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Original Article

Toll-like Receptors 2 and 4 and Their Mutations in Patients with Otitis Media and Middle Ear Effusion

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Objectives. Toll-like receptors (TLRs) detect microbial infections and they can directly induce innate host defense responses. TLR 2 has been shown to be primarily involved in the recognition of peptidoglycans and lipoteichoic acid of gram positive bacteria. TLR 4 recognizes lipopolysaccharides and lipoteichoic acids from both gram-negative and gram-positive bacteria. Both mutations lead a reduced capacity to elicit inflammation and they increase the risk for gram-positive and negative infections. This study was performed to investigate the expressions of TLR 2 and 4 and their mutations in patients suffering with otitis media and middle ear effusion.

Methods. Middle ear fluid samples were collected from 40 otitis media effusion (OME) patients who had ventilating tubes inserted. Bacteria in the effusion fluid were detected by standard bacterial culture. The secreted IgG, IgA and IgM were measured by Enzyme-linked immunosorbent assay. TLR 2 and 4 were assessed by performing RT-PCR. The genomic DNA from each patient was isolated from the middle ear fluid samples that were collected from 60 OME patients, and the presence of mutations was determined by performing restriction digestion and DNA sequencing analysis.

Results. Among the 40 middle ear fluid samples, bacteria were detected in 13 middle ear fluid samples. The amounts of IgM, IgA, and IgG were 151.20±60.94 ng/mL, 21.59±7.96 ng/mL and 11.55±16.98 ng/mL, respectively. TLR 2 and 4 were expressed in the middle ear fluid and the expression of TLR 2 was higher than that of TLR 4. However, there was no correlation between the expressions of TLR 2 and 4, and the concentration of immunoglobulin or the presence of bacteria (P>0.05). There ware no mutations of TLR 2 (Arg753Gln, Arg677Trp) and TLR 4 (Asp299Gly, Thr399Ile).

Conclusion. TLR 2 and 4 were expressed in all the middle ear fluid samples of OME, but the mutations of TLR 2 and 4 were not detected. TLR 2 and 4 may play a vital role in the immunological responses of patients with OME.

Key Words. *Otitis media, Toll-like receptor, Mutation*

INTRODUCTION

Otitis media with effusion (OME) is one of the most common ear diseases in children and it is a major cause of hearing loss (1, 2). Etiologically, OME has been associated with upper respiratory infection, Eustachian tube dysfunction, allergic rhinitis and immunological and environmental factors (3), and bacterial infection caused by Eustachian tube dysfunction is considered one of the most important causes (4). In addition to the bacterial infection itself, various inflammatory mediators released after infection and persistent immunological reactions have been considered as major causes of OME (5).

The innate immune system is involved first during host reactions to pathogens. Subsequently, antigen presenting cells (APC) have been shown to secrete various cytokines, chemokines and costimulatory signals, leading to the activation of the adaptive immune system (3). Among the factors involved in the activation of the innate immune system are pattern recognition receptors, pathogen-associated molecule patterns (PAMPs) and Toll-like receptors (TLRs). TLRs are membrane proteins that have been detected in the defense system of mammalians and Drosophila. To date,

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11 TLR subtypes have been identified in humans and they have been shown to be involved in the recognition of PAMPs in the innate immune system (6, 7). Upon the recognition of ligands by TLRs, NF-κB is activated through the MyD88-dependent and MyD88-independent pathways, and this induces the expression of inflammatory genes that encode cytokines and cell conjugation molecules (8, 9). Among the TLR subtypes, TLR 2 and TLR 4 have been reported to recognize pathogenic bacteria. TLR 2 has been found to be the receptor for the cell wall components of gram positive bacteria, such as peptidoglycans, lipoteichoic acid and lipoproteins, and TLR 4 has been observed to bind to the toxic pneumolysin ligand produced by gram positive bacteria, as well as binding to the major component of gram negative bacteria lipopolysaccharide (LPS) (10-12).

Two single nucleotide polymorphisms (SNPs) of TLR 2, Arg 677Trp and Arg753Gln, and two SNPs of TLR 4, Asp299Gly and Thr399Ile have recently been shown to be associated with the susceptibility to pathogenic bacteria because the TLRs with these polymorphisms have a decreased ability to bind to ligands (13, 14). So, we have assessed the role of these TLRs in the development of OME by assaying the expression of wild type and mutant TLR 2 and TLR 4, and the secretion of immunoglobulin in the fluid of the middle ear.

