Regulatory effect of dexamethasone on aquaporin 5 in human middle ear epithelium

Young Joon Seo

Department of Medicine

The Graduate School, Yonsei University

Regulatory effect of dexamethasone on aquaporin 5 in human middle ear epithelium

Directed by Professor Jae Young Choi

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Young Joon Seo

December 2014

This certifies that the Doctoral Dissertation of Jae Young Choi is approved.

Thesis Supervisor : Jae Young Choi

Thesis Committee Member#1 : Kyungwon Lee

Thesis Committee Member#2 : Joo Young Kim

Thesis Committee Member#3: Ji Hwan Ryu

Thesis Committee Member#4: Mee Hyun Song

The Graduate School
Yonsei University

December 2014

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my principal investigator, Professor Jae Young Choi, who has the attitude and the substance of a genius: he continually and convincingly conveyed a spirit of adventure in regard to research and scholarship, and and excitement in regard to teaching. Without his guidance and persistent help this dissertation would not have been possible.

I would like to thank my committee members, Professor Kyung Won Lee, Professor Joo Young Kim, Professor Ji Hwan Ryu, and Professor Mee Hyun Song, whose work demonstrated to me that concern for global affairs supported by an "engagement" in comparative literature and modern technology should always transcend academia and provide a quest for our times.

I would like to thank my family, mother, father, my older sister and my daughters (Lovely Yeo Won and Cutely Yeo Jeoung), who always trust me without any doubts and give me a happiness.

Finally, I would like to express thanks, from the bottom of my heart, to my wife Bo Yeon, who is always an anchor in my life. With her, I could overcome every hardship. With her, I could learn a happiness. With her, I finally completed my own family. With her, I will be.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Regulatory effect of dexamethasone on aquaporin 5 in human middle ear epithelium

Young Joon Seo

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jae Young Choi)

Purpose

To evaluate the effect of dexamethasone on the expression of aquaporin 5 (AQP5) in cultured human middle ear epithelium cells in vitro, which is related closely with water transports in the middle ear cavity

Methods

With dexamethasone, the expression of AQP5 was assessed by reverse transcriptase and real time PCR. We evaluated it's protein level using western blot and immunofluorscence methods. Functionally, the absorption of water from the surface treated with texas red was evaluated through special own confocal microscopy methods.

Results

Transcripts for AQP5 were expressed consistently in cultured cells from 3 to 5 days after the treatment of dexamethasone (a 16 fold increase at day 5 compared with day 0). Similar patterns of increase in proteins were demonstrated on western blot (a 2.5 fold increase at day 5 than day 0), too. We visualized the expressions of AQP5 in the surface of dexamethasone treated cells with immunofluorescence, which was not shown in the control cultured cells. Absorption of fluid occurred on the surface of the epithelium cells functionally in the presence of dexamethasone.

Conclusions

With dexamethsone, the expressions of AQP5 in the middle ear epithelium were increased. The role of AQP5 may be related to the absorption of water from the middle ear cavity to subepithelial spaces in otitis media. This is the first study to suggest the pathophysiology and the potential therapeutic effect of dexamethasone on the AQP5 in otitis media.

Key words : Aquaporin, Middle ear epithelium, Otitis media with effusion, Dexamethasone

Regulatory effect of dexamethasone on aquaporin 5 in human middle ear epithelium

Young Joon Seo

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jae Young Choi)

I. INTRODUCTION

Otitis media with effusion (OME) is one of the most common infectious diseases in childhood. Around 30% of children aged under three years visit their general practitioner with OME each year. About one in 10 children will have an episode of otitis media with effusion by three months of age[1]. It is important to study the causal mechanism of OME. Fluid absorption occurs as a consequence of an osmotic gradient generated by vectorial Na^+, K^+ transport and the ion and water transport that may counterbalance the flux of water to prevent an inundation of the middle ear cavities. Whether the eliminating system acts normally depends on the working state of the water and ion channels.

Aquaporins (AQPs) are specific water channels that allow the rapid transcellular movement of water in response to osmotic/hydrostatic pressure gradients. Therefore AQPs are considered important for the water balance of the middle ear[2]. When invading pathogens cause disorders of homeostasis of the middle ear, the ion channels are affected; thus the ion flows would be changed, which can result in alteration of the function and even the expression of transmembrane AQPs. Currently, 13 mammalian AQP isoporins, AQP0 to AQP12, have been identified[3]. One of the AQPs, AQP5, is present in the water transporting epithelia of the lacrimal gland, trachea,

eye, lung, and nasal cavity[4,5]. In our previous study, we revealed that AQP1, 3 and 5 existed in the human middle ear epithelium and the AQP5 could have an important role for absorption and secretion of water on the surface of the cells. In the normal human middle ear epithelium cells (NHMEECs), AQP5 has been topographically localized to the apical membranes of the cells but not basolaterally[6]. To facilitate the transport of water, AQPs are expressed throughout the respiratory tract, but the roles of AQPs under pathological conditions of the middle ear cavity still remain unclear.

Conventional medical therapy for OME includes antibiotics, decongestants, antihistamines and combinations of these agents. Decongestants and antihistamines are not effective in resolving effusion. Some authors offer glucocorticoids in the treatment of middle ear effusion, but there is no consensus on this issue. Various mechanisms have been proposed for the action of glucocorticoids: (1) a direct anti- inflammatory action on the middle ear and eustachian tube by reducing arachidonic acid concentration, thereby inhibiting the cyclooxygenase and lipooxygenase pathways for synthesis of inflammatory mediators; (2) an increase in eustachian tube surfactant concentration, facilitating better tube function; (3) shrinkage of peritubal lymphoid tissue, again allowing better tube function; and (4) reduction of middle ear fluid viscosity via an effect on mucoproteins[7]. Consequently, an understanding of the effects of glucocorticoids on AQP5 in the middle ear cavity may provide new insights into the molecular mechanisms involved in transcellular water transport in OME.

In the present study, we examined the expression of AQPs in the human middle ear with reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry using light. Then we examined AQP5 expression in immortalized NHMEEC (NHMEEC-I) in order to determine whether or not glucocorticoids induce this type of expression,

and we investigated the mechanisms involved in the increasing of AQP5 expression by glucocorticoids in NHMEEC-I.

II. MATERIALS AND METHODS

1. Cell culture and reagents

Specimens of normal human middle ear epithelium (NHMEE) were isolated and used to prepare primary cultures and perform immunohistological studies. Primary cultures of NHMEE cells were prepared as previously described[8]. All procedures were approved by the Institutional Review Board of Yonsei Medical Center. Passage-2 NHMEE cells were plated on a collagen-coated semi-permeable membrane with a pore size of 0.45 μm Transwell-clear culture inserts, (Costar Co., Cambridge, MA) at a density of 1.0×10^4 cells/cm^2. The cells were maintained in a 1:1 mixture of bronchial epithelial growth medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and all supplements, and kept at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. Cultures were grown submerged for the first 10 days, at which time an air–liquid interface was created by removing apical media. Cells were cultured for 2 weeks after reaching confluence for full differentiation.

