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Lower Nasopharyngeal Epithelial Cell Repair and Diminished Innate Inflammation Responses Contribute to the Onset of Acute Otitis Media in Otitis-Prone Children

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Abstract

About 30% of young children experience excessive, frequent episodes of middle ear infection and are classified as acute otitis media prone (OP). *Streptococcus pneumoniae* (*Spn*) is a predominant otopathogen in OP and non-OP (NOP) children. The pathogenesis of middle ear infection involves otopathogen nasopharyngeal (NP) colonization followed by an upper respiratory viral infection that modifies the NP environment to allow a sufficient inoculum of bacteria to reflux via the Eustachian tube into the middle ear space. Here, we analyzed the NP mucosal repair response between age-matched OP and NOP children who progressed to middle ear infection caused by *Spn*. We found lower epidermal growth factor, epidermal growth factor receptor, and angiogenin cytokine concentrations in nasal washes of OP compared to NOP children. Despite higher expression of TLR2/4 transcript expression in nasal epithelium and in polymorphonuclear cells present in nasal secretions in OP children, OP children had lower expression of proinflammatory cytokines such as IL-6 and IL-8 in the NP. Chemotaxis associated cytokine expression at onset of AOM in OP children was also lower compared to NOP children, possibly indicating a lower capacity to signal the innate immune system. We conclude that lower epithelial cell repair responses during viral infection in the NP combined with diminished innate inflammatory responses potentiate *Spn* pathogenesis in the OP child.

Keywords

Streptococcus pneumoniae; Nasal Epithelial Repair; Innate immune responses; Acute Otitis Media; Otitis-prone

Introduction

Acute otitis media (AOM) is a common infection of young children and is often caused by *Streptococcus pneumoniae* (*Spn*) [1,2]. The pathogenesis of AOM begins in the nasopharynx (NP) with colonization of the nasal passages by potential otopathogens [3,4]. Then in the context of a viral upper respiratory infection (URI), multiple changes occur in the NP that promote an increase in density of bacteria such that a sufficient inoculum of *Spn* refluxes via the Eustachian tube into the middle ear to cause AOM [5–7].

The otitis prone (OP) child is defined by experiencing 3 episodes of AOM within a 6-month time span or 4 episodes within 12 months [8,9]. We have identified within the 30% of all young children that meet the above definition, a subpopulation of OP children (5–6% of all children) who have recurrent AOMs despite tympanocentesis drainage and individualized

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antibiotic treatment; we have named these children stringently-defined OP (sOP) [10]. We have previously determined that sOP children in our study population have lower antibody [9,11], B cell memory [12], and CD4 T-cell memory responses [8] after *Spn* colonization and AOM infection thereby providing explanations within the adaptive immune response that contribute to a propensity for multiple recurrent infections. Here, we studied the role of epithelial and innate mucosal responses to NP colonization in our sOP child population during a viral URI that proceeded to cause AOM due to *Spn* and compared the convergent and divergent responses observed in non-OP (NOP) children during an AOM event.

The NP airway epithelium serves as a primary anchor for *Spn* colonization and respiratory viral infections can significantly enhance bacterial attachment to this epithelium [13–15]. Epidermal growth factor (EGF) and related cytokines can dramatically affect epithelial responses damaged by a viral URI, and also affect the local innate immune response to bacterial infections [16–19]. We hypothesized that deficient repair of NP epithelia and innate responses occurring in the local mucosal environment could be important mechanisms contributing to the frequency of recurrent AOM in the sOP child subpopulation.

In the studies described here, at onset of AOM, lower epithelial repair processes in sOP children were identified during a viral URI compared to NOP children. Despite higher expression of TLR2/4 on nasal epithelium and on polymorphonuclear cells present in nasal secretions in sOP children, sOP children had lower expression of proinflammatory cytokines such as IL-6 in the NP compared to NOP children. We evaluated chemotaxis associated cytokine expression at onset of AOM in children to determine whether the lower cytokine expression found in sOP children was associated with a lower capacity to signal the innate immune system.

