

Decreased expression of autophagy markers in culture-positive patients with chronic otitis media

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Abstract

Objective: Abnormal autophagy plays a role in the pathogenesis of various diseases. This study aimed to evaluate associations between the clinical manifestations of chronic otitis media (COM) and expression of autophagy markers.

Methods: Associations between presence of bacteria, otorrhea, and conductive and sensorineural hearing loss and levels of autophagy-related mRNAs were investigated in 47 patients with COM.

Results: Autophagy-related mRNAs were detected in all inflammatory tissues of COM patients. LC3-II showed the highest level of expression, followed by Beclin-1, PI3KC3, Rubicon, and mTOR. Beclin-1 mRNA levels were significantly lower in culture-positive than in culture-negative patients.

Conclusion: Autophagy is involved in the pathogenesis of COM. The finding that expression of autophagy markers, especially Beclin-1, was lower in culture-positive than in culture-negative patients suggested that these markers are closely associated with the clinical features of COM.

Keywords

Otitis media, autophagy, Beclin-1, mTOR, bacteria, gene expression

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Introduction

Otitis media (OM) is a general term describing inflammatory changes in the middle ear cavity. Irreversible histological changes observed in patients with chronic OM (COM) include granulation tissue formation, cholesterol granuloma, cholesteatoma, fibrosis, and osteogenesis. COM is caused by multiple factors, the most important of which are dysfunction of the eustachian tubes and bacterial infection.^{1,2}

Autophagy plays an important role in removal of entangled protein masses, damaged intracellular organelles, and extracellular pathogens. During autophagy, cell debris and denatured proteins are sequestered within cells in double-membrane vesicles called autophagosomes. These vesicles fuse with lysosomes, where their contents are metabolized by digestive enzymes. The raw materials consumed during autophagy are used as sources of energy for cell survival or to produce new organelles. During nutrient starvation, autophagy maintains energy balance in cells by decomposing proteins, lipid droplets and glycogen into amino acids, fatty acids, and glucose to produce adenosine triphosphate. By contrast, when sufficient nutrients are present, the degradation of energy sources (proteins, fat particles, and sugars) by autophagy is suppressed and storage is increased. Thus, autophagy acts as a recycling system within cells.³⁻⁶ Because excess removal of cellular material for recycling may lead to cell death, autophagy is often referred to as the third cell death mechanism or as a type of self-predation that manifests in dying cells.^{6,7} More than 30 autophagy-related genes (Atgs) have been identified to date. Dysregulation of autophagy may play a major role in various diseases, including cancer, degenerative brain disease, infections, aging, Crohn's disease, and heart

disease. Autophagy may effectively eliminate pathogens or phagosomes that have entered the cytoplasm directly through the cell membrane.⁸⁻¹⁰

The close relationship between bacterial infection and autophagy suggests that autophagy may be involved in COM. The expression of Atgs in patients with COM may be affected by the presence or absence of bacteria in the middle ear cavity, otorrhea, or the type or degree of hearing loss. To date, however, no studies have assessed the potential roles of bacterial infection-associated autophagy in the pathogenesis of COM. The present study therefore investigated the expression levels of autophagy markers and their associations with bacterial infection, the main cause of COM. In addition, we assessed the relationships between clinical manifestations and expression of autophagy markers in patients with COM.

Subjects and methods

Subjects

The study enrolled patients with COM who visited the Department of Otorhinolaryngology and Head and Neck Surgery of Kyung Hee University Medical Center, Seoul, Korea. COM was diagnosed when: (i) symptoms such as hearing loss, otorrhea, tinnitus, otalgia, or dizziness persisted for longer than 3 months; (ii) lesions were identified in the middle ear and mastoid process on temporal bone computed tomography (TBCT); and (iii) chronic inflammation, granulation tissue, and cholesteatoma were determined histologically from a biopsy of middle ear inflammatory tissue. A detailed history of otorrhea, hearing disturbances, otalgia, tinnitus, dizziness, and ear fullness was obtained from each patient. The presence or absence of otorrhea was confirmed by otoscopy and

endoscopy. Pus was cultured from patients with otorrhea in the external ear canal or tympanic membrane of the middle ear. The presence and severity of hearing disturbances were assessed by pure tone audiometry (PTA), and the presence and severity of middle ear lesions was assessed by preoperative TBCT. Inflammatory tissue was obtained by biopsy during surgery to confirm the diagnosis of COM. Patients with suspected acute OM, OM with effusion, head and neck anomalies, systemic diseases, or congenital or acquired immune deficiencies were excluded. The study protocol was approved by the Institutional Review Board of Kyung Hee University Medical Center (KMC IRB 2017-12-30). All patients or their guardians provided written informed consent.