NHMEEC-I, which was immortalized with the E6/E7 genes of human papilloma virus type 16, were kindly provided by Dr. David J. Lim (House Ear Institute, Los Angeles, CA). NHMEEC-I was stored in liquid nitrogen; aliquots were thawed and grown in a 1:1 mixture of Bronchial Epithelial Cell Basal Medium (Gibco, Carlsbad, CA) and Dulbecco's modified Eagle's medium (Gibco), supplemented with bovine pituitary extract (52 μg/ml), hydrocortisone (0.5 μg /ml), hEGF (0.5 ng/ml), epinephrine 0.5 (μg /ml), transferrin (10 μg /ml), insulin (5

μg /ml), triiodothyronine (6.5 ng/ml), retinoic acid (0.1 ng/ml), penicillin (100 U/ml), and streptomycin (100 g/ml) at 37 ℃ in a 5% CO_2- humidified chamber. When the cells reached approximately 70% confluency, they were removed from the culture dish using trypsin / EDTA passaging. NHMEEC-I were seeded into six-well plates (BD Falcon, San Jose, CA) at a density of 1.0×10^4 cells/cm^2, cultured for 24 hours to confluence, and serum starved in the same medium with 0.2% FBS (serum-starved medium) for 24 hours. Serum- starved medium was used throughout the study. The cells were treated with biologically active form of dexamethasone (9α-fluoro-16α-methyl-1,4-pregnadien-11β,17,21-triol-3,20-dione, Sigma-Aldrich, St. Louis, MO) at different concentrations (0–2 μM).

2. RNA Isolation, RT-PCR, and Quantitative Real-Time PCR

Total RNA was isolated from NHMEE cells using TRIzol (Invitrogen) and cDNA was synthesized with random hexamers (Perkin Elmer, Roche, Branchburg, NJ) using MMLV reverse transcriptase (RT; Perkin Elmer Life Sciences). Human AQP 0–12 primers were designed using National Centre for Biotechnology Information (NCBI)/Ensembl (Table 1) with β-actin used as transcript control. PCR conditions were as follows: 7 min at 95 ℃, followed by 35 cycles (except for AQP 3, 5 and β-actin; 25 cycles) at 95 ℃ for 30 s, annealing for 30 s (temperatures specified in Table 1), extension at 72 ℃ for 40 s, and 10 min elongation at 72 ℃. PCR products were analysed by 2% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator. To verify that the amplified products were from mRNA and did not result from genomic DNA contamination, negative controls were produced by omitting RT from the RT-PCR. In the absence of RT, no PCR products were observed.

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) oligonucleotide primers

Gene	Direction	Sequence (5'–3')	Annealing temperature (°C)
AQP0	Forward	TGTTCTGCAGGTGGCTATG	59.9
	Reverse	TGCTAGGTTTCCTCGGACAG	
AQP1	Forward	TCATCAGCATCGGTTCTGC	58.4
	Reverse	CAAGCGAGTTCCCAGTCAG	
AQP2	Forward	TAGCCTTCTCCAGGGCTGT	58.8
	Reverse	CGTGATCTCATGGAGCAGAG	
AQP3	Forward	GTCACTCTGGGCATCCTCAT	59.8
	Reverse	CTATTCCAGCACCCAAGAAGG	
AQP4	Forward	GCCCATCATAGGAGCTGTC	58.4
	Reverse	GGTCAACGTCAATCACATGC	
AQP5	Forward	CCACCCTCATCTTCGTCTTC	59.9
	Reverse	GTAGAAGAAAGCCCGGAGC	
AQP6	Forward	GTGCTGGCTAGGACAGGAAG	61.4
	Reverse	CTAGGAGAGGGCCTCCAAGT	
AQP7	Forward	TGCCACCTACCTTCCTGATC	59.9
	Reverse	GACGGGTTGATGGCATATCC	
AQP8	Forward	TGAGCCTGAATTTGGCAATG	60.0
	Reverse	CAGCGTGGCAATCACGAGC	
AQP9	Forward	CTCAGTGTCATCATGTAGTG	59.7
	Reverse	GACTATCGTCAAGATGCCG	
AQP10	Forward	GCACTGGGATGCTGATTGT	60.0
	Reverse	CCAGCCACGTAGGTGAAGAG	
AQP11	Forward	GACGCTGACGCTCGTCTACT	59.7
	Reverse	TCTGTGATGACCGCTTTGAG	
AQP12	Forward	GAACCTGTTCTACGGCCAGA	59.3
	Reverse	GTTCCAGGGTCCAGCTACAA	
β -actin	Forward	ATCATGTTTGAGACCTTCAA	56.0
	Reverse	CATCTCTTGCTCGAAGTCCA	

Real-time PCR was performed to determine the expression level of AQP5. The transcripts of β-actin and AQP5 were amplified using gene-specific primers that were retrieved from GenBank (β-actin forward 5'-GCACCACACCTTCTACAATG and β-actin reverse 5'-TGCTTGCTGATCCACATCTG-3', AQP5 forward 5'-CATCTTCGCCTCCACTGACT-3' and AQP5 reverse 5'-CCCTACCCAGAAAACCCAGT-3'). Real-time PCR was performed with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and the DyNAmoHS SYBRGreen qPCR Kit (Finnzymes, Espoo, Finland) with 10 μl of cDNA in each well. The thermocycler parameters were 95 ℃ for 2 minutes, followed by 40 cycles of 95 ℃ for 20 seconds and 60 ℃ for 30 seconds. PCR was

followed by melting at 60 ℃ to 95 ℃. The specificity for primers was checked by the presence of single melt peak temperature. To exclude the possibility of genomic DNA amplification during PCR, no-template controls were performed and accepted when the Ct value was at least nine cycles greater than the template run. Measurements were performed in duplicate and accepted if the difference in Ct value between the duplicates was less than 1. The real-time PCR data were normalized to the level of β-actin, and the relative quantity of mRNA was obtained using the comparative cycle threshold method.

3. Western Blot Analysis

The cells in each well were washed three times in phosphate buffered saline (150 mM NaCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 2 mM KH_2PO_4, pH 7.4) and then lysed with 2 x sample buffer (250 mM Tris-HCl [pH 6.5], 2% sodium dodecyl sulfate (SDS), 1% DTT, 0.02% bromophenol blue, and 10% glycerol). Protein was quantified by comparing the absorbance of the lysate with serially diluted bovine serum albumin (0, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml) on a VersaMax ELISA plate reader (Molecular Devices, Sunnyvale, CA). Samples were heated for 5 minutes at 95 ℃. Equal amounts of protein were loaded into each gel lane. A colored marker mixture was used for estimation of molecular weight. Proteins were separated using 10% SDS-polyacrylamide gel at 125 V for 4 hours using a running buffer (25 mM Tris-Base, 250 mM glycine and 0.1% SDS), and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using semidry transfer cell (Bio-Rad, Hercules, CA) for 2 hours at 200 mA using transfer buffer (25 mM Tris-Base [pH 8.0], 192 mM glycine containing 20% methanol). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) for 2 hours at room temperature. The blot was then incubated

overnight with 1:100 primary AQP5 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or a rabbit anti-human β-actin antibody (Abcam Ltd, Cambridge, UK), in 0.5% Tween-20 in TBS (TTBS). After washing with TTBS, the blot was incubated with a secondary anti-rabbit antibody (Cell signaling) in TTBS for 45 minutes at room temperature and visualized by enhanced chemiluminescence (Amersham Biosciences). Antibody against β-actin was used to confirm equal loading of the protein.

