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Complement activation in pediatric patients with recurrent acute otitis media

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ABSTRACT

Objective: Otitis media (OM) is one of the most common childhood diseases. The relative contribution of complement activation in protection and pathogenesis during OM remains largely unknown. The purpose of this study was to investigate the beneficial and pathogenic contributions of complement activation in the middle ear of pediatric patients with recurrent acute otitis media (rAOM), and therefore to provide a rational approach to prevent sequelae of OM such as hearing loss.
Methods: Twenty children undergoing pressure equalization tube placement with or without adenoidectomy for rAOM were enrolled in the study. Bacterial cultures, enzyme-linked immunosorbent assay (ELISA) for complement components and cytokines and western blot for complement activation were performed on middle ear effusion (MEE) and serum samples. The levels of complement C3a, C5a and sC5-b9 in MEEs and serum samples were compared. The levels of these factors were also examined in regards to length of episode. Pearson's correlation coefficients were calculated on variables between C5a and IL-6 or IL-8. Complement gene expression in human middle ear epithelial (HMEE) cells induced by otopathogens was evaluated. Data were analyzed with Student's *t* test or the Mann–Whitney rank sum test. In all cases, a P value of <0.05 was set as the measure of significance.
Results: Our data demonstrated that the complement classical/lectin, alternative and terminal pathways were activated in the middle ear of children with rAOM. Increased complement components of C3a, C5a and sC5-b9 in MEEs were detected in patients with the episode lasting more than six weeks. There was a strong correlation between C5a and IL-6 or IL-8 in the MEEs. Additionally, otopathogens induced enhanced gene expression of factor B and C3 in HMEE cells, which is beneficial for host defense against invading pathogens.
Conclusion: Our studies provided important new insights on how complement activation contributes to inflammatory process during rAOM. Knowledge of the activity of the complement pathway in patients with rAOM may stimulate the development of new strategies to prevent middle ear inflammatory tissue destruction by directing treatment to specific pathways within the complement cascade.

1. Introduction

Otitis media (OM) is one of the most common diseases in infants and young children in the United States and other developed countries. The incidence of acute OM is 10.85% (709 million cases each year) with 51% of these occurring in children under 5 years of age [1]. OM-related hearing impairment has a prevalence of 30.82 per ten-thousand [1]. *Streptococcus pneumoniae* (Spn) and nontypable *Haemophilus influenzae* (NTHi) are the two major pathogens to cause OM. In addition, the synergistic effect between influenza A virus (IAV) and Spn plays a significant role in the pathogenesis of OM [2,3].

The complement system is one of the major components of host innate immune systems in the defense against invading pathogens. Complement activation through the classical, alternative and lectin pathway results in the proteolytic cleavage of C3 to C3a and C3b. Once C3 is cleaved, the terminal pathway is activated, leading to the generation of the anaphylatoxin C5a and the assembly of the membrane attack complex (MAC) on the target surface or complexed with S-protein in plasma (sC5b-9) [4]. Complement proteins have previously been detected in human middle ear

Abbreviations: MEEs, middle ear effusions; Spn, *Streptococcus pneumoniae*; NTHi, nontypable *Haemophilus influenza*; IAV, influenza A virus.

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effusions (MEEs), and intense complement activation has been demonstrated in chronic otitis media with effusion (OME); maintaining ongoing inflammation in the middle ear [5–7]. In addition, the same authors observed strong staining for complement fragments iC3b/C3c and weaker labeling for C3d and C9, on the surface of the middle ear mucosa from chronic OME patients [6]. These findings suggest that intense complement activation could contribute to chronic inflammation resulting in effects on the tympanic membrane and middle ear such as development of myringosclerosis, middle ear adhesions, thinning and retraction of the tympanic membrane.

Our recent studies have demonstrated that the expression and production of alternative pathway components factor B and C3 in the middle ear epithelium are upregulated during the early stage of acute pneumococcal OM in mice [8]. Activation of the alternative pathway in the middle ear is a local phenomenon, which is essential for middle ear defense in bacterial clearance through the opsonophagocytosis mechanism [8]. Although our knowledge of the roles of the complement during OM has increased considerably during recent years, the beneficial and detrimental contributions of the complement system to this complex process remain largely unknown. To extend our findings in experimental animal studies into the human model, we analyzed the complement components of the classical/lectin and alternative and terminal pathways and cytokine levels in MEEs from pediatric patients with recurrent acute OM (rAOM) undergoing tympanostomy tube insertion. In addition, to examine whether complement factor B and C3 are produced locally by middle ear epithelial cells upon infection, we investigated the complement gene expression in primary culture of human middle ear epithelial (HMEE) cells infected with otopathogens. We showed that activation of the complement system significantly contributes to the pathogenesis of rAOM although it is inducted by otopathgens for protection.

