



Original Article

Lower Beclin-1 mRNA Levels in Pediatric Compared With Adult Patients With Otitis Media With Effusion

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OBJECTIVES: The role of autophagy in the pathophysiology of otitis media with effusion (OME) remains unclear, particularly regarding the difference between pediatric and adult patients. The present study analyzed the expression levels of autophagy-associated mRNAs in effusion fluids obtained from pediatric and adult patients with OME.

MATERIALS and METHODS: Middle ear fluid samples were collected from 76 pediatric patients and 41 adult patients with OME, and the levels of mRNAs encoding autophagy-related genes were measured using real-time reverse transcription-polymerase chain reaction. The relationships between the levels of autophagy-associated mRNAs and the frequency of ventilation tube insertion, the characteristics of middle ear fluid, and the results of bacterial culture were analyzed.

RESULTS: Autophagy-associated mRNAs were present in the effusion fluid of all patients. The level of Beclin-1 mRNA was significantly lower in pediatric than in adult patients, regardless of the frequency of surgery or fluid characteristics ($p < 0.05$).

CONCLUSION: Autophagy-associated mRNAs were expressed in effusion fluids of both pediatric and adult patients with OME. However, the level of Beclin-1 mRNA was significantly lower in the effusion fluid of pediatric than adult patients.

KEYWORDS: Otitis media with effusion (OME), autophagy, effusion fluid, child, adult

INTRODUCTION

The word autophagy derives from the Greek words *auto*, meaning “self,” and *phagy*, meaning “eating.” Autophagy is the process by which intracellular molecules are transported to lysosomes for degradation. Since the first identification of an autophagy-related (Atg) gene from yeast, > 30 Atg genes have been discovered [1–3]. Autophagy is an adaptive process that provides metabolites to cells under stressful situations. This process acts to inhibit tumor growth, eliminate toxic substances, and destroy invading microbes, and it is also involved in antigen presentation to lymphocytes by macrophages and dendritic cells [4, 5]. The mechanisms by which autophagy is directly associated with the maintenance of human health and the occurrence of diseases remain unclear. It is also unclear whether agents that control the level of expression of autophagy-associated mRNAs can be used in the prevention or treatment of various diseases. To date, however, there have been no studies that investigated the roles of autophagy in otitis media or the difference in expression of autophagy-associated mRNAs in pediatric and adult patients with otitis media with effusion (OME). The present study, therefore, compared the levels of expression of autophagy-associated genes between pediatric and adult patients with OME.

MATERIALS and METHODS

The present study included 61 pediatric and 36 adult patients evaluated at our hospital and diagnosed with OME. The diagnosis of OME was based on the amber color of effusion fluid, the air-fluid level, air bubbles, or opacity of the tympanic membrane and

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B- or C-type tympanograms. Patients diagnosed with OME received medication for 1-2 weeks and were observed for 3 months. If no improvement was observed, patients underwent ventilation tube insertion, with effusion fluid samples obtained during surgery. Patients with retraction of the tympanic membrane or pure-tone audiometry >40 dB also underwent tube insertion, as did patients whose parents desired the operation.

The external auditory meatus was thoroughly disinfected without bleeding, and the exudate was collected in an Eppendorf tube using a collector (Xomed Trace Products, Jacksonville, FL, USA). Samples were stored at $\leq -70^{\circ}\text{C}$. The fluid in the middle ear cavity was suctioned out, and ventilation tubes were inserted by aseptic manipulation. Patients suspected of having acute otitis media (AOM), head or neck anomalies, systemic diseases, or congenital or acquired immunodeficiencies were excluded. All patients provided written informed consent, and the study protocol was approved by the medical ethics committee of our hospital.

Bacterial culture

Effusion fluid samples obtained using sterile cotton swabs (Xomed Trace Products) were immersed in Stuart's transport medium. Aliquots of these samples were added to dishes of solid blood agar and flasks containing liquid thioglycollate medium (Hangang, Kun-po, Korea). The cultures were incubated for 24 h at 35°C . Bacteria that formed colonies were identified by Gram staining and biochemical testing, and fungi successfully cultured in solid blood agar were subsequently grown in Sabouraud medium.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was purified from homogenized tissue samples using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was synthesized from 1- μg total RNA using a reverse transcription system with random hexamers (Promega, Madison, WI, USA), as described by the manufacturer. The real-time reverse transcription-PCR was performed on a StepOnePlus real-time PCR system. Each 20- μl sample contained 1 μl of cDNA, 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μl of primers (Table 1), and 7 μl of PCR-grade water. The amplification conditions comprised an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The crossing points of the target genes with β -actin were calculated using the formula $2^{-(\text{target gene} - \beta\text{-actin})}$, and relative expression was quantified.