Materials and Methods

Patients

The study was approved by the Rochester General Hospital Research Subjects Review Board and written informed consent was obtained for participation and all procedures. Three cohorts of children were studied comprising sOP, NOP, and healthy controls (No URI or *Spn* colonization within 6 months of the sample). Children were enrolled at age 6 months from a private pediatric middle-class practice (Legacy Pediatrics in Rochester NY) and followed prospectively until 30 months of age. In the current study, NP samples were used from children approximately 9–15 months of age who experienced AOM caused by *Spn* (See table 1) representing both sOP and NOP children (n=6 per cohort) with a current clinical viral URI and AOM and calibrated against children without clinical viral URI or AOM during a healthy visit (n=4). We examined the URIs in children at a similar time-point (December 2009 and 2010) as the children used in this study and found that 90% were infected with either RSVa or RSVb as determined by qRT-PCR from nasal wash samples per the manufacturer's instructions (Primer Design, UK).

During the study period whenever a child experienced an AOM, middle ear fluid (MEF) was obtained by tympanocentesis to assure the diagnosis of AOM, confirm the presence of otopathogens in MEF and to provide the potential therapeutic benefit of drainage of bacteria [20]. Then, an antibiotic was given to the child based on the antibiotic susceptibility of the isolate [21,22].

Spn identification in middle ear fluid and NP washes was accomplished as previously reported [10]. Children experiencing 3 episodes of AOM within a 6-month time span or 4 episodes within 12 were classified as sOP while children with less frequent (1–2 events within 6–12 months) were classified as NOP.

Cytokine assays

The total protein concentration in 1 ml of NP wash was determined by Bradford assay and samples were adjusted to 1mg/ml of total protein. 1ml of NP wash was incubated overnight at 4 degrees C with a filter array and processed according to the manufacturer's directions (Ray Biotech, Norcross GA). The arrays measure a multitude of proinflammatory, growth, and effector cytokines per film array. Arrays were developed with Trueblue solution (KPL, Gaithersburg MD) and calculated by EC software using an inverse image. Cytokine values were then computed using the array manufacturer's software.

Cellular isolations from nasal washes

Nasal washes were held on ice until processing. Epithelial cells were enriched in washes using antibodies specific for CD14, CD15, CD45, and CD107 (Biolegend, San Diego CA) followed by anti-mouse IgG magnetic depletion (Polysciences, Warrington PA). Supernatants or isolated beads were spun at 2,000 RPM for 5 minutes and resuspended in RNeasy lysis buffer (Qiagen, Crawley UK) and held overnight at 4 degrees C prior to freezing at -20 degrees C. Polymorphonuclear cells were enriched by using antibodies for CD14 and CD326 (Biolegend) followed by anti-mouse IgG magnetic depletion with RNA isolated as previously mentioned.

RNA extraction, IVT, and qRT-PCR

RNA was isolated from total cell extracts from Trizol (Invitrogen, Carlsbad CA) according to the manufacturer's directions followed by DNase and column purification (Qiagen, Gaithersburg MD). RNA was extracted from isolated cell populations using a RNeasy spin kit (Qiagen) and was calculated using a nanodrop (ThermoFisher, Waltham MA). RNA from isolated epithelial cells was then amplified with two rounds of IVT (Epicenter, Madison WI) according to the manufacturer's instructions. qRT-PCR was used to amplify from 45ng of cDNA (reverse transcribed from aRNA using Superscript III) or 30ng of cDNA (reverse transcribed from mRNA) using G3PDH as an internal calibrator or TLR2 primers or TLR4 primers designed using PrimerBank (Harvard, Massachusetts MA). Data was calculated by the $\Delta\Delta CT$ method and expressed as fold change between groups.

Statistical Analysis

Data was analyzed by two tailed Mann Whitney U test and results with a $p < 0.05$ considered significant.