MATERIALS AND METHODS

The study was performed on 100 pediatric patients who underwent insertion of ventilation tubes for treating chronic OME in the Department of Otorhinolaryngology at our hospital between March 2005 and August 2006. The B and C types of OME were diagnosed by impedance audiometry, as well as by the presence of an air-fluid level and air bubbles behind the tympanic membrane and an amber colored tympanic membrane by otoscopic examination. Prior to surgery, the external auditory canal was cleaned with potadine solution, and the exudate was sterilely collected in an effendorf tube with using a collector (Jhun typ. Tap), and this was stored at -70℃. The use of the samples and the purpose of experiment were explained to the parents and guardians, and written informed consent was obtained. The children suspected of having head and neck anomalies, systemic disease or congenital or acquired immunodeficiency were excluded from the study. The 100 pediatric patients were divided into 40 and 60 OME patients. In 40 samples, the bacteria in the effusion fluid were detected by standard bacterial culture, and the secreted IgG, IgA and IgM were measured by enzyme-linked immunosorbent assay, and the TLR 2 and 4 were assessed by RT-PCR. They were 23 boys and 17 girls in this group. Their mean age was 4.5 yr. In the 60 samples, the genomic DNA from each patient was isolated and the presence of mutations was determined by restriction digestion and DNA sequencing analysis. They were 27 boys and 33 girls in this group. Their mean age was 4.9 yr.

Bacterial culture

The exudates were mixed with Stuart's transport medium, transported to a microbiology laboratory, inoculated onto blood agar medium and thioglycollate liquid medium, and then they were cultured at 35℃ for 24 hr. The bacterial colonies were identified by gram staining and biochemical tests.

Enzyme-linked immunosorbent assay (ELISA)

All the samples of middle ear effusion were stored at -80℃, and their IgG, IgA, and IgM concentrations were measured by ELISA. Briefly, 50 μL of 1:100 goat anti-human IgG, anti-human IgA and anti-human IgM in coating buffer (1.59 g Na_2CO_3+2.93 g $NaHCO_3$+5% NaN_3, pH 9.6) were placed in each well of a 96 well plate and this was allowed to incubate overnight at 4℃. The wells were washed 6 times, blocking antibody was added and 50 μL of each sample was added to each well, and the plates were incubated at room temperature for 3 hr. The wells were washed 6 times, and purified goat anti-human IgG, anti-human IgA, and anti-human IgM conjugated to horseradish peroxidase in PBS/Tween/BSA solution was added; the plates were then incubated at room temperature. The plates were washed 6 times, substrate solution (2,2′-AZINO-Bis) was added and the optical absorbance was measured at 450 nm (Bethyl, Montgomery, TX, USA).

RNA extraction

The cultured cells were lysed by adding 1 mL RNA-Bee solution (Tel-test, Inc, Friendswood, TX, USA) to a 60 mm culture dish. Each sample of cells was mixed with 0.2 times volume of chloroform, they were kept on ice for 5 min and next centrifuged at 12,000 × g for 15 min at 4℃ (Centrifuge5402, Eppendorf, Germany). An equal volume of isopropanol was added to each supernatant, and these were allowed to precipitate at room temperature for 10 min. Following centrifugation at 12,000 g for 5 min at 4℃, the supernatant was discarded, and each pellet was washed with 800 μL of 75% ethanol, centrifuged at 7,500 × g for 5 min at 4℃ and then air dried for 10 min. Each pellet was resuspended in DEPC-water, and the RNA concentration of each sample was measured at 260 nM with using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at A_{260}/A_{280} of each sample was 1.5-1.8, and the RNA samples were stored at -70℃.

Reverse transcription-polymerase chain reaction

To synthesize the complementary deoxynucleotide acid (cDNA), 1 μg RNA was adjusted to 10 μL with DEPC-treated water; this was then heated at 70℃ for 5 min and kept on ice for 5 min. To each sample was added 2 μL 10 × reaction buffer (100 mM Tris-HCl, pH9.0, 500 mM KCl, 1% Triton X-100), 4 μL 25 mM $MgCl_2$, 2 μL 10 mM deoxynucleoside triphosphates (dNTPs), 0.5 μL (40 units/μL) ribonuclease (RNase) inhibitor and 15 units AMV reverse transcriptase (Promega, Madison, WI, USA), after which the volume was adjusted to 20 μL and the samples were incubated at

42℃ for 1 hr, 9℃ for 5 min and 4℃ for 5 min.