Comparison of clinical features

The frequency of ventilation tube insertion, the characteristics of middle ear fluid, and the results of bacterial culture were compared in pediatric and adult patients.

Statistical analysis

Data were analyzed statistically using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp., Armonk, NY, USA). Between-group comparisons were performed using Student's t-test, and the levels of mRNA were compared using the Levene homogeneity of variance test and Pearson's correlation test. Statistical significance was set at $p\text{-value} < 0.05$.

RESULTS

The 61 pediatric patients with OME included 36 boys and 25 girls (age range: 1–17 years; mean age, 5.9 ± 3.9 years). The 36 adult patients with OME included 16 men and 20 women (age range: 20–80 years; mean age, 56.9 ± 17.5 years). Autophagy-associated mRNAs were present in the effusion fluid of all patients. Among the four autophagy mRNAs tested, the level of Beclin-1 mRNA was significantly lower in the effusion fluid of pediatric than adult patients (Table 2).

Table 1. Primers for real-time RT-PCR

Name	Sequences	Annealing temperature	Size (bp)
mTOR	5'-CCTGCCACTGAGAGATGACA-3' 5'-TCCGGCTGCTGTAGCTTATT-3'	60	168
Beclin-1	5'-AGGTTGAGAAAGGCGAGACA-3' 5'-AATTGTGAGGACACCCAAGC-3'	60	112
FLIP	5'-GCAGAGTTTCTGCCAAGGAG-3' 5'-CTCCCAAAGTGCTGGGATTA-3'	60	123
Rubicon	5'-CAGATTCTGCTGCCTCTTCC-3' 5'-AGTGTCTGCCCCCTCTGAGAA-3'	60	105
β -Actin	5'-GCGAGAAGATGACCCAGATC-3' 5'-GGATAGCACAGCCTGGATAG-3'	60	77

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; mTOR, mechanistic target of rapamycin; FLIP, FLICE-inhibitory protein; FLICE, FADD-like IL-1 β -converting enzyme

Table 2. Expression levels of mTOR, Beclin-1, FLIP, Rubicon mRNAs in the middle ear effusion fluid of children and adults with OME

	Children	Adults	p
mTOR	0.266 \pm 0.268	0.301 \pm 0.328	0.514
Beclin-1	0.072 \pm 0.072	0.204 \pm 0.117	0.001*
FLIP	1.270 \pm 2.564	1.931 \pm 8.750	0.638
Rubicon	0.279 \pm 0.553	0.500 \pm 2.200	0.530

Abbreviations: mTOR, mechanistic target of rapamycin; FLIP, FLICE-inhibitory protein; FLICE, FADD-like IL-1 β -converting enzyme.

* $p < 0.05$

Table 3. Culture results of middle ear effusion fluid

Bacteriology	Children No. (%)	Adult No. (%)
No growth	44 (72.1%)	30 (83.4%)
Growth	17 (27.9%)	6 (16.6%)
<i>Haemophilus influenzae</i>	10 (13.1%)	0 (0.0%)
<i>Streptococcus pneumoniae</i>	3 (3.9%)	0 (0.0%)
<i>Coagulase-negative staphylococci</i>	2 (2.6%)	3 (7.3%)
<i>Methicillin-resistant staphylococcus aureus</i>	2 (2.6%)	1 (2.4%)
<i>Micrococcus spp.</i>	2 (2.6%)	0 (0.0%)
<i>Corynebacterium spp.</i>	1 (1.3%)	1 (2.4%)
<i>Streptococcus viridans</i>	1 (1.3%)	0 (0.0%)
<i>Staphylococcus aureus</i>	1 (1.3%)	0 (0.0%)
<i>Gram negative rod</i>	0 (0.0%)	1 (2.4%)
Total	61 (100%)	36 (100%)

Table 4. Levels of mTOR, Beclin-1, FLIP, Rubicon mRNAs in cultures of middle ear effusion fluid from children and adults positive and negative for bacteria

Culture	(+)			(-)		
	Children (n=17)	Adult (n=6)	p	Children (n=44)	Adult (n=30)	p
mTOR	0.339±0.274	0.282±0.314	0.722	0.251±0.264	0.322±0.335	0.304
Beclin-1	0.077±0.076	0.132±0.097	0.240	0.073±0.072	0.216±0.117	0.001*
FLIP	0.680±1.637	0.375±0.242	0.409	1.493±2.818	2.198±9.464	0.670
Rubicon	0.166±0.423	0.141±0.137	0.822	0.322±0.592	0.562±2.380	0.563

Abbreviations: mTOR, mechanistic target of rapamycin; FLIP, FLICE-inhibitory protein; FLICE, FADD-like IL-1 β -converting enzyme.