Results

sOP children have lower nasal epidermal related cytokine expression at onset of AOM

Divergent levels of epidermal growth factor expression could delay the NP epithelial cell repair process in sOP children and contribute to an increased density of bacterial colonization and persisting inflammatory conditions in the NP. To explore deficits in epidermal growth/repair related cytokines in the nasal epithelium, we selected children at onset of AOM caused by *Spn* and assessed epidermal related expression by film array. We found higher expression of EGF and EGFR in children with AOM versus healthy controls (data not shown) and subtracted healthy control values from those of NOP and sOP samples. When expression levels of EGF, EGF receptor, and angiogenin were compared between sOP and NOP children, we found that sOP had lower levels compared to NOP children (Fig 1). During a follow-up visit for both sOP and NOP children after an AOM, EGF was no longer detectable above baseline for both groups (data not shown). These data suggest that sOP children may have a lower capacity for epithelial repair during a viral URI when *Spn* concurrently colonizes the NP at onset of AOM.

sOP children have higher expression of toll-like receptor complexes in the nasal epithelium

EGF receptor ligation by EGF can have a dramatic effect on TLR expression during respiratory bacterial infection and dramatically effect innate sensing of secondary bacterial pathogens during a viral infection. To explore this association, we determined the level of TLR RNA transcript expression in cells obtained from nasal washes of sOP and NOP children. Surprisingly, we found higher expression of TLR2/4 transcripts in nasal cells in sOP children as compared to NOP children (Fig 2A). We next examined TLR2 expression on individual cell types isolated from the nasal mucosa and again found higher expression on both neutrophils (Fig 2B) and epithelial cells (Fig 2B) in sOP compared to NOP children.

sOP children and NOP children have divergent levels of proinflammatory cytokines in nasal washes

Pro-inflammatory conditions in the nasal mucosa could affect Eustachian tube drainage from the middle ears or affect mucus drainage from the middle ear allowing for bacterial buildup in the nasal mucosa. Therefore, we examined the levels of pro-inflammatory cytokines in the NP passage of sOP and NOP children by cytokine film array. We found significantly lower levels of IL-8, IL6, and MIP1 β cytokines in sOP (Fig 3) as compared to NOP children.

Innate signaling by nasal mucosa is impaired in sOP children

We evaluated chemotaxis associated cytokine expression at onset of AOM to determine if lower cytokine expression in sOP children was associated with a lower capacity to signal the innate immune system. Lower levels of cytokines such as ENA-78 and ICAM-1 in sOP children as compared to NOP children (Fig 4) suggest deficits in cytokine responses in the OP child that could contribute to prolonged and/or increased density of *Spn* during NP colonization.

Discussion

Following NP colonization, *Spn* may proceed to cause local infections at mucosal sites (AOM, sinusitis, chronic obstructive pulmonary disease and pneumonia in children and adults) or systemically (septicemia and meningitis) [23,20]. Regarding AOM, children fall into two categories, those that experience infrequent infections and children that experience repeated infections in the first few years of life [10]. Viral URI precedes the development of AOM in about 95% of cases [24,25]. Differential up-regulation of NP epithelial cell receptors and modulation of innate immune responses caused by viral URIs can lead to proliferation or enhanced adhesion of *Spn* and other otopathogens in the NP to yield differences in AOM-causing inocula in OP children [26–28,14]. Damage to NP epithelial cells, followed by slowed repair, combined with a diminished innate immune response in the NP likely would facilitate a higher bacterial inoculum in NP secretions more capable of causing AOM when the bacteria and nasal mucus mix are effluxed from the NP to the middle ear space via the Eustachion tube [29]. Thus our findings of lower epidermal repair processes and higher inflammation in sOP over NOP children provide another mechanism to explain the predisposition of the sOP child to repeated AOM events.

Epithelial cells lining the nasal mucosa are the primary anchor point for *Spn* adherence during NP colonization [29,30]. The cytolytic damage caused by viral URI in the nasal passages significantly disrupts the epidermal lining and increases the availability of epithelial cell receptors for bacterial adherence proteins [31]. The epithelial cells respond by releasing proinflammatory mediators and innate signaling, while simultaneously undergoing repair to reestablish mucosal barrier integrity. Lower EGF-related cytokine expression in sOP children would likely result in a delay or slower capacity to repair damaged airway

epithelium. Viral replication kinetics observed in sOP and NOP children are similar for a multitude of viral species [25]; therefore it is unlikely that the differences observed were due to differences in viral species causing the URI. Instead the results suggest that the sOP child is deficient in their ability to generate as robust an epithelial repair process during a viral URI.

