Expression of Lysozyme and Aquaporins mRNA in Otitis Media

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Lysozyme is known as a key substance of the innate immunity and have antibacterial effect in the mucosal tissues, especially middle ear. Aquaporin (AQP) functions as water movement in the tissue and has been expected to be participated in the inflammatory responses. In the present study, we investigated to reveal association of lysozymes and AQPs in otitis media. The gene expression of lysozyme genes, homo sapiens lysozyme (hLYZ), homo sapiens lysozyme M (hLYZ M), and homo sapiens lysozyme G like-2 (hLYGH), and AQP genes (AQP 0 - AQP 12) were measured from postauricular skin, mastoid mucosa, inflamed mastoid mucosa, and middle ear mucosa. The hLYZ, hLYZ M and hLYGH gene were expressed in mastoid mucosa, inflamed mastoid mucosa, middle ear mucosa. Of AQP genes, all AQP gene except AQP 3 gene were expressed in the tissue of middle ear. Among them, AQP 4, AQP 8, AQP 9, AQP 10, AQP 11 and AQP 12 were highly expressed in the inflamed mastoid mucosa and normal mastoid mucosa ($P < 0.001$). Interestingly, expression levels of AQP 4, AQP 9, and AQP 12 gene were significantly higher in the inflamed mastoid mucosa compared to normal middle ear mucosa ($P < 0.05$). These results suggest that lysozyme and AQPs could be associated with inflammatory response in the middle ear.

Key Words: Otitis media, Lysozyme, Aquaporin (AQP), Inflamed mastoid mucosa

INTRODUCTION

Otitis media is the inflammatory disease of the middle ear. Otitis media is a main reason for doctor visits and the most common cause of hearing loss (Klein, 2000). Otitis media can be classified on the characteristics of the effusion; serous, mucoid, suppurative, which indicates infectious or inflammatory response in the mucosa. The main factors of otitis media are Eustachian tube dysfunction and microbial infection (Rovers et al., 2004). Bacterial infection is likely cause otitis media than viral infection, and the immune response tends to depend on congenital immune mechanisms rather than acquired immunity.

Lysozyme is found in most of tissue of mammals, especially mucosal membrane such as respiratory, digestive and reproductive system as well as middle (Konstan et al., 1982; Prager and Jolles, 1996; Lim et al., 2000; Ganz, 2004), which functions as a key substance of the innate immunity and exhibit bactericidal activity (Park et al., 1992). Lysozyme M is known to exert excellent bactericidal effects against gram-negative bacteria (Markart et al., 2004). Homo sapiens Lysozyme G like-2 (hLYGH) is distributed in ovipara and show bactericidal activity as lysozyme (Kim, 2005; Giacomello et al., 2006).

Aquaporin (AQP), also called waterway, was first reported in 1993 (Agre et al., 1993). Aquaporin is the most rapid piping system that enable water move in a structured way

