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Age-dependent changes in pattern recognition receptor and cytokine mRNA expression in children with otitis media with effusion

Sang Hoon Kim [a], Sung Ho Cha [b], Young Il Kim [c], Jae Yong Byun [a], Moon Suh Park [a], Seung Geun Yeo [a,c,*]

[a] Department of Otorhinolaryngology - Head and Neck Surgery, School of Medicine, Kyung Hee University, Seoul, Republic of Korea
[b] Department of Pediatrics, School of Medicine, Kyung Hee University, Seoul, Republic of Korea
[c] Medical Science Research Institute, Kyung Hee University Medical Center, Seoul, Republic of Korea

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ABSTRACT

Objective: To investigate age-dependent changes in expression of pattern recognition receptors (PRRs) and cytokines in pediatric OME.
Materials and methods: Ninety five pediatric patients with OME were divided into 4 age groups: 0–2, 2–4, 4–7, and over 7 years. The presence of bacteria, and the levels of expression of mRNAs encoding Toll-like receptor (TLRs), NOD like receptors (NLRs) and cytokines in middle ear fluid were assessed, as were their correlations with age, gender, presence of bacteria and accompanying disease.
Results: Bacteria were detected in 32.6% of patients. The levels of expression of PRR and cytokine mRNAs tended to be lower in children aged 2–4 and 4–7 years. The levels of expression of TLR-2, TLR-9, NOD-1, NOD-2, IL-1, IL-6, and TNF-α mRNAs in effusion fluid were significantly lower in these two groups than in children aged 0–2 and over 7 years ($p < 0.05$ each). The levels of expression of TLR-4, TLR-5, TLR-9, and NOD-1 mRNAs were significantly lower in culture positive than in culture negative patients ($p < 0.05$ each). However, the expression levels of PRR and cytokine mRNAs were unrelated to gender and accompanying disease ($p > 0.05$ each).
Conclusions: The levels of expression of PRR and cytokine mRNAs differed by age in children with OME.

1. Introduction

Upper respiratory tract infection and immune responses to infection are important etiological factors of otitis media in children with undeveloped Eustachian tubes. Otitis media with effusion (OME) may result in conductive hearing loss and be a precursor to retraction and perforation of the tympanic membrane. Although most patients recover spontaneously, some show frequent recurrence of otitis media. At any time, 5% of children aged 2–4 years have hearing loss due to middle ear effusion lasting 3 months or longer. About 80% of children under 10 years have at least one episode of OME, and boys are slightly more affected more than girls. The prevalence of OME is highest in children aged 2 years or younger, but sharply declines in children older than 6 years [1,2].

Pattern recognition receptors (PRRs) play a key role in innate immune responses by recognizing pathogen associated molecular patterns (PAMPs) present on microbial pathogens, including bacteria, viruses, parasites, fungi, and protozoa [3,4]. PRRs are present on various cellular compartments, including the cell surface, lysosomes, cytoplasm, and endosomes; following their recognition of various pathogenic motifs, they activate various cell signal pathways [5]. Cytokines regulate chronic infection in the middle ear, resulting in histopathological changes associated with OME and based on molecular, pathological processes. Nitric oxide (NO) may cause sensorineural hearing loss (SNHL) secondary to OME; enhance local inflammatory reactions together with other inflammatory factors; and mediate LPS induced mucociliary dysfunction in the middle ear [6].

The immunologic roles of PRRs and cytokines underlying the pathogenesis of OME have been investigated, but their age-associated changes in expression have not been determined. Moreover, the expression of these molecules may also differ by gender or by the presence or absence of bacteria or comorbidities. This study was therefore designed to determine whether the levels

* Corresponding author at: Department of Otorhinolaryngology - Head & Neck Surgery, School of Medicine, Kyung Hee University, #1 Hoegi-dong, Dongdaemun-gu, Seoul 130-702, South Korea. Tel.: +82 2 958 8474; fax: +82 2 958 8470.
E-mail address: yeo2park@gmail.com (S.G. Yeo).