TLR expression on innate immune and epithelial cells plays a significant role in initiation of the innate responses to viral and bacterial infections. Disruption to TLR signaling or loss of TLRs, like TLR4 or TLR2, on the surface of cells impairs bacterial clearance [32,33]. In fact, disruption of MyD88 leads to a disruption in nontypeable *Haemophilus influenzae* (NTHi) clearance and granulocyte recruitment to the infected middle ears of experimental infected animal models [32]. To our surprise, we found increased expression of TLR2/4 on the surface of epithelial and innate immune cells infiltrating the nasal mucosa in sOP children. Higher TLR expression could signal more functional pathogen surveillance; however, the level of EGF related cytokines could also play a role in the amount of TLR expression in the NP or vice versa [34,35]. Ligation of EGF receptors can down regulate TLR2 expression during NTHi infections [36]. Higher TLR2 signaling, which may correlate with the level of TLR2 expression, can significantly increase the level of inflammation in the NP thereby enhancing pathogen clearance or contributing to inflammation of the NP [37,38]. *Spn* synergizes with NTHi to induce inflammation via upregulating TLR2 [39]. TLR signaling also plays a role in EGFR mediated epithelial repair signaling during *Staphylococcus aureus* infection and therefore lower EGFR expression in sOP children during a *Spn* infection could affect epithelial repair and indirectly bacterial adhesion [39]. TLR2 stimulation is associated with IL-17a/IL-23 production, a cytokine associated with protection from *Spn* [40,41]. In previous studies, we did not find a difference in the production of IL-17a transcripts or IL-17 recall responses from memory CD4 T-cells or PBMCs in sOP and NOP children [8,42]. Given this, and the observed lower expression of inflammatory cytokines in the nasal mucosa of sOP children, our data suggest that sOP children may have deficiencies downstream of TLR signaling or polymorphisms in TLR genes that affect function [33].

Interestingly, we found lower expression levels of inflammatory markers and chemotactic cytokines in the nasal mucus of sOP versus NOP children. Moreover, we found lower expression of IL-8 that may indicate a defect in neutrophil recruitment to the nasal mucosa, although the requirement for IL-8 in chemotaxis, at least in the middle ear, during AOM is not clear [43,44]. Neutrophils of children with recurrent AOM have been shown to have defective chemotaxis which coupled with our observed lower production of chemokines could contribute to a more limited innate response in OP children [45]. In tandem, we found significantly lower expression of ENA-78 (CXCL5), a cytokine released from inflamed epithelial cells for chemotaxis of neutrophils. The divergent expression this cytokine further demonstrates higher inflammation in NOP children as compared to sOP children. We also found trends in chemokines associated with T-cell trafficking downregulated in sOP children as compared to NOP children. If the lower observed expression of MIP1- α , MIP1- β , or MIP1- δ corresponds to lower monocyte/macrophage trafficking to or activation in the nasal mucosa of sOP children, an impact on the level of memory Th1 and Th17 cytokine production [40] or pneumococcal phagocytosis and subsequent clearance [46] could occur. Importantly, expression of MIP1-b, which we found significantly lower in the nasal mucosa of OP children, has an effect on neutrophil recruitment providing further evidence that frequent AOMs in sOP children may be an effect of diminished neutrophil recruitment and activation. Further examination of the divergent cellular infiltration into the nasal mucosa could be important to better understand how differences in chemotactic chemokines direct the innate responses in sOP children.