The TLR 2 and TLR 4 primers are shown in Table 1. Each PCR reaction consisted of 2 μL cDNA, 5 μL 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 4 μL 2.5 mM dNTPs, 20 pmol of sense and antisense primer and 2 units Taq DNA polymerase (Takara, Shiga, Japan), and this was adjusted to 50 μL with sterile distilled water. The amplification protocol consisted of an initial denaturation at 95℃ for 5 min, followed by 30 cycles (25 for β-actin) of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 1 min, followed by a final extension at 72℃ for 10 min (Biometra, Gottingen, Germany). A 10 μL aliquot of each PCR product was mixed with 2 μL of 6× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% sucrose) and this was electrophoresed on a 2% agarose gel containing 0.5 μg/mL ethidium bromide with using 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 90 volts for 1 hr. The bands were assessed using the Gel Doc 1000 gel documentation system (Bio-Rad, Hercules, CA, USA).

Real-time PCR

To a 1.5 mL test tube was added 2.4 μL 25 mM $MgCl_2$, DNA master SYBR green I containing 2 μL Lightcycler fast start enzyme and 10 pmol of the sense and antisense primers, respectively, with the final volume adjusted to 18 μL with distilled water. To each tube was added 2 μL cDNA, and the samples were aliquoted into capillary tubes and centrifuged at 700×g for 5 sec and then they were placed in a Lightcycler amplifier (Roche Applied Science, Mannheim, Germany). The annealing temperature for TLR 2, TLR 4 and β-actin was 55℃, and real-time PCR was performed at 25 bp/sec, depending on the size of the PCR products. The crossing point of TLR 2 or TLR 4 with β-actin was applied to the formula, $2^{-(TLR2/4-\beta\ actin)}$ and the relative amounts of TLR 2 or TLR 4 were quantitated.

Table 1. Primers for real-time RT-PCR

Gene	Sequence	Product size (bp)
TLR 2	5′-GCCAAAGTCTTGATTGATTGG-3′	347
	5′-TTGAAGTTCTCCAGCTCCTG-3′	
TLR 4	5′-TGGATACGTTTCCTTATAAG-3′	507
	5′-GAAATGGAGGCACCCCTTC-3′	
β-actin	5′-CTTCTACAATGAGCTGCGTG-3′	305
	5′-TCATGAGGTAGTCAGTCAGG-3′	

RT-PCR: reverse transcriptase-polymerase chain reaction; TLR: toll-like receptors.

Genotyping of the TLR-2 and TLR-4 genes

The TLR-2 and TLR-4 genes were amplified using the primers shown in Table 2 (15). To 100 ng genomic DNA was added 1 ×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 10 pmol of each primer and 1 unit of AmpliTaq Gold DNA polymerase (Roche, Applied Biosystems, Foster City, CA, USA), with the final volume adjusted to 25 μL with distilled water. Following initial denaturation at 94℃ for 14 min, the samples were subjected to 35 cycles of denaturation at 94℃ for 30 sec, the proper annealing temperature for 30 sec (64℃ for Arg677Trp and Arg753Gln, 65℃ for Asp299Gly and 62℃ for Thr399Ile), and extension at 72℃ for 30 sec, with a final extension step at 72℃ for 10 min.

Restriction enzyme digestion of the PCR products

To 15 μL of each PCR product was added 1×enzyme reaction buffer and 10 units of each restriction enzyme (Table 3; New England Biolabs, Beverly, MA, USA), with the volume adjusted to 20 μL with distilled water, and this was reacted at 37℃ (Nco I, Hinf I, and Msp I) or 60℃ (Mwo I) for 16 hr. Each reaction product was mixed with 4 μL 6X gel loading dye (30% sucrose, 0.05%

Table 2. PCR primer sequences

Gene	Polymorphism	Primers
TLR 2	Arg677Trp	F:5′-CCCCTTCAAGTTGTGGCTTCATAAG-3′
		R:5′-AGTCCAGTTCATACTTGCACCAC-3′
	Arg753Gln	F:5′-CATTCCCCAGCGCTTCTGCAAGCTCC-3′
		R:5′-GGAACCTAGGACTTTATCGCAGCTC-3′
TLR 4	Asp299Gly	F:5′-AGCATACTTAGACTACTACCTCCATG-3′
		R:5′-GAGAGATTTGAGTTTCAATGTGGG-3′
	Thr399Ile	F:5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′
		R:5′-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3′

PCR: polymerase chain reaction; TLR: toll-like receptors.