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of expression of PRRs and cytokines in children with OME are altered by age, gender, the presence of bacteria, or the occurrence of comorbidities.

2. Methods

2.1. Subjects

Effusion fluid samples were obtained from 95 pediatric patients who visited the Department of Otorhinolaryngology, School of Medicine, Kyung Hee University, Seoul, Korea, and who underwent ventilation tube insertion to treat chronic OME between September 2011 and August 2013. OME was diagnosed by the presence of an amber-colored tympanic membrane on otoscopic examination and by the presence of B- or C-type tympanograms on impedance audiometry. Surgery was performed on chronic OME patients who did not show improvement after a 2–3 month follow-up, showed progressive retraction of the eardrum, or experienced progression of hearing loss as shown by an increase in pure tone threshold. Children suspected of having acute otitis media, head and neck anomalies, systemic diseases, or congenital or acquired immunodeficiencies were excluded. Written informed consent to use patient samples was obtained from each child's parents or guardians, and the study protocol was approved by the Institutional Review Board of Kyunghee University Medical Center.

2.2. Middle ear effusion fluid

When surgery was required, the external auditory canal was washed with a potadine solution and a radial incision was made in the anterior inferior quadrant of the tympanic membrane. Effusion fluid was aseptically collected with the aid of Juhn Tym-Tap collectors (Medtronic Xomed; Jacksonville, FL, USA); care was taken to avoid bleeding. Fluid samples were transferred to Eppendorf tubes and stored at −80 °C. Effusion fluid samples, in their original collectors, were sampled using sterile cotton swabs (Xomed Trace Products, Jacksonville, FL, USA); the swabs were immersed in Stuart transport medium. These samples were used to inoculate solid blood agar and liquid thioglycollate medium (Hangang, Kun-po, Korea). Cultures were incubated for 24 h at 35 °C, and bacteria that formed colonies were identified by Gram staining and biochemical testing.

2.3. Amplification

Total RNA was extracted from effusion fluid using RNA-Bee solution kits (Tel-Test, Friendswood, TX, USA), according to the manufacturer's protocol. First-strand cDNA was synthesized by reverse transcription in a 20 μl reaction mixture containing 1 μg of RNA, 1× reaction buffer, 1 mM dNTP, 5 μM random primers, 20 units RNase inhibitor, and 20 units AMV reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture was incubated at 42 °C for 1 h, and the reaction was terminated by heating at 95 °C for 5 min. The primers specific for Toll-like receptors (TLRs)-1, -2, -4, -5, -6, and -9; NODs-1 and -2; interleukins (IL)-6, -8, -10, and -12; interferon (IFN)-γ; tumor necrosis factor (TNF)-α are shown in Table 1. Real-time polymerase chain reactions (PCR) were performed using a Chromo4 Detector real-time system (Bio-Rad, Hercules, CA, USA) and SsoFast EvaGreen supermix (Bio-Rad). Each 20-μl PCR mixture contained 2 μl of cDNA, 10 μl SsoFast EvaGreen supermix, 2 μl of each primer and 6 μl PCR grade water. The amplification protocols consisted of an initial denaturation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 5 s and annealing and extension at 55–64 °C for 12 s (Table 1). The point at which the expression of each cDNA crossed with that of β-actin cDNA was applied to the formula, $2^{-(\text{target gene} - \beta\text{-actin})}$, and the relative amounts were quantitated.

Table 1
Primers for real-time RT-PCR.