Our study has limitations. Differences in fluid volumes extracted during nasal washing could affect the levels of protein detection. We sought to mitigate this by using samples from our repository with similar total protein concentrations. To minimize variability attributable to other aspects of using human samples, we selected samples that had similar ages and URI time points during a AOM event with the same otopathogen. Our data could be attributed to responses to viral infection, bacterial colonization, or a combination of the two. The study design did not allow us to dissect those possibilities. Nonetheless, we detected multiple differences between the sOP and NOP children and achieved significance with a relatively small sample numbers used in our study. In addition, we studied TLR mRNA expression rather than proteins which could diverge although a good correlation between TLR2/4 mRNA transcripts and TLR2/4 proteins in human cells have been observed in other studies [47–49]. Although we directly compared sOP and NOP children at onset of AOM rather than during a URI without AOM, priming or alterations of the memory adaptive response during the response to AOM could affect future NP colonization events with similar serotypes thus reducing the number of future colonizations/AOMs in NOP children. Future studies could seek to determine the contributions of the adaptive immune memory responses to nasal and middle ear colonizations and how they influence the innate inflammatory responses.

In summary, we found divergent nasal mucosal responses in sOP and NOP children with respect to epithelial repair and innate inflammation responses that could enhance the ability of *Spn* to bind to NP epithelium during a viral URI, a key step in pathogenesis. We found lower levels of chemotaxis associated cytokine expression at onset of AOM in sOP children that was associated with a lower capacity to signal the innate immune system. Further work on the interface between the TLRs and EGF signaling could be important for therapeutic discovery to prevent AOM in OP children. Differences in the ability to restrict *Spn* to nasal colonization without advancement to the middle ear with respect to the innate response to infection could be important for further study to better define immune deficits in the OP child. Understanding how the mucosal microenvironment is more conducive to colonization during viral infections in sOP and NOP children could identify pathways to interventions applicable to multiple types of *Spn* infections in children and adults.

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Figure 1. Lower epithelial repair responses are characteristic of the sOP child response to *Spn* infection

We examined the protein expression levels of epidermal repair response cytokines in nasal secretions of sOP and NOP children at onset of AOM by film array. (A) The mean concentrations of epidermal related cytokines in the nasal washes are shown with (B) individual cytokine distributions shown for cytokines found to significantly diverge between groups (n=6 each group, mean + SE). Healthy, uninfected controls (n=4) were used to subtract the background levels of cytokines. *p<0.05. EGF: Epidermal Growth Factor, FGF: Fibroblastic Growth Factor, VEGF: Vascular Endothelial Growth Factor.

Figure 2. Divergent expression levels of TLRs in sOP and NOP children

We assessed the transcriptional expression levels of TLR4/TLR2 in (A) total cells obtained from nasal mucosal washes, or TLR2 expression in (B) isolated nasal neutrophils, and nasal epithelial cells in sOP and NOP children at onset of AOM. Data is expressed as relative fold change in expression of sOP over NOP children. (n=4 each group for Fig2A or n=5 each group for Fig 2B, mean + SE). * p=0.049 for calibrated $\Delta\Delta\text{CT}$ values.

Figure 3. The inflammatory mucosal response is limited in sOP children during AOM

We examined the protein expression levels of inflammatory cytokines in nasal secretions of sOP and NOP children at onset of AOM by film array. (A) The mean concentrations of inflammatory related cytokines in the nasal washes are shown with (B) individual cytokine distributions shown for cytokines found to significantly diverge between groups (n=6 each group, mean + SE). Healthy, uninfected controls were used to subtract the background levels of cytokines.

Figure 4. Limited chemotactic cytokines in the nasal mucosa may impair local immune responses

We examined cytokines in the nasal secretions of sOP and NOP children during AOM with respect to chemokines associated with immune trafficking during infection. (n=6 each group, mean + SE). (A) The mean concentrations of chemokine related cytokines in the nasal washes are shown with (B) individual cytokine distributions shown for cytokines found to significantly diverge between groups (n=6 each group, mean + SE). Healthy, uninfected controls were used to subtract the background levels of cytokines.

Table 1

Demographics of children in this study.

	OP	NOP
Average Age	14.8 months	15 months
Median Number of AOM Events	4.5	2
Smoking	N	N
Breastfed	Y	Y