*p<0.05

Table 5. Levels of mTOR, Beclin-1, FLIP, Rubicon mRNAs in middle ear effusion fluid from children and adults relative to the frequency of surgery

Frequency of surgery	Once			More than twice		
	Children (n=14)	Adult (n=13)	p	Children (n=47)	Adult (n=23)	p
mTOR	0.226±0.220	0.154±0.101	0.221	0.293±0.281	0.408±0.376	0.180
Beclin-1	0.036±0.011	0.181±0.112	0.001*	0.087±0.080	0.218±0.120	0.001*
FLIP	1.247±2.216	0.800±0.847	0.419	1.277±2.688	2.583±10.994	0.555
Rubicon	0.253±0.489	0.222±0.263	0.812	0.288±0.576	0.661±2.763	0.319

Abbreviations: mTOR, mechanistic target of rapamycin; FLIP, FLICE-inhibitory protein; FLICE, FADD-like IL-1 β -converting enzyme.

*p<0.05

Table 6. Levels of mTOR, Beclin-1, FLIP, Rubicon mRNAs in serous-type and mucoid/mucopurulent/purulent-type middle ear effusion fluid from children and adults

Effusion fluid type	Serous-type			Mucoid/mucopurulent/purulent-type		
	Children (n=13)	Adult (n=24)	p	Children (n=48)	Adult (n=12)	p
mTOR	0.237±0.221	0.314±0.365	0.389	0.288±0.281	0.323±0.238	0.662
Beclin-1	0.036±0.011	0.203±0.128	0.001*	0.086±0.079	0.207±0.094	0.001*
FLIP	1.160±2.241	2.634±10.571	0.480	1.304±2.673	0.417±0.402	0.239
Rubicon	0.232±0.494	0.675±2.657	0.397	0.293±0.573	0.123±0.164	0.295

Abbreviations: mTOR, mechanistic target of rapamycin; FLIP, FLICE-inhibitory protein; FLICE, FADD-like IL-1 β -converting enzyme.

*p<0.05

The standard culture of effusion fluid samples showed the presence of bacteria in samples from 27.9% (17/61) of pediatric patients and 16.6% (6/36) of adult patients (Table 3). The levels of all autophagy mRNAs in otitis media were higher in adult patients negative than in positive for bacteria and the level of Beclin-1 mRNA was significantly increased (p<0.05; Table 4). Similarly, when comparing pediatric with adult patients according to the frequency of operation, we found that the level of Beclin-1 mRNA was significantly higher in the latter (p<0.05; Table 5). The level of Beclin-1 mRNA was significantly increased in serous and mucoid/mucopurulent/purulent-type fluid in adults than in children (p<0.05; Table 6).

DISCUSSION

Despite the post-natal development of the innate and adaptive immune systems, infections by pathogenic viruses, bacteria, and parasites can be fatal. The uterus is a relatively sterile environment. As the fetus passes through the birth canal, the oral, skin, and respiratory systems become exposed and respond to external stimuli. Although the immune system of newborns matures and strengthens as they develop, the strength of the immune system eventually plateaus. More than 1600 genes are involved in the innate and adaptive immune systems; these genes are involved in protecting the host from

hostile environments [6-8]. Because children are not small adults, their immune responses are not necessarily proportional to their weight, height, body mass index, body fat, or age. Because the stage of development may contribute to human immune responses, samples should be classified as being from infants, children, adolescents, adults, and elderly persons. The relatively small sample size in the present study meant that patients were divided into two groups, children aged 0-17 years and adults aged ≥ 18 years.

OME is a condition wherein exudates accumulate in the middle ear cavity, without otalgia or fever. Factors associated with its etiology include Eustachian tube dysfunction, bacterial or viral infections, immune system immaturity or deficiency, genetic factors, breast-feeding, sex, race, age, communal living, second-hand smoking, obesity, allergy, and cleft palate [9,10]. The chronic nature of OME can be caused by defects in blood antibody production, ciliary dysfunction, mucosal edema or hypertrophy, increased viscosity of secretions, fibromatous changes of edema, overproduction of mucus from increased the number of goblet cells and glandular tissues, and defects of the Eustachian tube. The immunological mechanisms underlying OME include infections with bacteria and viruses, cells in effusion fluid, mucosal alterations, chemokines, cytokines, antibodies, B and T cells,

and innate and adaptive immunity^[11–14]. To date, however, the role of autophagy in OME and the difference between the pathophysiology of otitis media in pediatric and adult patients have not yet been determined.