Bacterial culture

Samples were obtained for bacterial culture when otorrhea was observed. After cleansing of the external auditory canal, the otorrhea sample was aseptically collected using sterilized cotton swabs (Xomed Trace Products, Jacksonville, FL, USA) and immersed in Stuart's transport medium.

An antiseptic otoscope was used to prevent contact with the external auditory canal. Solid blood agar and fluid thioglycollate medium (Hangang, Gunpo, South Korea) were inoculated with these samples, and the cultures were incubated for 24 hours at 35°C. Bacteria that formed colonies were identified by Gram staining and biochemical testing.

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

Total RNA was purified from patient samples using TRIzol solution (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was isolated from tissues using RNeasy Mini kits (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed by reverse transcribing 1 µg of total RNA with random hexamer primers (Promega, Madison, WI, USA) according to the manufacturer's protocol. Real-time PCR was performed on a StepOnePlus real-time PCR system. A 1.5-µL aliquot of cDNA was mixed with 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA,

Table 1. Primer sequences for real-time PCR.

Name	Sequences	Size (bp)
β -Actin	5'-GCGAGAAGATGACCCAGATC-3' 5'-GGATAGCACAGCCTGGATAG-3'	77
mTOR	5'-CCTGCCACTGAGAGATGACA-3' 5'-TCCGGCTGCTGTAGCTTATT-3'	168
PI3KC3	5'-GGAACACCGACCTCACAGTT-3' 5'-CACAGCACCTCCTCTGTGAA-3'	128
Beclin-1	5'-AGGTTGAGAAAGGCGAGACA-3' 5'-AATTGTGAGGACACCCAAGC-3'	112
LC3-II	5'-AGCAGCATCCAACCAAAATC-3' 5'-CTGTGTCCGTTACCAACAG-3'	187
Rubicon	5'-CAGATTCTGCTGCCTCTTCC-3' 5'-AGTGTCTGCCCTCTGAGAA-3'	105

mTOR, mammalian target of rapamycin; PI3KC3, class III phosphatidylinositol 3-kinase; LC3-II, microtubule-associated protein 1A/1B-light chain II; Rubicon, RUN and cysteine-rich domain containing Beclin-1 interacting protein.

USA), 2 μ L of primers designed as previously described^{9–11} (Table 1), and 7 μ L of PCR-grade water in a total reaction volume of 20 μ L. The amplification conditions consisted of an initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 minute. Relative expression of target genes compared with expression of β -actin was calculated using the formula $2^{-(\text{target gene} - \beta\text{-actin})}$.

Evaluation of hearing level and type of hearing loss

PTA was performed at the time of diagnosis by measuring air conduction and bone conduction hearing. We calculated the hearing threshold using a quadratic method with 2 \times weighting at 1,000 Hz using the average of the values at frequencies of 500 Hz, 1,000 Hz, and 2,000 Hz. Sensorineural hearing loss (SNHL) was diagnosed if the bone conduction threshold was more than 30 dB and the air–bone gap was less than 10 dB. Conductive hearing loss (CHL) was diagnosed if the bone conduction threshold was less than 25 dB and the air–bone gap was more than 10 dB. Cases with mixed hearing loss (i.e., bone conduction \geq 25 dB with air–bone gap \geq 10 dB) were included in the SNHL group.

Statistical analysis

Sample size was not calculated in this study but was instead estimated based on the results of similar studies. Normally distributed data were compared using Student's *t*-tests, and non-normally distributed data were compared using Mann–Whitney U tests. All statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Demographics and results of bacterial culture

Of the 47 COM patients enrolled in this study, 20 (42.6%) were men and 27 (57.4%) were women. Twenty-four patients (51.1%) had lesions in the right ear and 23 (48.9%) had lesions in the left ear. *Pseudomonas aeruginosa* was the most frequently detected bacterium, followed by methicillin-resistant *Staphylococcus aureus* (MRSA), coagulase-

Table 2. Demographic and clinical characteristics of patients with COM.