Table 3. Restriction enzymes and the length of the restriction fragments

Gene	Polymorphism	Restriction enzyme	Restriction temp (℃)	Length of the restriction fragments
TLR 2	Arg677Trp	MwoI	60℃	Wild type (allele C): 130 bp+22 bp
				Arg677Trp (allele G): 152 bp
TLR 2	Arg753Gln	MspI	37℃	Wild type (allele G): 104 bp+25 bp
				Arg753Gln (allele G): 129 bp
				Wild type (allele A): 188 bp
TLR 4	Asp299Gly	NcoI	37℃	Asp299Gly (allele G): 168 bp+20 bp
TLR 4	Thr399Ile	HinfI	37℃	Wild type (allele C): 124 bp
				Thr399Ile (allele T): 98 bp+26 bp

TLR: toll-like receptors.

bromophenol, 0.05% xylene cyanol) and this was electrophoresed on a 2.5% agarose gel containing 0.5 μg/mL ethidium bromide in 0.5× electrophoresis buffer (45 mM Tris-borate, 1 mM EDTA) at 90 volts for 1 hr. The polymorphisms were assessed by a gel imaging analysis (Gel Doc 1000 Gel Documentation System, Bio-Rad, Hercules, CA, USA).

The differences in the immunoglobulin and TLR expressions between the patients from whom bacteria were not isolated and were isolated were compared by the Mann-Whitney test. Correlations between IgA, IgG, IgM, and TLR 2 and TLR 4 were analyzed by the Spearman correlation test. All the calculations were performed using SPSS 11.5 for Windows. *P* values less than 0.05 considered statistically significant.

RESULTS

We attempted to culture bacteria from the exudates taken from the 40 patients. Of these 40 samples, 13 were positive for bacteria and 27 were negative. The detected bacteria were *S. pneumoniae, H. influenzae, M. catarrhalis,* MRSA, coagulase negative Staphylococcus, *S. pyogenes,* Bacillus and Gram-negative rods.

The concentrations of IgA, IgG, and IgM in the exudates from the 13 patients who were positive for bacteria (21.59±7.96 ng/mL, 11.55±16.98 ng/mL, and 151.20±60.94 ng/mL, respectively) were higher than the concentrations in the exudates from the 27 patients who were negative for bacteria (20.98±5.99 ng/mL, 10.96±4.27 ng/mL, and 145.32±48.70 ng/mL, respectively), but the differences were not statistically significant (*P*>0.05), (Fig. 1).

TLR 2 and TLR 4 mRNA were detected in the middle ear exudates, with the level of TLR 2 mRNA being higher than the level of TLR 4 mRNA. However, the levels of TLR 2 and TLR 4 mRNA were similar in the 13 patients who were positive for bacteria and the 27 patients who were negative for bacteria (*P*>0.05) (Fig. 2, 3).

We did not detect the TLR 4 gene mutations, TLR 4 (Asp299Gly) and TLR 4 (Thr399Ile), or the TLR 2 gene mutations, TLR 2 (Arg 753Gln) and TLR 2 (Arg677Trp), in any of these samples (Fig. 4).

The IgA, IgG, and IgM levels in the exudates did not correlate with the TLR 2 and TLR 4 mRNA (*P*>0.05), (Table 4).

