and SP in the up-regulation of GFM expression.

Figure 4. Impact of multiple microbial whole cell lysate on gel forming mucin expression

HMEEC were exposed to the combination of NTHi and SP that displayed synergistic effect on mucin expression together with Mcat WCL. The addition of Mcat WCL did not participate in the synergistic effect between NTHi and SP but demonstrated a trend of reduction of synergistic response of both *MUC2* ($p=0.0042$) and *MUC5AC* ($p=0.9673$) to that of the response to Mcat alone.