Autophagy is intricately regulated by proteins secreted during the process^[15, 16]. The entire process of autophagy can be divided into several steps: initiation, vesicle nucleation, vesicle elongation, fusion and degradation, and termination. Genetic screening using yeast has identified > 35 Atg genes to date. Our study focused on the expression patterns of genes encoding four proteins that are secreted during autophagy in patients with OME: mechanistic target of rapamycin (mTOR), which is involved in induction and initiation; Beclin-1, which is involved in nucleation; the death proteases caspase 8 inhibitory protein (FLIP), which is involved in vesicle elongation and also antagonizes Atg3; and Rubicon, which inhibits maturation.

A more frequent upper airway infection was found to be linked to an increased prevalence of OME^[9, 10, 13]. In these studies, most patients had a history of upper airway infections, with the detection of bacteria in middle ear effusion fluids, highlighting the importance of bacterial infection as a cause of OME. Therefore, understanding bacterial infection and immune responses within the middle ear may be important in determining the etiology of OME and the role of autophagy. Autophagy may be involved in the removal of pathogens that enter the cytoplasm through the cell membrane or through phagosomes, suggesting that a bacterial infection may activate autophagy^[17]. Similarly, our finding that autophagy-associated mRNAs are expressed in the effusion fluid from both pediatric and adult patients strengthens the likelihood that bacterial infection is one of the causes of OME. In the present study, only the level of Beclin-1 mRNA increased in adult patients, with this level being significantly higher than that in pediatric patients, regardless of the presence of bacteria, the frequency of surgery, and the characteristics of effusion fluid. However, the reason that the level of Beclin-1 mRNA alone was higher in adults than in pediatric patients remains unclear, although several factors may have influenced our findings. First, only six samples were positive for bacteria. Second, many pediatric patients were started on antibiotics prior to admission. Third, effusion samples of adult patients were more frequently serous-type than mucoid-, mucopurulent-, or purulent-type. Finally, the results may have been confounded by testing samples whose status had already become subacute or chronic after 1–3 months. Therefore, it may be necessary to obtain and compare the effusion fluid samples from untreated patients during the early stages of AOM and OME.

Beclin-1 acts as a director during the signaling process involved in autophagy. Following its direct phosphorylation by ULK-1, in response to inhibition by mTOR in mammals, Beclin-1 activates hVPS34. When cells experience hypoxia or when infection occurs, Beclin-1 and Bcl-2 are released from the autophagy-inhibiting complex and activate hVPS34. Several Beclin-1-interacting proteins have been implicated in starvation-induced autophagy. Positive regulators of autophagy include VPS34, VPS15, UVRAG, ATG14, AMBRA1, HMGB1, and Bif-1, with AMBRA1 promoting autophagy as well as being involved in the nutrient-dependent localization of Beclin-1. Negative regulators of autophagy include Rubicon, Bcl-2, Bcl-xL, and IP3R. Bcl-2 participates in the

inhibition of autophagy and Beclin-1-containing VPS34 complexes, and Bcl-xL and IP3R participate in the inhibition of autophagy and the binding of Beclin-1 complexes at the endoplasmic reticulum^[18].

Of the four autophagy-associated genes assayed, only the level of Beclin-1 mRNA differed significantly between children and adults. In contrast to Beclin-1, which acts as an agonist, the other three genes, mTOR, FLIP, and Rubicon, act as antagonists. However, further studies are required to determine the reason for the difference in Beclin-1 expression in children and adults.

Our study had several limitations^[19, 20]. First, despite the comparison between pediatric and adult patients, these comparisons would have been more accurate if they were between the same individuals at different ages. Second, because the subject population was small, the subjects were divided into only two groups. More accurate results may have been obtained if these patients were further subdivided into groups of fetuses, infants, adolescents, adults, and elderly persons. Third, the time allowed to observe the progression of symptoms prior to surgery was 3 months for pediatric patients but only 1–2 months for adults. Fourth, instead of obtaining results during the early stages of OME, our results were obtained after 1–3 months of inflammation, which may have influenced the timing of innate and acquired immune responses. Fifth, only mRNAs were measured; because all of these mRNAs may not have been translated, the levels of mRNA and protein expression may have differed. Sixth, although the results may have been more accurate if both the middle ear mucosa and effusions were analyzed, only middle ear effusion fluids were analyzed for ethical reasons. Because these exudates contained only a few partially exfoliated mucosal epithelial cells and inflammatory cells, they may not fully reflect immune responses and inflammation in the middle ear mucosa during infection.

CONCLUSION

Autophagy-associated genes were expressed in the middle ear fluid of both pediatric and adult patients, suggesting that autophagy may be highly associated with the pathogenesis of OME in both children and adults. However, pediatric patients who tested negative in bacterial cultures, regardless of the frequency of surgery and characteristics of the exudate, showed lower expression levels of Beclin-1 mRNA than those observed in adult patients.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethic committee of Kyung Hee University Hospital (KMC IRB 2012-10-304-014).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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