DISCUSSION

In patients with OME, exudates are retained in the middle ear cavity. Most children have at least one episode of OME before the age of 4 yr, and it is the most common cause of hearing loss in children (2). Eustachian tube dysfunction and inflammatory reactions induced by viruses and bacteria are important causes of OME, and the exudates produced within the middle ear cavity are thought to be due to inflammatory reactions. Thus, analy-

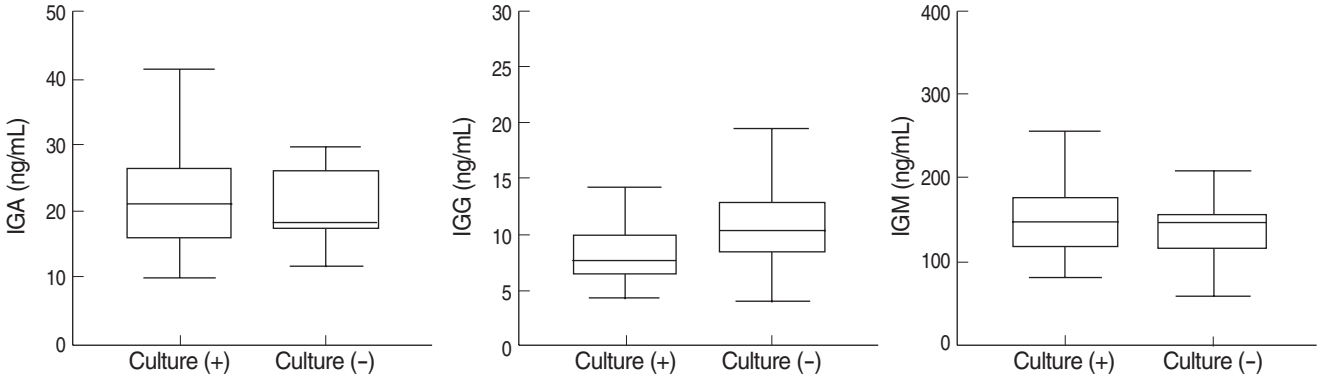

Fig. 1. Concentrations of IgA, IgG, and IgM in the effusion fluid of OME according to the culture results.

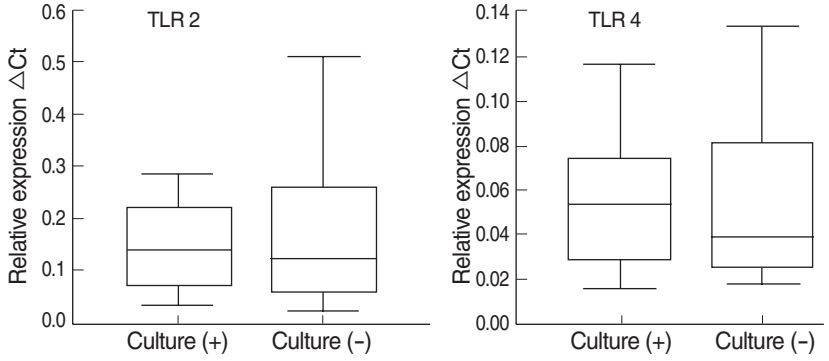

Fig. 2. Comparison of the TLR 2, TLR 4, and TLR 9 expressions according to the culture results.

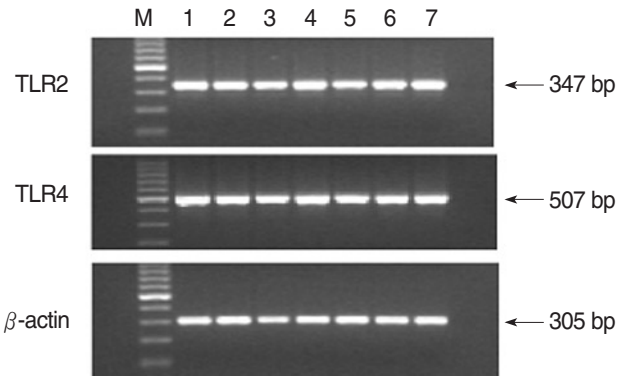

Fig. 3. The expression of TLR2, TLR4, and TLR9 mRNA in the middle ear fluid from patients with otitis media with effusion. The mRNA expression was measured by RT-PCR and it was resolved on 2% agarose gel by electrophoresis.
Lane 1: 100 bp DNA ladder; lane 2: normal PBL; lane 3-7: patients. TLR 2, TLR 4, and TLR 9 and β-actin mRNA were expressed in all the patients.