4. Immunohistochemistry

To examine the expression pattern of AQPs in NHMEE, tissues and cells were fixed with 4% paraformaldehyde for 24 hours and then dehydrated and embedded in paraffin. Paraffin blocks were sectioned into 5-μm-thick slices and fixed with a chilled 1:1 mixture of methanol and acetone for 5 minutes after pretreatment with 0.3% H_2O_2 for 20 minutes at room temperature. Dewaxed tissue sections were subjected to antigen retrieval by boiling for 10 minutes in tris-ethylene -diaminetetraacetic acid (Tris-EDTA; pH 9.0 at AQP 4) and citric acid (pH 6 at AQP 1, 3, and 5). The slides were treated with 5% BSA for 1 hour to block nonspecific reactions and then incubated with a monoclonal mouse antibody against the human AQPs (Table 2.).
The slides were then incubated with biotinylated antimouse rabbit immunoglobulin G (1:200; Vector Laboratories, Burlingame, CA, USA). Peroxidase was attached to the secondary antibody by avidin-biotin peroxidase complex formation. Specimens were incubated in diaminobenzidine tetrahydrochloride to detect primary antibody binding sites.

Table 2. Antibodies used for immunohistochemistry (IHC) studies

Antibody	Host	Antigen	Concentration, dilution	Source (Cat. No.)
Anti-AQP 1	Mouse	Human AQP 1, C-term.	1:100	Abcam(ab9566)
Anti-AQP 3	Rabbit	Human AQP 3, N-term.	1:100	Santa Cruz(sc-20811)
Anti-AQP 4	Mouse	Rat AQP 4, C-term.	1:500	Abcam(ab11026)
Anti-AQP 5	Rabbit	Human AQP 5, C-term.	1:100	Abcam(ab9566)

5. Localization of AQP5 Protein by Fluorescence Staining and Confocal Laser Microscopy

The cells were grown on plain coverglasses (half were controls with PBS and the other were treated with 2 μM dexamethasone for 3 days). Cells grown on plain coverglasses were washed with PBS three times, fixed in liquid containing acetone and methanol at the ratio of 6:4 at 41 ℃ for 10 min and incubated for 1 hour at 37 ℃ with goat anti-human AQP5 antibody (Santa Cruz) at a dilution of 100. After three rinses with PBS containing 1% bovine serum albumin, the cells were incubated for 1 hour with fluorescence-conjugated donkey anti-goat IgG (1:50 dilution, Rockland, Gilbertsville, PA, USA).
DAPI (4′, 6-diamidino-2-phenylindole) was used as a blue-fluorescent nuclear counterstain. Coverglasses were mounted with 80% glycerol in PBS for confocal laser microscopy (Carl Zeiss Micro Imaging Inc., LSM 700).

6. Measurement of ASL thickness

The cells were grown in 6 well plates (half were controls with PBS and the other were treated with 2 μM dexamethasone for 3 days). The thickness of the airway surface liquid (ASL) was measured in NHMEEC-I using a modified of the method described by Terran et al[9]. Briefly, the cells were washed, and 20 μl of PBS containing 0.2% (v/v)

Texas Red-dextran (Invitrogen, San Diego, CA, USA) was added onto the apical cell surface to label the ASL layer. A 100-μl aliquot of perfluorocarbon (FluorinertFC-770; 3M,St. Paul, Minn., USA) was then added to the apical surface to prevent evaporation of the ASL. After 6 hours, the cultures were transferred to the stage of an inverted confocal microscope (Carl Zeiss MicroImagingInc., LSM 700), and the height of the ASL was measured at five predetermined points in the cultures (one central, four circumferential) via XZ scans. Images were reconstructed 3-dimensionally and analyzed using Imaris 7.1 software (Bitplane Co, Zurich, Switzerland). We standardized the values of 5 points on each well (15 values in control and 15 values in treated cells).

III. RESULTS

1. AQPs Screening in the NHMEEC

The expression of AQP 0-12 mRNAs in the human middle ear was studied by RT-PCR . The middle ear epithelium expressed AQP1, 2, 3, 4, 5, 6, 8, 10, and 11 but not detectable levels of AQP 0, 7, 9, and 12 subtypes (Figure 1). Failure of PCR amplication in the absence of RT indicates that the PCR products were derived from the transcribed cDNAs, but not from the genomic DNA in the tissues.

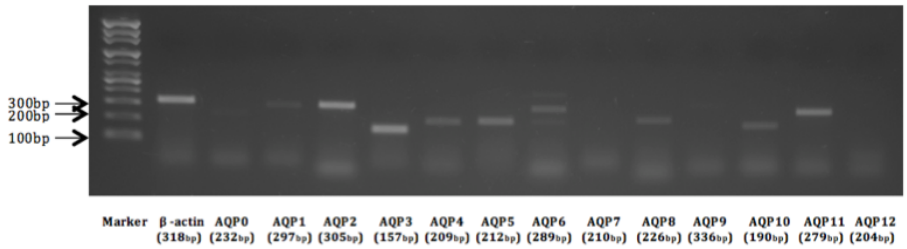

Figure 1. Expression of Aquaporin (AQP) mRNA in cultured NHMEE cells. Transcripts for AQP 1, 2, 3, 4, 5, 6, 8, 10, and 11 were consistently expressed. But AQP 0, 7, 9, and 12 subtypes were not detected. The expected sizes of RT-PCR product of AQPs are described in each bracket under the name.

Of the nine AQP subtypes examined in this study, three subtypes (AQP1, AQP3, and AQP5) were detected in the human middle ear (Figure 2). AQP1 was detected in subepithelial fibroblasts and endothelial cells of capillary vessels. AQP3 was distinctly detected in the lateral and basal membrane of the epithelial cells in the NHMEE tissue. AQP4 was not detected in the specimen of the NHMEE. In contrast to AQP3, AQP5 was expressed in the apical side of the epithelial cells. We could see more clearly that there were the difference between the locations of AQP3 and AQP5 in the cultured cells of the NHMEE (Figure 3); AQP3 was observed at the lateral and basal membranes of the ciliated epithelial cells, whereas AQP5 was localized to the apical portion of the epithelial cells.