Age, years, mean \pm SD	52.85 \pm 14.71
Sex, male: female, n (%)	20 (42.6): 27 (57.4)
Disease onset, months, mean \pm SD	9.31 \pm 10.10
Affected side, right: left: both, n (%)	24 (51.1): 23 (49.9): 0 (0)
Culture positive, n (%)	22 (46.8)
Otorrhea positive, n (%)	35 (74.5)
Revision surgery, n (%)	7 (14.9)
Audiologic configuration	
PTA (AC), dB, mean \pm SD	52.63 \pm 20.91
PTA (BC), dB, mean \pm SD	26.05 \pm 16.40
Hearing loss type	
Conductive, n (%)	19 (40.1)
Sensorineural, n (%)	13 (27.6)
Normal, n (%)	15 (31.9)

PTA, pure tone audiometry; AC, air conduction; BC, bone conduction; SD, standard deviation.

Table 3. Bacterial culture results in patients with chronic otitis media.

No growth	25 (53.2%)
Growth	22 (46.8%)
<i>Pseudomonas aeruginosa</i>	7
MRSA	6
<i>Staphylococcus aureus</i>	3
Coagulase-negative staphylococci	3
<i>Candida</i> spp.	2
<i>Achromobacter xylosoxidans</i>	1

MRSA, methicillin-resistant *Staphylococcus aureus*. All data are shown as n or n (%).

negative *Staphylococcus*, *Staphylococcus aureus*, and *Achromobacter xylosoxidans* (Tables 2 and 3).

Expression of mammalian target of rapamycin (mTOR), class III phosphoinositide 3-kinase (PI3KC3), microtubule-associated proteins 1A/1B light chain 3B (LC3)-II, Beclin-1, and Run domain Beclin-1-interacting and cysteine-rich domain-containing protein (Rubicon)

In all 47 COM patients, inflammatory middle ear tissue (granulation tissue and cholesteatoma matrix) was positive for expression of mTOR, PI3KC3, LC3-II, Beclin-1, Fas-associated death domain-like interleukin-1 β -converting enzyme inhibitory protein (FLIP), Rubicon, Baculoviral IAP repeat-containing protein (BIRC)2, and BIRC5 mRNAs. LC3-II showed the highest expression level, followed by Beclin-1, PI3KC3, Rubicon, and mTOR (Table 4).

Relationship between mTOR, PI3KC3, LC3 II, Beclin-1, and Rubicon expression and the presence or absence of bacteria

The level of Beclin-1 mRNA was significantly lower in culture-positive patients than

Table 4. Abundance of Atg mRNAs.

Genes	COM
mTOR	0.0009 \pm 0.0005
PI3KC3	0.009 \pm 0.010
LC3-II	0.385 \pm 0.349
Beclin-1	0.119 \pm 0.137
Rubicon	0.006 \pm 0.005

Relative expression Δ Ct (mean \pm SD) values are shown. mTOR, mammalian target of rapamycin; PI3KC3, class III phosphatidylinositol 3-kinase; LC3-II, microtubule-associated protein 1A/1B-light chain II; Rubicon, RUN and cysteine rich domain containing Beclin-1 interacting protein; COM, chronic otitis media.

in culture-negative patients ($p=0.001$) (Table 5).

Relationship between mTOR, PI3KC3, LC3 II, Beclin-1, and Rubicon expression and the presence or absence of otorrhea

There were no significant differences in mRNA levels of any autophagy marker between patients with and without otorrhea.

Relationship between mTOR, PI3KC3, LC3 II, Beclin-1, and Rubicon expression and type and severity of hearing loss

There were no significant differences in mRNA levels of any autophagy marker between subjects with CHL, SNHL, and normal hearing. In addition, there were no significant differences in mRNA levels of any autophagy marker associated with degree of hearing loss.

Discussion

Development of COM depends on host factors including genes, ethnicity, gender, and age, as well as on external factors including environmental, social, and cultural triggers. Most patients with acute OM can be cured

Table 5. Abundance of mRNAs encoding Atgs in patients with positive and negative bacterial cultures.

Atg	Positive	Negative	p-value
mTOR	0.002 \pm 0.002	0.003 \pm 0.004	0.548
PI3KC3	0.037 \pm 0.054	0.110 \pm 0.200	0.842
LC3 II	0.629 \pm 0.823	0.908 \pm 0.867	0.484
Beclin-1	0.149 \pm 0.172	0.296 \pm 0.182	0.001
Rubicon	0.043 \pm 0.066	0.065 \pm 0.074	0.065

Relative expression Δ Ct (mean \pm SD) values are shown. mTOR, mammalian target of rapamycin; PI3KC3, class III phosphatidylinositol 3-kinase; LC3 II, microtubule-associated protein 1A/1B-light chain II; Rubicon, RUN and cysteine rich domain containing Beclin-1 interacting protein; Atg, autophagy-related gene.