Table 4. Correlation between TLR 2 and TLR 4, and IgG, IgA, and IgM expressions

	TLR2	TLR4
IgG	r=0.117 (*P*=0.562)	r=0.207 (*P*=0.301)
IgA	r=-0.184 (*P*=0.358)	r=-0.171 (*P*=0.394)
IgM	r=-0.226 (*P*=0.256)	r=-0.324 (*P*=0.099)

r: spearman correlation coefficient.

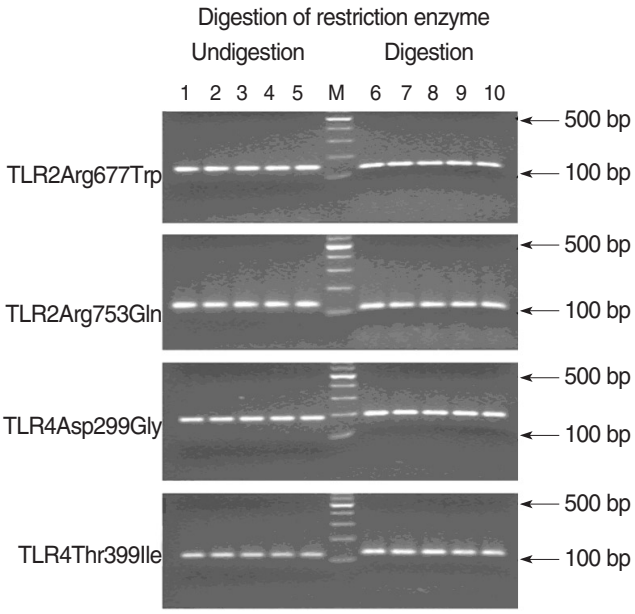

Fig. 4. Polymorphism of the TLR2 (Arg677Trp and Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) genes in the middle ear fluid from patients with OME. The genomic DNA was amplified by PCR, and the products were digested with MwoI (TLR2 Arg677Trp), MsPI (TLR2 Arg753Gln), NcoI (TLR4 Asp299Gly), and HinfI (TLR4 Thr399Ile) restriction enzymes, and then they were separated on 3% agarose gel by electrophoresis.
Lane 1: normal PBL; lane 2-5: patients; lane 6: 100 bp DNA ladder; lane 7: normal PBL; lane 8-11: patients. TLR2 (Arg677Trp and Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) were absent from all the patients.

sis of exudates is important for assessing the immunological reactions in middle ear infections.

The human immune system is composed of the innate and acquired immune systems, which act together against pathogens. The innate immune system recognizes microbial non-self, as well as molecules that are present only in microorganisms. The receptors of the innate immune system that recognize the molecular patterns pertinent to pathogens are called pattern-recognition receptors. These receptors sense invading pathogens and they recognize pathogen-related molecular patterns and signals that induce the expression of cytokines, chemokines and other signaling molecules, and they activate biological defense mechanisms such as reactive nitrogen, reactive oxygen and anti-peptide molecules (16). In addition, upon the recognition of a pathogen-associated molecular pattern, CD80 and CD86 are induced on antigen presenting cells, indicating that the adaptive immune system is derived from the innate immune system (17).

Among the receptors that recognize patterns in the innate immune system are the TLRs, of which 11 types have been currently identified in humans. The TLRs have been reported to be expressed primarily on primary defense cells, including dendritic cells, macrophages, neutrophils, T cells and B cells (18). Each TLR consists of an intracellular domain, a transmembrane domain and an extracellular domain. Upon the recognition of the pathogen-associated molecular pattern of a microorganism by the extracellular domain of a TLR, NF-κB is activated through the MyD88-dependent and -independent pathways. NF-κB subsequently migrates to the nucleus where it acts as a transcription factor, and it induces the activation of cytokines or co-stimulatory signals, thus bridging the innate immune system and the acquired immune system (8, 9).

TLR 2 has been reported to be stimulated primarily by lipoproteins and peptidoglycans of gram positive bacteria, as well as it reacts with the lipoprotein that's present on the surface of Mycobacterium tuberculosis, Borrelia burgdorferi, Treponema pallidium, Mycoplasma fermentans, fungus cell walls and mycobacterial lipoarabinomannan (10).

TLR 4 has been reported to recognize the major component of gram negative bacteria cell wall LPS, and it induces innate immune reactions. Moreover, TLR 4 has been found to react with the toxic factor pneumolysin that's produced by gram positive bacteria, thereby inducing inflammatory reactions (11, 19).