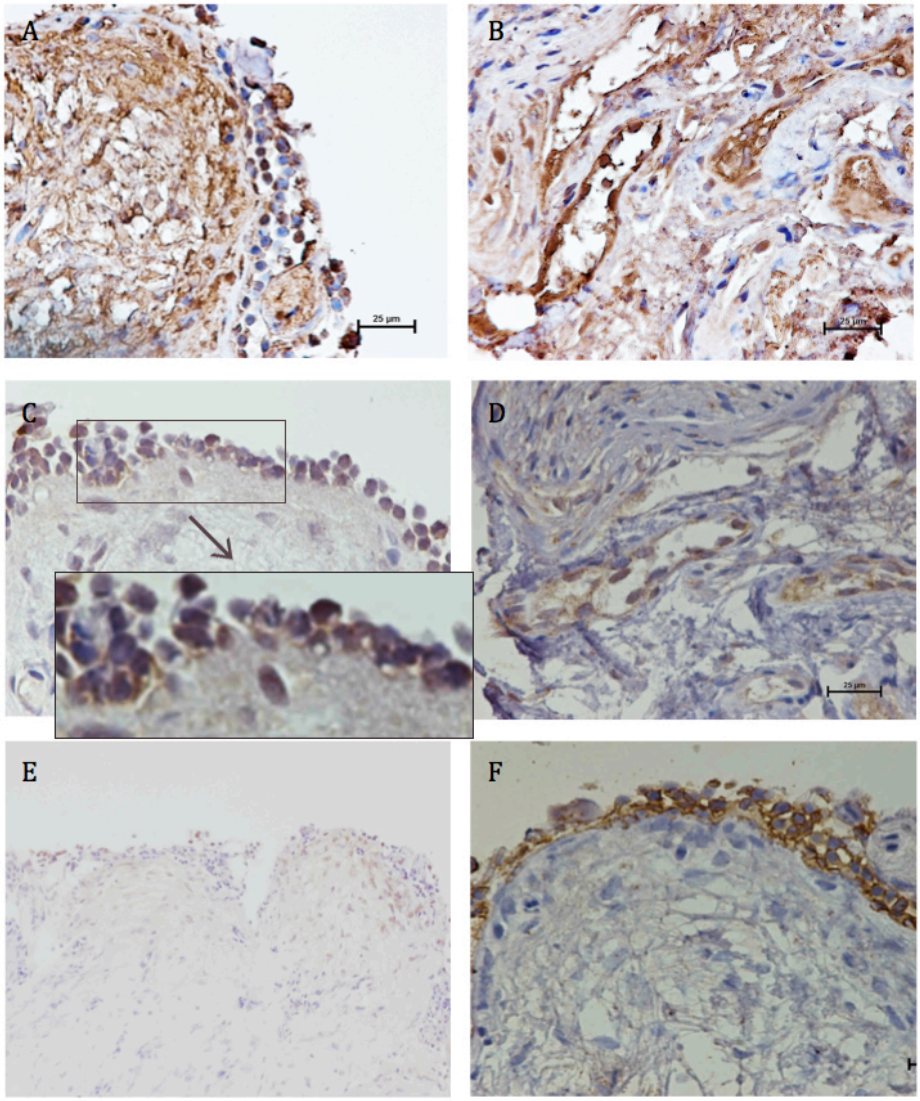

Figure 2. Aquaporin (AQP) immunohistochemistry results of NHMEE tissue in situ. (A, B) AQP1 was located in the fibroblasts in subepithelium (A), capillary endothelial cells (B). (C, D) AQP3 was located in the basolateral membrane of the stratified epithelium (C) with expression in capillary endothelial cells (D). AQP4 was not detected in the specimen of the NHMEE (E). In contrast to AQP3, AQP5 was expressed in the apical side of the epithelial cells (F).

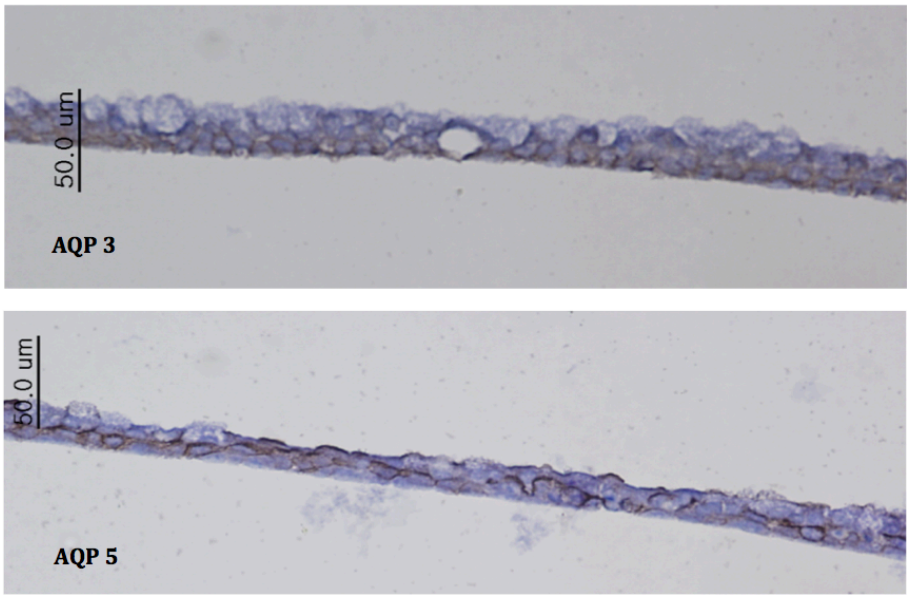

Figure 3. Aquaporin (AQP) immunohistochemistry results of cultured NHMEE cells in vitro. AQP3 was expressed on the basal layers of the ciliated stratified epithelial cells, while AQP5 was expressed on the apical surface of the epithelial cells.

2. The changes of AQP5 expression in NHMEEC-I after dexamethasone treatment

A. Effects of dexamethasone on Cell Growth

The growth kinetics of NHMEEC-I treated with various concentrations of dexamethasone were investigated by MTT assay for up to 6 days. As shown in Figure 4, no remarkable cytotoxicity was observed until the 6th day when NHMEEC were treated with 0, 500 nM, 1 μM, and 2 μM of dexamethasone. Thus, we selected a concentration of 2 μM and a safe duration of 5 days (120 hours) for the following experiments.

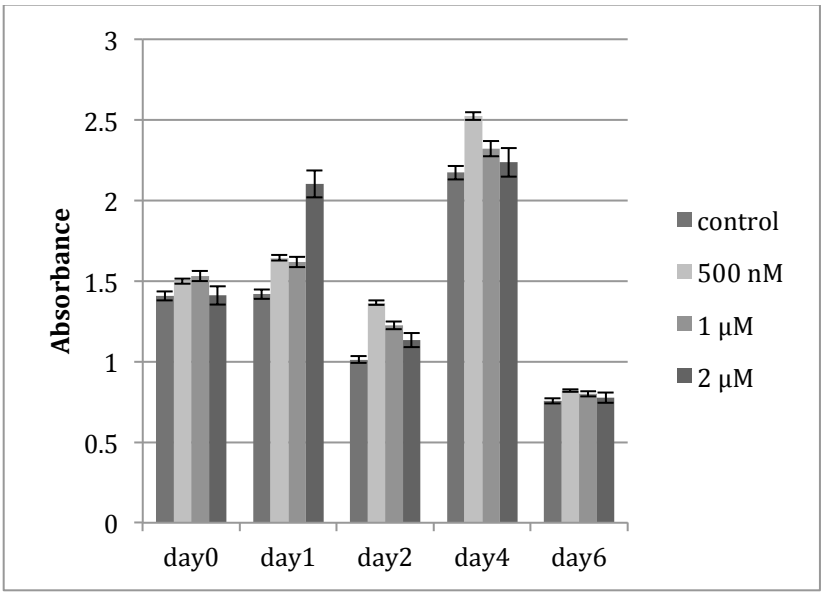

Figure 4. Growth properties of NHMEEC-I cultured in the presence or absence of dexamethasone at concentrations of 0, 500 nM, 1 μM, and 2 μM for up to 6 days. Viable cells were estimated by CCK-8. No significant suppression of cell growth was detected in any of the concentrations of dexamethasone up to 5days. On the sixth day, cell growth was decreased with dexamethasone.