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in the tissue cells. Thirteen Aquaporin-related genes, AQP 0 to AQP12 have been reported up to now (Seo and Choi, 2015). The AQP 0 gene is known to be essential to maintain the transparency of the lens in the eye, which has holes to enable water to move within the protein (Nemeth-Cahalan et al., 2013). The AQP 1 gene is inherently present and expressed in the cell membrane of the erythrocyte and kidney, but its expression is not regulated by Vasopressin (Denker et al., 1988). The AQP 2 gene was expressed in the collecting duct of the kidney, and its expression was regulated by Vasopressin. In the starvation state, expression level of AQP 2 gene was decreased (Dibas et al., 1998). AQP 3 and AQP 6 protein are found in the basal cell membrane of renal collecting ducts, where play a role of water excretion (Sasaki et al., 1998). AQP 4 gene was also expressed in the basal cell membrane of the renal collecting duct and was a target of autoimmunity temporally in the neuromyelitis optica (Lennon et al., 2005). AQP 5 is involved in the production of saliva and tear, and also involved in the secretory mechanism of the lungs. The function of AQP 5 is very similar to AQP 0, AQP 2, and AQP 6 (Lee et al., 1996). AQP 7 is involved in the transport of water, glycerol, and urea and AQP 8 is widely distributed in the digestive system (Ishibashi et al., 1997; Elkjaer et al., 2001). AQP 9 is genetically very similar to AQP 3 and AQP 7, and is known to promote the permeability of osmotic pressure and urea transportation. In addition, AQP 9 facilitates immune responses in lymphocytes and neutrophils (Rojek et al., 2007; Moniagal et al., 2007). AQP 10 is found in the human small intestine and appears to have selective absorption and release of water and glycerol in the epithelium of small intestine (Li et al., 2005). AQP 11 distributed in the kidney, liver, testis, and brain, but its exact mechanism is not well known (Gorelick et al., 2006; Saitoa et al., 2018). AQP 12 is present in the acinar cells of the pancreas and is presumed to play a role in the secretion of digestive enzymes (Itoh et al., 2005). In the case of otitis media, it is presumed that the water movement was necessary because a tympanum was found in the middle ear, which contain inflammatory substances.

In the present study, we examined the expression of lysozyme genes, a key substance of innate immunity, in the middle ear. And AQP genes, possible factors of inflammation,

were also investigated in the middle ear, and found that most of AQP genes might be associated with inflammatory status in the middle ear.

MATERIALS AND METHODS

Subjects and specimens

Specimens were obtained from patients with otitis media who visited the Department of Otorhinolaryngology, Chungju Hospital, Kunkuk University School of Medicine, Chungju, Republic of Korea between August 2015 and July 2017. Ethical approval was obtained from the Institutional Ethics Committee of Kunkun University School of Medicine (KUCH 2015-08-037). All subjects provided written informed consent. The specimens, postauricular skin, mastoid mucosa, inflamed mastoid mucosa and middle ear mucosa were collected and stored at -70 °C until used.

Total RNA extraction

To examine the expression of lysozyme and Aquaporin genes, the total RNA was extracted from specimens using RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. Briefly, approximately 20 mg of tissue was taken and 1 mL of RNAiso Plus reagent was added to the tissue, and then tissue was completely disrupted using a homogenizer. After adding 200 µL of chloroform (Sigma, USA), the mixture was vigorously mixed and centrifuged at 13,000 rpm for 10 minutes using a refrigerated centrifuge (Labogene, Korea). Five hundred µL of the supernatant was removed into a new tube, and the same amount of isopropanol (Sigma, USA) was added, and then mixed gently. The tube was kept at room temperature for 10 minutes, and then centrifuged at 13,000 rpm for 10 minutes. After the supernatant was discarded, the precipitated pellet was rinsed gently with 1 mL of 70% ethanol and centrifuged again at 13,000 rpm for 10 minutes. After discarding the supernatant, the pellet was dried at room temperature for 10 minutes and then, dissolved with 30 µL of DEPC-treated RNase- and DNase-free water (WEL GENE, Korea). The extracted RNA was kept at -70 °C until used.

Reverse transcription PCR

Complementary DNA (cDNA) was synthesized using PrimeScript 1st strand cDNA synthesis kit (Takara Clontech, Japan) according to manufacturer's recommendations. In brief, the first mixture containing 1 μ L of Oligo dT Primer, 1 μ L of dNTP mixture, 1 μ L of extracted RNA, 7 μ L of DEPC-treated RNase- and DNase-free water was incubated at 65°C for 5 minutes and immediately chilled on ice. Then, the second mixture containing 4 μ L of 5 $\times$ PrimeScript buffer, 0.5 μ L of RNase inhibitor, 1 μ L of PrimeScript RTase, 4.5 μ L of DEPC-treated water were incubated at 50°C for 1 hour and at 95°C for 5 minutes.