TLRs recognize invasive viruses, bacteria and fungi at early stages, and by inducing the expression of innate immune mediators such as the complement cascade, the TLRs play a role as sentinels for the activation of local inflammation. TLR 2 and TLR 3 are expressed on airway epithelium and, following stimulation by their ligands, they help produce β-defensins (20). Moreover, in the sinus mucosa, TLR 4 is expressed less than TLR 1, TLR 2,

TLR 3 and TLR 5 (21). In addition, defensins were found to be more abundantly expressed in palatine tonsils and adenoid tissues than in the non-mucosal cells in response to TLR stimulation (22). Further, the TLR 2 expression was increased in epithelial cells of an otitis media model that was caused by Hemophilus influenzae (23). We have shown here that TLR 2 and TLR 4 mRNA were expressed in the middle ear fluids of patients with OME, with TLR 2 expression being higher than that of TLR 4. These findings confirm that the presence of TLR is involved in the development of OME. However, when we compared the TLR 2 and TLR 4 mRNA expressions in patients for whom we did and did not identify bacteria, we found that the differences were not statistically significant. For about 3 months prior to surgery, these patients had been treated with antibiotics, which may have had an effect on the expression of these TLRs.

TLRs on B cells recognize pathogen-associated molecular patterns, they help activate B cells and they induce the production of low affinity IgM (24). LPS and CpG-DNA activate the signal transduction pathways of B cells and also TLR 4 and TLR 9, and so they play an important role in the proliferation of B cells and immunoglobulin synthesis, whereas bacterial lipoproteins activate TLR 2 and they stimulate B cells (25). Moreover, TLR signal transduction is important for the production of IgM, IgG1, and IgG2c, but it does not influence the production of IgA (26). When we measured the concentrations of IgG, IgA, and IgM in the exudates, we found that the level of IgM was the highest among the 3 types of antibodies. However, there were no differences in expressions of IgG, IgA, and IgM in the exudates that did and did not contain bacteria, suggesting there no association between the antibody concentration in the middle ear and the presence or absence of bacteria. Furthermore, we did not observe a significant association between the expressions of IgG, IgA, and IgM, which are associated with the adaptive immune system, and the expressions of TLR 2 and TLR 4 mRNA, which are associated with the innate immune system, suggesting the absence of a quantitative association between these systems.

Abnormalities in TLRs caused by SNPs may change the susceptibility to infection and inflammatory diseases. For example, many individuals with a lowered response to inhaled LPS have TLR 4 polymorphisms, and THP 1 cells transfected with mutated TLR 4 have a lower response to LPS; in both systems, the ability to recognize ligands was decreased (13). Among the SNPs of TLR 4 are Asp299Gly and Thr399Ile, and both of these have been associated with the increased susceptibility to infection by gram negative bacteria (14), and the former was reported to be a risk factor for the development of haematogenous osteomyelitis (27). Among the TLR SNPs are Arg677Trp and Arg753Gln, and both of these have been reported to be associated with a lowered ability to recognize bacterial cell wall components (28). Yet when we attempted to assess the association of each of these TLR SNPs with OME, we did not observe any correlation. The frequency of each of these polymorphisms has been reported to be low. For example, in patients with periodontitis, the frequency of TLR 4 (Asp299Gly) and TLR 2 (Arg753Gln) has been reported to be 2.9% and 4.1%, respectively (15), and in osteomyelitis patients, the frequency of TLR 4 (Asp299Gly) and TLR 2 (Arg753Gln) has been reported to be 3.8% and 0%, respectively (27). Since we only assessed 60 patients, it was possible that none of these SNPs was present.

Although TLRs are highly expressed on monocytes, they are also expressed on other human tissues. Therefore, TLR polymorphisms may not be expressed in middle ear fluid (18). In addition, we did not assess TLRs on the immunocytes in the middle ear cavity, but on the retained fluid. Thus, the effects of immunocytes could not be ruled out.

CONCLUSION

We have shown here that TLR 2 and TLR 4, which are both responsible for innate immunity, are expressed in the fluid in the middle ear cavity. This suggests that TLRs play an important role in the immunological process during OME. However, the TLR SNPs involved in the susceptibility to infection were not detected in this study's samples.