B. AQP5 Gene Expression in NHMEEC and NHMEEC-I

RT-PCR was used to examine AQP5 and AQP3 mRNA expression in NHMEEC and NHMEEC-I to test the compatibility of two cell types, because it was too hard to get enough cells in normal humans and we should continue to keep the same characteristics of the cells. Both normal human middle ear tissues employed as a positive control and immortalized cells apparently expressed AQP5 mRNA (212 bp) as well as AQP3 (157 bp) (Figure 5). In the above RT-PCR experiments, equal loading of RNA samples was demonstrated by measurement of the housekeeping gene β-actin.

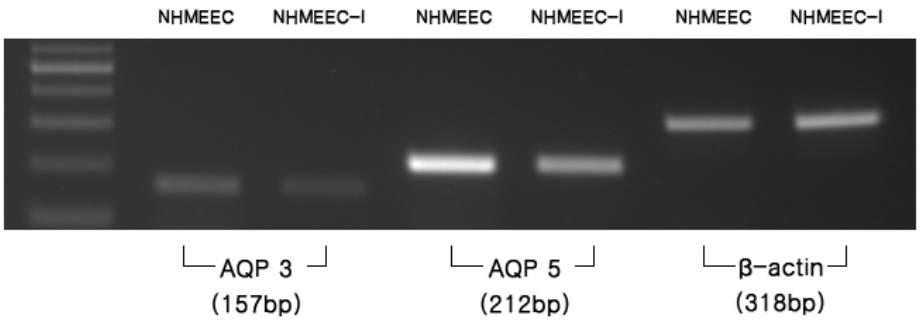

Figure 5. RT-PCR analysis for the expression of AQP5 and AQP3 mRNA in NHMEEC and NHMEEC-I. Similarly like in NHMEEC, AQP5 and AQP3 mRNA were expressed in NHMEEC-I. Despite 35 cycles of PCR on AQP5, AQP5 mRNA was not well detected in NHMEEC-I without dexamethasone, but AQP3 mRNA was well visualized in both cells. Equal loading of RNA samples was demonstrated for the housekeeping gene β-actin.

C. Induction of AQP5 mRNA and Protein in NHMEEC-I in Response to Dexamethasone

Time course analysis was used to investigate the induction of AQP5 mRNA expression in NHMEEC-I by treatment with dexamethasone. A significant increase in the AQP5 mRNA level was detected in NHMEEC-I after 72 hours (Figure 6). This high level continued for up to 120 h. Densitometric analysis (NIH Image software, ver. 1.63) revealed that the expression level of AQP5/ β-actin mRNA in dexamethasone-treated NHMEEC-I at each time point relative to that of NHMEEC was as follows: 1.28 at 0 hour of NHMEEC, 0.72 at 0h of NHMEEC-I, 0.64 at 24 hours, 0.67 at 48 hours, 1.03 at 72 hours, 0.92 at 96 hours and 2.57 at 120 hours. Quantitative RT-PCR was employed to further examine the expression levels of AQP5 mRNA by dexamethasone in NHMEEC-I. As shown in Figure 7, a statistically significant increase in AQP5 mRNA expression was also detected after 72 hours with treatment of dexamethasone: in NHMEEC-I, the

expression level of AQP5 mRNA was near the 20% of that of normal middle ear epithelium tissues, and with the time of dexamethasone treatment, NHMEEC-I expressed greater amounts of AQP5 mRNA than that in the normal middle ear tissues. To detect the production of AQP5 protein, NHMEEC-I was subjected to Western blot analysis during preparation. As can be seen in Figure 8, AQP5 expression was significantly augmented by the dexamethasone treatment. Densitometric analysis demonstrated that the expression level of AQP5/ β-actin in NHMEEC-I at each time point relative to that of the NHMEEC was as follows: 0.85 at 0 hour, 0.89 at 48 hours, 0.69 at 72 hours, 1.14 at 96 hours, and 1.96 at 120 hours. These values were similar to those of quantitative real-time RT-PCR analysis.

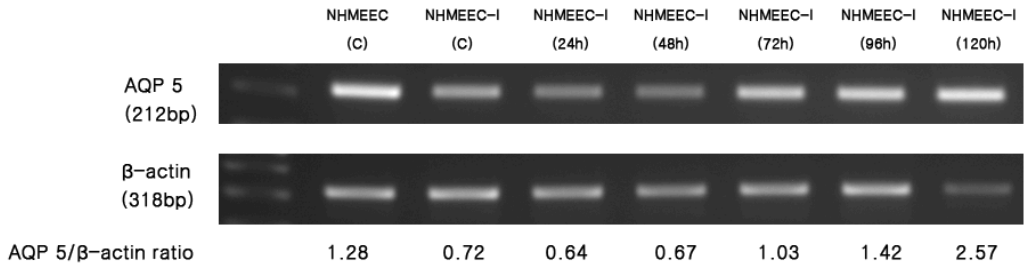

Figure 6. RT-PCR analysis for the expression of AQP 4 m RNA in dexamethasone treated NHMEEC-I. NHMEEC-Is were treated with dexamethasone (2 μM) for 0, 24, 48, 72, 96 and 120 hours. A rapid increasing of AQP5 mRNA was detected after 72 hours. β-actin mRNA was used as an equal loading of RNA samples. Densitometric analysis AQP5/ β-actin mRNA ratio revealed the expression level of AQP5 mRNA in dexamethasone treated NHMEEC-I relative to that of NHMEEC.