PCR primers were designed to target lysozyme genes, hLYZ, hLYZ M, hLYGH using the Primer 3 software (<http://frodo.wi.mit.edu/>) using the genomic information obtained from NCBI site (Table 1). PCR primers targeting AQP genes were synthesized according to the previous report (Seo and Choi, 2015). As an internal control, primers for β -actin gene were used.

PCR reaction for amplification was performed using 2 $\times$ TOPsimple DyeMix-multi HOT (Enzynomics, Korea). PCR reaction were carried out using SimpleAmp PCR machine (ABI, USA) with the PCR mixture composing of 2 μ L of cDNA, 0.5 μ L of each primer, 7 μ L of DEPC-treated water, and 10 μ L of 2 $\times$ TOPsimple DyeMix-multi HOT (Enzynomics, Korea) in a total volume of 20 μ L. The thermal cycling conditions were 10 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 1 minute at 72°C, and final extension for 5 minutes at 72°C. The PCR products were separated by electrophoresis on 1.5 % agarose gel at 100 V for 1 hour, and the gels were stained with ethidium bromide and photographed using a Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA). All experiments were performed in triplicate.

The mRNA expression level was calculated by quantifying the relative intensity to β -actin as a reference using ImageJ (National Institutes of Health, MD, USA) software.

Statistical analysis

The data was analyzed using SPSS (IBM SPSS, version 24, Chicago, IL, USA) and the nonparametric Wilcoxon-

test were used for statistical difference between experimental groups. Values with $P < 0.05$ were considered to be statistically significant. Graphpad prism version 6.0 (GraphPad Software, La Jolla, USA).

RESULTS

Subjects

The 27 patients having otitis media were grouped as post-auricular skin (n=9), mastoid mucosa (n=7), inflamed mastoid mucosa (n=4) and middle ear mucosa (n=7), and consisted of 15 males and 12 females, ranging in age from 19~71 years.

Lysozyme genes mRNA expression in otitis media specimens

The expression of lysozyme genes in otitis media specimens were assed using semi-quantitative RT-PCR (Fig. 1). The hLYZ mRNA expression level was very low in the post-auricular skin, but high level of hLYZ mRNA expression were detected in mastoid mucosa, inflamed mastoid mucosa and middle ear mucosa compared to that in the postauricular skin ($P < 0.001$, relative gene expression level of hLYZ mRNA in mastoid mucosa, middle ear mucosa, postauricular skin, 34.5, 81.4, 4.2, respectively). The expression levels of hLYZ M mRNA and hLYGH mRNA were high both in the inflamed mastoid mucosa and in the middle ear mucosa, but the hLYZH mRNA and hLYGH mRNA were expressed lowly in the mastoid mucosa. No significant expression of hLYZ mRNA, hLYZ M mRNA, and hLYGH mRNA was not detected in the postauricular skin. The expression level of hLYZ mRNA was higher than the expression levels of hLYZ M mRNA and hLYGH mRNA in mastoid mucosa, inflamed mastoid mucosa, and middle ear mucosa ($P < 0.05$, relative gene expression level in mastoid mucosa of hLYZ mRNA and hLYZ M mRNA, 35.0, 1.2, respectively). In the mastoid mucosa, the expression levels of hLYZ mRNA, hLYZ M mRNA, and hLYGH mRNA were lower than in the inflamed mastoid mucosa and middle ear mucosa ($P < 0.001$, relative gene expression level of hLYZ M mRNA in mastoid mucosa, inflamed mastoid mucosa, and middle ear mucosa, 5.5, 47.0, 35.0, respectively), but