without sequelae, but others may experience recurrent or exudative OM with persistent inflammation. Patients with untreated OM may develop COM. It remains unclear how acute infection in the middle ear and mastoid develops into chronic inflammation.¹¹⁻¹⁶

The presence or absence of appropriate inflammatory responses in OM plays an important role in the pathogenesis of acute OM and COM. This study was performed to investigate whether Atgs associated with inflammation were expressed in patients with COM, and whether the expression of these genes was associated with the clinical manifestations of COM. The Atgs assessed included mTOR, which is associated with the initiation of autophagy; P13KC and Beclin-1, which are associated with the vesicle nucleation process; LC3-II, which is associated with the vesicle elongation process; and Rubicon, which is associated with the fusion and degradation process.¹⁷⁻¹⁹

The biological role of autophagy differs depending on the disease,^{20,21} being activated in some diseases and inactivated in others. Activation of autophagy following infection activates cellular defenses against bacteria and viruses, whereas inactivation of autophagy following infection allows pathogens to establish a replicative niche. Although autophagy associated with infection may play an essential role in COM, there have been no studies to date of associations between the clinical manifestations of infection and the activation or inactivation of autophagy.

In this study, all COM samples were positive for autophagy marker expression, suggesting that autophagy is involved in the pathogenesis of COM irrespective of whether bacteria are detected. In addition, the expression levels of all Atgs were lower in bacterial culture-positive patients than in bacterial culture-negative patients. Beclin-1 mRNA levels were significantly lower in

culture-positive patients ($p=0.001$). In the presence of hypoxia or infection, Beclin-1, along with Bcl-2, is freed from autophagy inhibitory complexes, promoting the activity of hVp34.^{11,22} Therefore, bacterial infection in COM tissue may reduce the expression of Beclin-1, preventing the activation defenses against bacteria in the middle ear cavity. These findings suggest that reduced Beclin-1 mRNA levels were associated with the pathogenesis of COM and were triggered in response to infection.

There are several possible explanations for the lack of significant differences in Atg expression between patients with and without otorrhea. Patients in our study were treated with prophylactic antibiotics for 1 week to reduce preoperative inflammation. This may have reduced inflammatory responses in these patients, even those presenting with otorrhea at their initial hospital visit. Patients may also have differed in terms of otorrhea and inflammatory responses in the external auditory canal and mastoid process, which may have affected our results.

Hearing loss is a common symptom of COM, with CHL occurring more commonly than SNHL. However, severe or persistent inflammation may be complicated by labyrinthitis, resulting in mixed hearing loss or SNHL. The degree of hearing loss is determined by the size and location of the perforation of the tympanic membrane, as well as the state and mobility of the ossicle chain.^{4,16}

The incidence of ossicular lesions is dependent on both the type of COM and the presence or absence of otorrhea. Incidence is higher in patients with the active form of COM and otorrhea than in patients with the inactive form of COM without otorrhea.^{23,24} No studies to date have assessed the association between hearing loss and autophagy in animals or humans. The expression of Atgs is related to the presence, degree, and type of hearing

loss, suggesting that the expression of these genes may be altered by increasing or decreasing the expression of specific aquaporin mRNAs.^{11,25,26} We found that SNHL was not associated with the expression level of Atgs.

Our study had several limitations. First, we could not obtain samples of the normal middle ear mucosa for ethical reasons. Second, we measured Atg expression at the mRNA and not the protein level. Differences in levels of mRNAs may not be reflected by differences in protein expression. Third, although none of the patients studied had otorrhea in the auditory canal at the time of initial screening, some patients experienced inflammatory reactions and otorrhea in the middle ear and mastoid process during surgery. Fourth, bacterial culture is not the most sensitive method for detecting bacteria in middle ear samples. Molecular approaches such as PCR or fluorescent *in situ* hybridization may be more reliable. Fifth, only six Atgs were assessed, as there were insufficient amounts of cDNA to measure expression of all Atgs. Sixth, Atg expression may have been affected by administration of antibiotics for 1 week before surgery to reduce inflammation. Seventh, we did not identify any associations between Atg expression and expression of the autophagy-related adaptor molecules, nor could we determine the relationships of these phenomena to clinical manifestations. Eighth, we could not assess changes in lesions and clinical features of OM that may depend on Atg activation and inactivation. Finally, the limited number of samples may have affected the statistical significance of the results.

Conclusion

Our findings indicated that autophagy may be involved in the pathogenesis of COM. In particular, Beclin-1 expression was lower in

culture-positive patients than in culture-negative patients, suggesting that different Atgs may be involved in COM pathogenesis and that each autophagy marker may be differentially associated with clinical features.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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