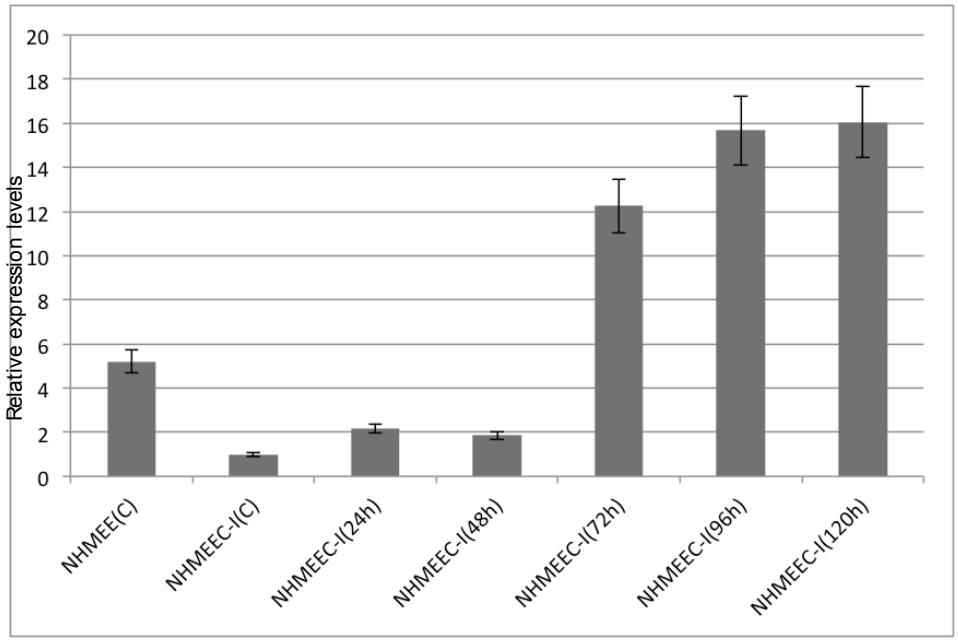

Figure 7. Steady-state levels of AQP5 mRNA measured using quantitiative real-time PCR. Relative quantification of gene expression was performed as described by the manufacturer by using β-actin mRNA as an internal standard. Induced expression levels of AQP5 mRNA in NHMEEC-Is were compared with the expression level (one fold) in the 0 day of NHMEEC. Each bar represents at least three separate mRNA isolations performed in duplicate. NHMEEC-I expressed a relatively small amount (1/5 fold less) of AQP5 mRNA as compared to NHMEEC. Dexamethasone (2 μM) treated NHMEEC-I acquired large amounts of AQP5 mRNA at 120 H after treatment (16 folds more). * Statistically significant at p value<0.05 (Mann-Whitney U-test).

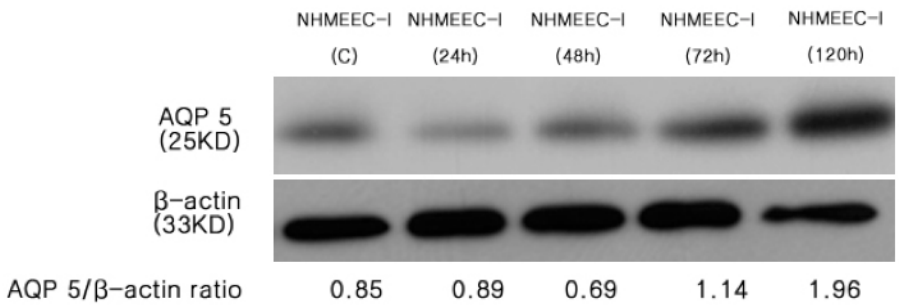

Figure 8. Western blot analysis of AQP5 and β-actin proteins in dexamethasone treated NHMEEC-I. Increased expression of AQP5 protein with a molecular weight of 25 kDa was clearly observed in NHMEEC-I with treatment with 2 μM dexamethasone (from 72 h to 120 h). Densitometric analysis (AQP5/β-actin ratio) revealed the expression level of AQP5 protein in dexamethasone treated NHMEEC-I. These results were similar to those observed with quantitative real-time RT-PCR analysis.

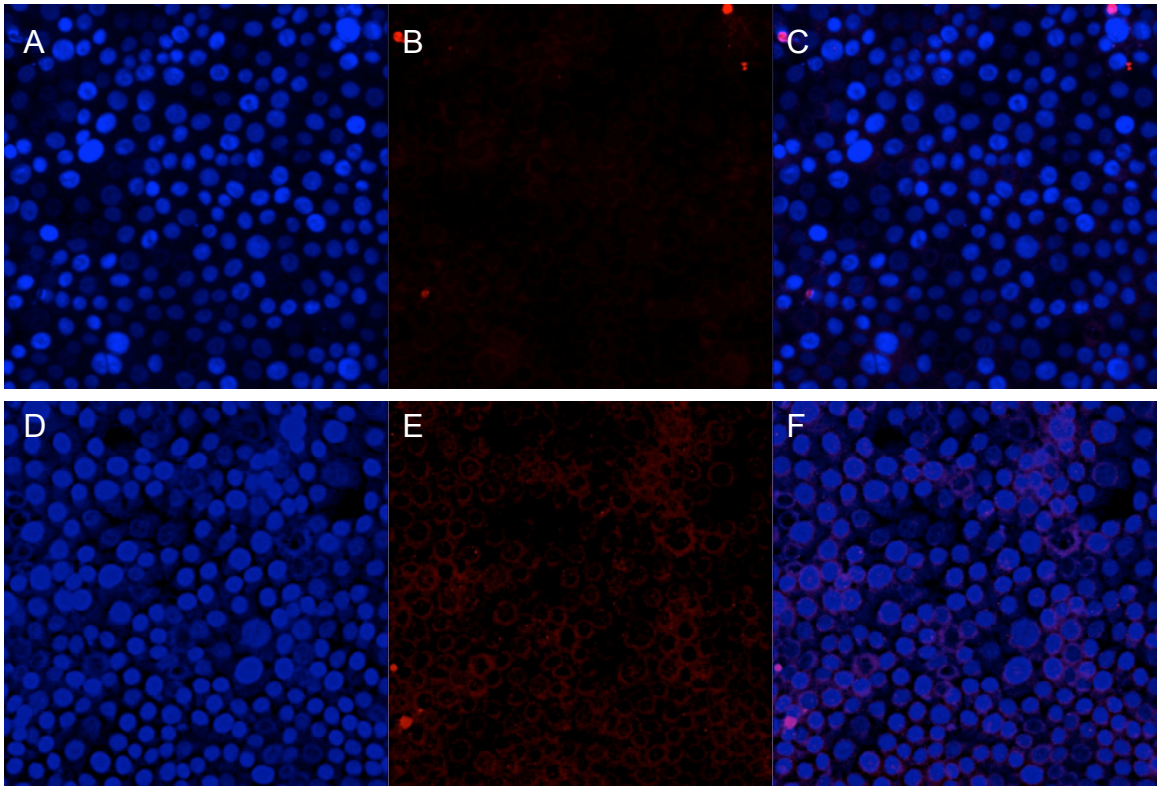

Figure 9. Localization of AQP5 in NHMEEC-I, grown on coverglasses, were treated with dexamethasone (2 μM) for 72 hours (A: DAPI in control, B: AQP5 in control, C: DAPI and AQP5 in control, D: DAPI in treated cells, E: AQP5 in treated cells, F: DAPI and AQP5 in treated cells). DAPI was used as a blue-fluorescent nuclear counterstain in A and D. Confused images for AQP5 (red) and DAPI (blue) are showed in C and F. AQP5 staining at the surfaces of the plasma membrane can be seen in NHMEEC-I (E). However, no apparent staining for AQP 5 was observed in untreated NHMEEC-I (B).