Table 1. Primers sequences for semi-quantitative RT-PCR

Target	Direction	Sequences (5' - 3')	Product size (bp)	References
AQP0	Forward	TGTTCTGCAGGTGGCTATG	232	Seo & Choi, 2015
	Reverse	TGCTAGGTTTCTCTCGGACAG		
AQP1	Forward	TCATCAGCATCGGTTCTGC	297	
	Reverse	CAAGCGAGTTCCCAGTCAG		
AQP2	Forward	TAGCCTTCTCCAGGGCTGT	305	
	Reverse	CGTGATCTCATGGAGCAGAG		
AQP3	Forward	GTCACCTCTGGGCATCCTCAT	157	
	Reverse	CTATTCCAGCACCCAAGAAGG		
AQP4	Forward	GCCCATCATAGGAGCTGTC	209	
	Reverse	GGTCAACGTCAATCACATGC		
AQP5	Forward	CCACCCTCATCTTCGTCTTC	212	
	Reverse	GTAGAAGAAAGCCCGGAGC		
AQP6	Forward	GTGCTGGCTAGGACAGGAAG	289	
	Reverse	CTAGGAGAGGGCCTCCAAGT		
AQP7	Forward	TGCCACCTACCTTCCTGATC	210	
	Reverse	GACGGGTTGATGGCATATCC		
AQP8	Forward	TGAGCCTGAATTTGGCAATG	226	
	Reverse	CAGCGTGGCAATCACGAGC		
AQP9	Forward	CTCAGTGTATCATGTAGTG	336	
	Reverse	GACTATCGTCAAGATGCCG		
AQP10	Forward	GCACTGGGATGCTGATTGT	190	
	Reverse	CCAGCCACGTAGGTGAAGAG		
AQP11	Forward	GACGCTGACGCTCGTCTACT	279	
	Reverse	TCTGTGATGACCGCTTTGAG		
AQP12	Forward	GAACCTGTTCTACGGCCAGA	204	
	Reverse	GTTCCAGGGTCCAGCTACAA		
β-actin	Forward	ATCATGTTTGAGACCTTCAA	318	
	Reverse	CATCTCTTGCTCGAAGTCCA		
hLYZ	Forward	AGGGGTTGCCAGCCTTCAG	259	AF_099029.1
	Reverse	AGCTGTACTCATGCCGTCCG		
hLYZ M	Forward	TGTTTCGGCATTCTATGCTCTACT	217	NC_000012.12
	Reverse	ATGCCACCCTGTAGAAAAAGATG		
hLYGH	Forward	GGAAGATTGTCCTGGAAAGCTA	136	NC_000002.12
	Reverse	CAATCGCTTCAATTCTGACTTA		

RT-PCR, reverse-transcription-polymerase chain reaction; AQP, aquaporin; hLYZ, homo sapiens lysozyme; hLYZ M, homo sapiens lysozyme M; hLYGH, homo sapiens lysozyme G like-2.

there was no significant difference in the expression level of hLYZ mRNA, hLYZ M and hLYGH mRNA between in the inflamed mastoid mucosa and in the middle ear mucosa.

Aquaporin (AQP) genes mRNA expression in otitis media specimens

The expression of AQP genes were measured in the otitis media specimens and was shown in Fig. 2. AQP 0 mRNA

Fig. 2. Expression of AQP genes in the middle ear tissue was analyzed by RT-PCR. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide (A-D). Results are representative of three experiments. The mRNA expression level was calculated by quantifying the relative intensity to β -actin using ImageJ (E). M: 100bp DNA ladder, PS: Postauricular skin, MM: Mastoid mucosa, iMM: Inflamed mastoid mucosa, MeM: Middle ear mucosa; AQP: Aquaporin.