Gene name	Sequences	Annealing temperature	Product size (bp)
TLR 1	F:5'-CTATACACCAAGTTGTCAGC-3' R:5'-GTCTCCAACTCAGTAAGGTG-3'	60	220
TLR 2	F:5′-GCCAAAGTCTTGATTGATTGG-3′ R:5′-TTGAAGTTCTCCAGCTCCTG-3′	64	347
TLR 4	F:5′-TGGATACGTTTCCTTATAAG-3′ R:5′-GAAATGGAGGCACCCCTTC-3′	56	507
TLR 5	F:5′-CTAGCTCCTAATCCTGATG-3′ R:5′-CCATGTGAAGTCTTTGCTGC-3′	56	438
TLR 6	F:5'-CCTCCCAGGATCAAGGTACTTG-3' R:5'-ATCAGGCCAGCCCTCTAACAC-3'	60	327
TLR 9	F:5′-CCCTCAACTTCACCTTGGATCT-3′ R:5′-CCACATATGGCCCAGTGCA-3′	64	408
NOD-1	5′-GTCACTGAGGTCCATCTGAAC-3′ 5′-CATCCACTCCTGGAAGAACCT-3′	60	59
NOD-2	5′-CATGTGCTGCTACGTGTTCTC-3′ 5′-CCTGCCACAATTGAAGAGGTG-3′	60	67
RIG-1	5′-GACCCTCCCGGCACAGA-3′ 5′-TCAGCAACTGAGGTGGCAATC-3′	60	84
IL-6	F:5'-GTGTTGCCTGCTGCCTTC-3' R:5'-AGTGCCTCTTTGCTGCTTTC-3'	60	194
IL-8	F:5'-GACATACTCCAAACCTTTCCAC-3' R:5'-CTTCTCCACAACCCTCTGC-3'	60	160
IL-10	F:5'-GAACCAAGACCCAGACATC-3' R:5'-CATTCTTCACCTGCTCCAC-3'	60	137
IL-12	F:5'-TCGGCAGGTGGAGGTCAGC-3' R:5'-CGCAGAATGTCAGGGAGAAGTAGG-3'	60	77
IFN-γ	F:5'-TGTGGAGACCATCAAGGAAGAC-3' R:5'-TGCTTTGCGTTGGACATTCAAG-3'	60	121
TNF-α	F:5'-ATCTTCTCGAACCCCGAGTG-3' R:5'-GGGTTTGCTACAACATGGGC-3'	60	51
β-Actin	F:5'-GCGAGAAGATGACCCAGATC-3' R:5'-GGATAGCACAGCCTGGATAG-3'	60	77

Abbreviations: RT-PCR, real time-polymerase chain reaction; TLR, Toll-like receptor; NOD, nucleotide-binding oligomerization domain; RIG, retinoic acid-inducible gene; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

2.4. Statistical analysis

The demographic characteristics of the four groups of patients were analyzed by Fisher's exact test, and the levels of expression of mRNAs were determined using the Mann–Whitney *U* test or the Kruskal–Wallis test with Bonferroni correction. All statistical analyses were performed using IBM SPSS version 20 (IBM Corp., Armonk, NY), with a p value <0.05 defined as statistically significant.

3. Results

The subjects consisted of 95 pediatric patients (59 males, 36 females) ranging in age from 0 to 10 yr (mean ± SD age, 4.6 ± 2.3 yr). Pediatric patients were divided into four age groups, those aged 0–2, 2–4, 4–7, and over 7 years. Evaluation of the characteristics of middle ear effusion (MEE) showed that 21 children had serous, 67 had mucoid and 7 had purulent MEE. There were no differences among the four groups in gender distribution, duration of effusion or characteristics of middle ear fluid, but the proportion of children with allergic rhinitis was significantly higher in those aged over 7 years than in the other three groups (Table 2).

Bacteria were detected in 31/95 (32.6%) effusion fluid samples by standard culture, with the remaining samples being negative for bacterial growth. Among the bacteria detected were coagulase negative *Staphylococcus*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, MRSA (*methicillin-resistant Staphylococcus aureus*), *Pseudomonas aeruginosa*, *Corynebacterium suppurative*, *S. aureus,*