D. Distribution of AQP5 in Dexamethasone-Treated NHMEEC-I

In an effort to examine the localization of AQP5 (red) in dexamethasone-Treated NHMEEC-I, we used laser microscopy with fluorescence staining. Untreated NHMEEC-I showed no AQP5 expression, compared with DAPI, blue-fluorescent nuclear counterstains. (Figure 9A, B and C). However, when NHMEEC-I was treated with 2 μM dexamethasone for 72 hours, AQP5 protein (red) was highly expressed. As shown in Figure 9E, AQP5 expressions in these cells were detected at surfaces of the plasma membrane.

E. Fluid Absorption in Dexamethasone Treated NHMEEC-I

To determine whether or not the AQP5 expressed in dexamethasone-treated NHMEEC-I affected their water permeability, we measured the transepithelial net fluid secretion of ASL in NHMEEC-I after treatment with dexamethasone (Figure 10). The thickness of ASL in dexamethasone-treated NHMEEC-I was thinner than that in the control (p-value<0.001). The mean thickness of ASL in dexamethasone-treated NHMEEC-I was 8.53±2.42 (μm), compared with 17.87±2.44 (μm) thickness of the control. Dexamethasone stimulated the absorption of water from the surface of the epithelium.

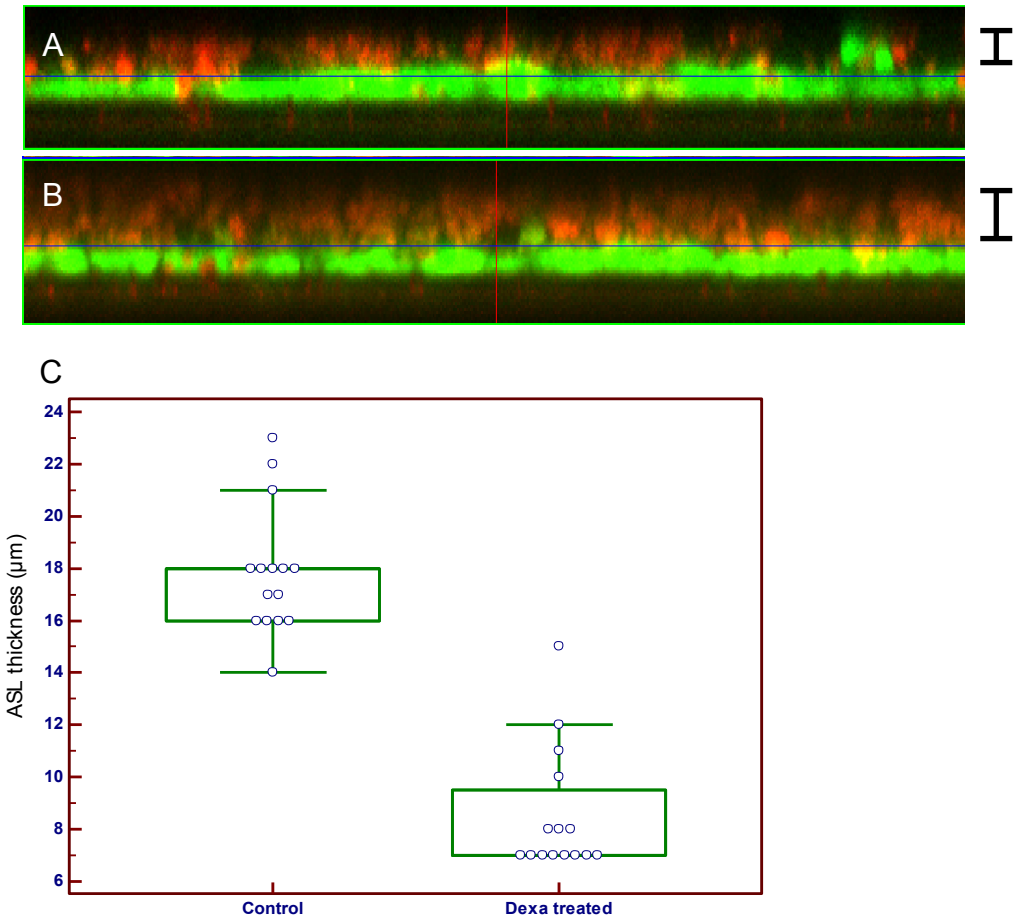

Figure 10. Confocal laser microscopy images for Measurement of ASL thickness. Texas Red-dextran was added onto the apical cell surface to label the ASL layer (red), compared with the epithelial layers (green). The heights of the ASL between no treated and dexamethasone treated NHMEEC-Is were consistently different (A; dexamethasone treated and B: no treated). The results are expressed as absorption in µm of ASL thickness. The mean thickness of ASL in dexamethasone-treated NHMEEC-I was 8.53±2.42 (µm), compared with 17.87± 2.44 (µm) thickness of the control (C). The results were analyzed using the Mann-Whitney U-test. The p-value was 0.001 between two groups

IV. DISCUSSION

In an attempt to elucidate the functional roles of AQPs in the normal and pathological functions of human middle ear epithelium, we previously studied the expression and distribution of AQPs in a human middle ear epithelium cell line (NHMEEC-I). We showed that AQP3 and AQP5, but not AQP1 and AQP4, were expressed in NHMEEC-I. This observation suggested that AQP3 and AQP5 might provide the principal route for fluid transport in human middle ear epithelia and middle ear cavity secretion[10]. We thought that AQP5, topographically localized to the apical membranes of the epithelium cells, could function to stimulate the absorption of water from the middle ear cavity. Because the expressions of AQP3 were not changed according to circumstances of hypoxia and dexamethasone, it might be feasible to hypothesize that middle ear epithelium cells could acquire the ability to absorb fluids when they express especially AQP5.

Otitis media with effusion is defined as chronic inflammation of the middle ear mucosa. It is a common reason for prescribing antibiotics, contributing to the growing problem of bacterial resistance[11]. The mechanism of OME is not clearly understood[12]. In the conservative treatment of OME, the role of corticosteroids is controversial. But Podoshin et al.[13], in a double blind, randomized study, showed that a group treated with amoxicillin and steroids had a much better response than groups treated with placebo or amoxicillin alone. Schwartz et al. reported that results for 40 children treated with steroids either initially or following crossover revealed that 70% demonstrated resolution of OME via pneumo-otoscopy and 64% via tympanometry. Persico et al.[14] found that 53 % of children treated with a combination of prednisone and ampicillin achieved resolution of OME; after two weeks of treatment, twice as many ears were cleared by dexamethasone treatment compared with placebo.

In order to understand the involvement of AQPs and their roles in

the pathological function of airways, we analyzed the expression of AQP5 in response to dexamethasone treatment. Our results showed that in NHMEEC-I, dexamethasone stimulated AQP5 expression at the mRNA and protein levels including the results of localization of AQP5 with immunofluorescence staining. Though there is no study of aquaporins in the middle ear, this observation is consistent with previous other reports in the lung. In Ben's study[15], dexamethasone and ambroxol upregulated the expression of AQP3 and AQP5 in A549 cells. Such upregulation suggested that AQP5 and AQP3 present in A549 cells may be involved in the regulation of the volume and composition of mucous through a water transport mechanism. Similarly, King et al.[16] reported that AQP1 expression was increased in corticosteroid treated rat lung, which suggests that maternal, internal corticosteroids may increase the expression of AQP1 in the fetal lung. Furthermore, we showed the role of AQP5 functionally to absorb the water in the confocal laser microscopy study. In dexamethasone treated NHMEEC-I, the net absorption of fluid on the epithelium of cells was significantly increased, demonstrating that the expressed AQP5 was functional.