mRNA, AQP 9 mRNA, AQP 10 mRNA, AQP 11, and AQP 12 mRNA were weakly expressed, and the expression level of AQP 12 mRNA was highest. In the inflamed mastoid mucosa, expression of AQP 0, AQP 1, AQP 2, AQP 4, AQP 5, AQP 6, AQP 7, AQP 8, AQP 9, AQP 10, AQP 11 and AQP 12 mRNA except AQP 3 mRNA were detected. The expression level of AQP 0 mRNA, AQP 1 mRNA, AQP 2 mRNA, AQP 5 mRNA, AQP 6 mRNA and AQP 7 mRNA were weak, but AQP 4 mRNA, AQP 8 mRNA, AQP 9 mRNA, AQP 10 mRNA, AQP 11 mRNA and AQP 12 mRNA were expressed very highly. In the middle ear mucosa, the expression of AQP 0 mRNA, AQP 1 mRNA, AQP 4 mRNA, AQP 5 mRNA, AQP 7 mRNA, AQP 8 mRNA, AQP 9 mRNA, AQP 10 mRNA, AQP 11 mRNA and AQP 12 mRNA were detected, but the expression of AQP 2 mRNA, AQP 3 mRNA and AQP 6 mRNA were not examined. The expression level of AQP 0 mRNA, AQP 1 mRNA, AQP 5 mRNA, and AQP 7 mRNA were expressed very dim, but the expression level of AQP 4 mRNA, AQP 8 mRNA, AQP 9 mRNA, AQP 10 mRNA, AQP 11 mRNA and AQP 12 was fairly high.

DISCUSSION

In the present study, lysozyme and AQPs were investigated to reveal the possible association with immune response in the middle ear. hLYZ M gene and hLYGH gene were expressed highly in the inflamed mastoid mucosa compared to the normal mastoid tissue. These results were consistent with previous report that hLYZ M has bacteriicidal activity, especially against gram-negative bacteria (Markart et al., 2004). In addition, the bactericidal activity of hLYGH was also reported previously (Giacemello et al., 2006; Kim, 2005). These might indicate that hLYZ M and hLYGH might be strongly related with inflammatory status as lysozyme in the mucosal membrane of middle ear, which might contribute to immunity of middle ear from bacterial infection.

AQPs are water channel that play a role in the intercellular water movement in the living tissues and have been regarded as potential factors of immunity. In the present study, aquaporin genes were examined to reveal their relevance with the immunity of middle ear. Of the 13 AQP genes, all tested AQP genes except AQP 3 gene were expressed in the tissue

of middle ear. The expression levels of AQP 0, AQP 2, AQP 5, AQP 6 and AQP 7 gene were weak in the otitis media, which suggest that these genes might be possibly associated with inflammatory response of the otitis media. Among them, AQP 4, AQP 8, AQP 9, AQP 10, AQP 11, and AQP 12 gene were highly expressed in the inflamed mastoid mucosa and middle ear mucosa, and their expression level in the inflamed mastoid mucosa was higher than that in the mastoid mucosa ($P < 0.001$). These results suggest that these AQP genes might be related with inflammatory responses, which imply their contribution to immunity in the middle ear. The high level of expression of AQP 9 gene supposed to be related with the accumulation of lymphocytes or neutrophils in the inflammatory site of mastoid mucosa, which could be analogize from the previous reports that AQP 9 facilitate immune response in lymphocytes and neutrophils (Rojek et al., 2007; Moniagal et al., 2007). The AQP 4 gene had been a target of autoimmunity in the neuromyelitis optica (Lennon et al., 2005), but there was rare report that AQP 4 is related with inflammatory response. Therefore, it might be a novel discovery that AQP 4 has a potential factor of the inflammatory response in the middle ear. The other AQP genes, AQP 8, AQP 10, AQP 11, and AQP 12, could be new factors as AQP 4 in the immunity of otitis media. In the future study, it would be necessary to investigate the mechanism of AQP genes including hLYZ, hLYZ M, and hLYGH, in the inflammation of the otitis media.

In the present study, a large number of patient subjects having otitis media were not enrolled, and especially, healthy subjects could not be enrolled by prohibition of Institutional Ethical Committee. Nevertheless, involvement of lysozyme genes and AQP genes in the otitis media could be statistically investigated.

In conclusion, lysozyme genes, hLYZ, hLYZ M and hLYGH gene were highly expressed in the inflamed mastoid mucosa. The expression of most of AQP genes were examined in the tissue of otitis media, and especially six AQP genes were highly expressed in the inflamed mastoid mucosa. These suggest that Lysozyme genes and AQP genes might be associated with immunity of otitis media.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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