REFERENCES

1. Klein JO, Tos M, Hussl B, Naunton RF, Ohyhyama M, Van Cauwenberge PB. Recent advances in otitis media: definition and classification. Ann Otol Rhinol Laryngol Suppl. 1989 Apr;139:10.
2. Kitajiri M, Sando I, Takahara T. Postnatal development of the eustachian tube and its surrounding structures: preliminary study. Ann Otol Rhinol Laryngol. 1987 Mar-Apr;96(2 Pt 1):191-8.
3. Tos M, Bak-Pedersen K. New aspects in the pathogenesis of chronic secretory otitis media. Acta Otolaryngol. 1973 Apr;75(4):269-70.
4. Senturia BH, Gessert CF, Carr CD, Baumann ES. Studies concerned with tubotympanitis. Ann Otol Rhinol Laryngol. 1958 Jun;67(2):440-67.
5. Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM. Otitis media. Lancet. 2004 Feb 7;363(9407):465-73.
6. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell. 1997 Oct 31;91(3):295-8.
7. Yang RB, Mark MR, Gurney AL, Godowski PJ. Signaling events induced by lipopolysaccharide-activated toll-like receptor 2. J Immunol. 1999 Jul 15;163(2):639-43.
8. Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the Toll signaling pathway. Nat Immunol. 2001 Sep;2(9):835-41.
9. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature. 2001 Sep;413(6851):78-83.
10. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science. 1999 Jul 30;285(5428):736-9.
11. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999 Oct;11(4):443-51.
12. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, et al. Recognition of pneumolysin by Toll-like receptor 4 confers

resistance to pneumococcal infection. Proc Natl Acad Sci USA. 2003 Feb 18;100(4):1966-71.
13. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001 Aug;2(8):675-80.
14. Hirschfeld M, Weis JJ, Toshchakov V, Salkowski CA, Cody MJ, Ward DC, et al. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect Immun. 2001 Mar;69(3):1477-82.
15. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002 May 1;168(9):4531-7.
16. Chaudhary PM, Ferguson C, Nguyen V, Nguyen O, Massa HF, Eby M, et al. Cloning and characterization of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. Blood. 1998 Jun 1;91(11):4020-7.
17. Diamond G, Legarda D, Ryan LK. The innate immune response of the respiratory epithelium. Immunol Rev. 2000 Feb;173:27-38.
18. Vandermeer J, Sha Q, Lane AP, Schleimer RP. Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and toll-like receptors. Arch Otolaryngol Head Neck Surg. 2004 Dec;130(12):1374-80.
19. Claeys S, de Belder T, Holtappels G, Gevaert P, Verhasselt B, van Cauwenberge P, et al. Human beta-defensins and toll-like receptors in the upper airway. Allergy. 2003 Aug;58(8):748-53.
20. Shuto T, Imasato A, Jono H, Sakai A, Xu H, Watanabe T, et al. Glucocorticoids synergistically enhance nontypeable Haemophilus influenzae-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. J Biol Chem. 2002 May 10;277(19):17263-70.
21. Toubi E, Shoenfeld Y. Toll-like receptors and their role in the development of autoimmune diseases. Autoimmunity. 2004 May;37(3):183-8.
22. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000 Dec 7;408 (6813):740-5.
23. Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. Nature. 2005 Nov 17;438(7066):364-8.
24. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000 Jun;25(2):187-91.
25. Agnese DM, Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE, et al. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis. 2002 Nov 15;186(10):1522-5.
26. Montes AH, Asensi V, Alvarez V, Valle E, Ocaña MG, Meana A, et al. The Toll-like receptor 4 (Asp299Gly) polymorphism is a risk factor for Gram-negative and haematogenous osteomyelitis. Clin Exp Immunol. 2006 Mar;143(3):404-13.
27. Bochud PY, Hawn TR, Aderem A. Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. J Immunol. 2003 Apr 1;170(7): 3451-4.
28. Schröder NW, Hermann C, Hamann L, Göbel UB, Hartung T, Schumann RR. High frequency of polymorphism Arg753Gln of the Toll-like receptor-2 gene detected by a novel allele-specific PCR. J Mol Med. 2003 Jun;81(6):368-72.