V. CONCLUSION

The present study showed that dexamethasone upregulated the expression of AQP5 in NHMEEC-I and the upregulation of AQP5 functions the absorption of water from the apical of the epithelium cells. Such upregulation suggested that AQP5 present in human middle ear epithelium may be involved in the regulation of the volume and composition of secretions through a water transport mechanism. The dynamic diversity of AQP5 expression in the whole course of OME using animal models and the role of AQP5 in clinical cases of OME will also be explored in a forthcoming study.

REFERENCES

1. Tos M, Stangerup SE, Holm-Jensen S, Sørensen CH. Spontaneous course of secretory otitis and changes of the eardrum. Arch Otolaryngol. 1984 May;110(5):281–9.
2. Lim DJ, Chun YM, Lee HY, Moon SK, Chang KH, Li JD, et al. Cell biology of tubotympanum in relation to pathogenesis of otitis media — a review. Vaccine. 2000 Dec.;19:S17–25.
3. Ishibashi K, Hara S, Kondo S. Aquaporin water channels in mammals. Clin. Exp. Nephrol. 2009 Apr.;13(2):107–17.
4. Preston GM. Molecular Cloning and Characterization of an Aquaporin cDNA from Salivary, Lacrimal, and Respiratory Tissues. Journal of Biological Chemistry. 1995;270(4):1908–12.
5. Sung-Ho Kang, Ki-Hong Chang, Shuji Ohcho, Haa-Yung Lee et al. Expression of water channel proteins (aquaporins) in the rat Eustachian tube and middle ear mucosa. Acta Oto-laryngologica. 2007;127:687-92.
6. Etsu Takahashi, Toshihiko Kikuchi, Yukio Katori, and Toshimitsu Kobayashi. Localization of aquaporins, water channel proteins, in the mouse eustachian tube. Acta Otolaryngol. 2009;129:66-70
7. Rosenfeld RM, Mandel EM, Bluestone CD. Systemic steroids for otitis media with effusion in children. Arch Otolaryngol HeadNeck Surg 1991;117:984 –9
8. Choi, J.Y., Kim, C.H., Lee, W.S., Kim, H.N., Song, K.S., Yoon, J.H., Ciliary and secretory differentiation of normal human middle ear epithelial cells. Acta Oto-Laryngol. 2002;122(1), 270–5.
9. Tarran R, Button B, Boucher RC. Regulation of normal and cystic fibrosis airway surface liquid volume by phasic shear stress. Annu Rev Physiol. 2006;68:543-61.
10. Qian Zhang, Changjian Liu, Jianqun Wang, Jianhe Sun,

Expression pattern of aquaporin 4 and 5 in the middle ear of guinea pigs with secretory otitis media. Acta Oto-laryngol. 2010;130(1), 68-74

11. Berman S, Casselbrant ML, Chonmaitree T, Giebink GS, Grote JJ, Ingvarsson LB et al. Recent advances in otitis media. 9. Treatment, complications, and sequelae. Ann Otol Rhinol Laryngol Suppl 2002;188:102–24

12. Rosenfeld RM, Mandel EM, Bluestone CD. Systemic steroids for otitis media with effusion in children. Arch Otolaryngol Head and Neck Surg. 1991;117:984 – 9

13. Podoshin L, Fradis M, Ben-David Y, Faraggi D. The effi- cacy of oral steroids in the treatment of persistent otitis media with effusion. Arch Otolaryngol Head Neck Surg 1990;116:1404 – 6

14. Persico M, Podoshin L, Fradis M. Otitis media with effusion: a steroid and antibiotic therapeutic trial before surgery. Ann Otol Rhinol Laryngol 1978;87:191–6

15. Yong Ben, Jie Chen, Rong Zhu, Lei Gao, and Chunxue Bai, Upregulation of AQP3 and AQP5 induced by dexamethasone and ambroxol in A549 cells. Respiratory Physiology & Neurobiology 2008;161(2):111-8

16. King, L.S., Nielsen, S., Agre, P., 1996. Aquaporin-1 water channel protein in lung: ontogeny, steroid-induced expression, and distribution in rat. J. Clin. Invest. 97, 2183–91.

ABSTRACT(IN KOREAN)

중이 점막에서 덱사메타손 (Dexamethasone) 이 아쿠아포린 5 (Aquaporin 5)의 발현 조절 양상에 미치는 영향에 대한 연구

<지도교수 최재영 >

연세대학교 대학원 의학과

성 명 서 영 준

목적

인체 중이 점막에서 덱사메타손이 중이강에서 물의 이동에 관여하는 아쿠아포린 5의 발현에 어떠한 영향을 미치는지 평가하기 위함이다.

방법

아쿠아포린의 발현양을 역전사 실시간 중합효소연쇄반응 기법을 사용하여 RNA의 발현양상을 알아보았고, 단백질의 발현 변화를 알기위해 Western blot 방법과 면형광염색법을 사용하여 알아보았다. 기능적으로 물의 흡수에 관여하는지 알아보기 위하여 Texas Red 염색법을 사용하여 공초점 레이저 현미경을 사용하여 기도상피액 (Airway Surface Liquid)의 두께를 측정해 보았다.

결과

사람 중이 점막에 덱사메타손을 처리하였을 때, 3일 이후에 아쿠아포린 5의 발현양이 급증하였으며, 이는 역시 단백질 레벨에서도 같은 발현양 증가 양상을 보였다. 면역형광 염색을 하였을 때에도, 세포 표면에 아쿠아포린5가 덱사메타손 처리 후에 발현양이 증가하는 것을 시각적으로 확인할 수 있었으며, 덱사메타손 처리후에 기도상피액 두께가 감소하는 것을 확인하여, 기능적으로 물의 흡수에 관여할 것으로 추측할 수 있었다.

결론

덱사메타손은 아쿠아포린 5의 발현양을 직접적으로 증가시키며, 아쿠아포린 5는 세포 표면에서 발현양을 증가시켜 물의 흡수에 관여함을 간접적으로 증명하였다. 중이염에서 아쿠아포린 5의 역할이 중요함을 알 수 있으며, 덱사메타손의 생태병리 작용을 밝힐 수 있는 중요한 단서가 될 것이다.

핵심되는 말 : 아쿠아포린, 중이 점막, 삼출성 중이염, 덱사메타손

PUBLICATION LIST

YJ Seo, SH Kim, IS Moon, JY Choi. A Melting Method for RNA Extraction from the Mucosal Membrane of the Mouse Middle Ear. Yonsei Medical Journal. 2015. be scheduled for publication.