2. Materials and methods

2.1. Patient population, clinical samples collection and processing

Children, between 5 and 38 months of age, undergoing pressure equalization (PE) tube placement with or without adenoidectomy for rAOM were enrolled in the study. rAOM is defined as more than six episodes during the first 2 years of life, or more than four episodes during the first year of life or more than three episodes in 6 months. A disease free interval of at least 30 days separates individual episodes of otitis media. Children with a known immunological disorders (including IgG subclass deficiency), craniofacial malformations or obstructive adenoid hypertrophy were excluded. To collect MEEs, a myringotomy was made in the standard fashion for tympanostomy tube placement. If a middle ear effusion was present it was suctioned with a JUHN TYMP-TAP Middle Ear Fluid Aspirator/Collector™ (Medtronic). Samples were placed on ice as soon as they were collected. Blood samples for sera were obtained prior to tympanostomy. Single use aliquots of the sera were stored at – 70 °C. Nasopharyngeal samples were then taken from the patients with BBL Culture Plus™ swabs (BD). The swabs were passed along the floor of the nose into the nasopharynx and kept there for 5 s and then withdrawn. MEE and NP samples were cultured overnight at 37 °C on chocolate agar and Columbia CNA agar plates in an incubator supplemented with humidity and 5% CO_2. A standard dilution assay and plate counting determined the number of CFU per milliliter. The remaining MEE and NP samples were centrifuged and single use aliquots of these samples were stored at −80 °C. Nationwide Children's Hospital Institutional Review Board approved this study.

2.2. Bacteria and virus

Spn serotype 6A (EF3114) with predominant transparent phenotype was kindly provided by Anderson, Department of Clinical Immunology, University of Goteborg. NTHi strain 2019 was obtained from Michael A. Apicella, University of Iowa College of Medicine. Influenza virus A (IAV)/Alaska (6/77) (H3N2) was propagated and its titer was determined by a plaque assay as previously described previously [9]. All these pathogens have been previously described and extensively used for OM studies [10–12].

2.3. Infection of HMEE cells

The primary culture of HMEE cells had been established from middle ear biopsy specimens taken near the orifice of the eustachian tube from a 19-year-old patient and was reported previously [11]. HMEE cells were infected with formalin-killed NTHi at a multiplicity of infection (MOI) of 10, with IAV at a MOI of 1, and live or ethanol-killed Spn with or without a prior IAV at a MOI of 10 as previously described [12]. Control cell cultures were incubated with medium alone. The experiment included an unstimulated negative control flask and a positive control flask incubated with TNF-α (20 ng/ml; Sigma–Aldrich), as described for a previous study that used cytokines in positive controls [11]. The cell culture supernatants were collected at each time point prior to harvesting cells, centrifuged at 500 g, and frozen at −70 °C.

2.4. Quantitation of complement component transcripts from the HMEE cells by real-time PCR

Real-time PCR assays were performed to quantitate C3, C4, C5, factor B, factor H and factor I transcripts. Total RNA from middle ear lysate sample pooled from five mice was reverse transcribed with random hexamers by using the Superscript preamplification system (Invitrogen, Carlsbad, CA). Real-time PCR primers were selected for each gene by using PRIMER EXPRESS software (Version 2.0, Applied Biosystems, Foster City, CA). Primer sets for the following genes were synthesized by Invitrogen: C3 (sense, 5′-GAACCAGCTTGGCGTCTTG-3′; antisense, 5′-TGGCCCATGTTGACGAGTT-3′), C4 (sense, 5′-GCGCAACCCTGTACGACTACT-3′; antisense, 5′-TACTTGGTGCCCCGTAAAACA-3′), C5 (sense, 5′-GCGAGCTGCACGGATTAGTT-3′; antisense, 5′-TGCGACGACACAACATTCAGT-3′), factor B (sense, 5′-GCCAGACTATCAGGCCCATTT-3′; antisense, 5′-AGCCTCAAAGCTCGAGTTGTTC-3′), factor H (sense, 5′-CCATCCTGGCTACGCTCTTC-3′; antisense, 5′-GGAGTAGGAGACCAGCCATTCTC-3′), and GAPDH (sense, 5′-ATGGAAATCCCATCACCATCTT-3′; antisense, 5′-CGCCCCACTTGATTTTGG-3′). Reactions were performed in a 50-μl volume that included diluted cDNA sample, primers, and SYBR Green PCR Master mix (Applied Biosystems) according to the manufacturer's protocol. Real time PCR amplifications were performed on an Applied Biosystem Prism 7900 HT Sequence Detector according to the manufacturer's instructions. All data were normalized to the GAPDH mRNA. Relative changes in gene expression were determined using the $2^{-\Delta\Delta CT2}$ method as previously described [8] and expressed as the *n*-fold difference relative to a cDNA from normal control mice prepared in parallel with the experimental cDNAs.

2.5. Immunofluorescent staining

The cells were cultured on four-chamber microscope slides. After Spn treatment, the cells were fixed in 4% paraformaldehyde solution and permeabilized with DPBS containing 0.5% TritonX-100, and then blocked with DPBS (pH 7.2) containing 1% bovine serum albumin (BSA). The cells were incubated with primary antibodies: goat anti-human factor B polyclonal antibody (1:500,