Expression of Tumor Necrosis Factor-α and Interleukin-6 in Chronic Suppurative Otitis Media

Kronik Süpüratif Otitis Media’da Tümör Nekroz Faktör-α ve İnterlökin-6 İfadeleri

Yan EDWARD1,2, Jacky MUNILSON1,2, Rossy ROSALINDA1,2, Hirowati ALİ3, Dolly IRFANDY1,2*, Delva SWANDA1,2

1Department of ENT-HNS, Faculty of Medicine, Andalas University, West Sumatra, Indonesia
2Dr. M. Djamil General Hospital Padang, West Sumatra, Indonesia
3Biomedical Laboratory, Faculty of Medicine, Andalas University, West Sumatra, Indonesia

Correspondence:
Yan EDWARD
Department of ENT-HNS, Faculty of Medicine, Andalas University
Jalan Universitas Andalas, Padang
25163, West Sumatra, Indonesia
E-mail: dr.yanedward@gmail.com
Phone: +62-751-71181

Received: Jan 01, 2018
Accepted: Jan 26, 2019

https://doi.org/10.25002/tji.2019.782
©2019 Turkish Journal of Immunology. All rights reserved.

Abstract

Introduction: Chronic suppurative otitis media (CSOM) with cholesteatoma is described by keratin epithelial invasion in the middle ear and osteolysis in both of the ear bones and the temporal bone. Some inflammatory factors involved in bone resorption stimulation are T umor Necrosis Factor Alpha (TNF-α) and Interleukin-6 (IL-6). The aim of this study is to determine the expression of TNF-α and IL-6 in CSOM patients with cholesteatoma.

Materials and Methods: The design of this study is cross-sectional comparative analytic in 16 responders or CSOM patients with cholesteatoma and 16 samples of normal ear skin. The gene expressions of samples were examined through Real-Time Polymerase Chain Reaction (RT-PCR) method and were analyzed with statistics software (p<0.05).

Results: The expression of TNF-α and IL-6 in CSOM patients with cholesteatoma were higher than their expression in normal ear skin. The expression of TNF-α was 0.1835±0.322 ng/ul in CSOM patients, while it is 0.005±0.006 ng/ul in normal ear skin (p=0.043). In addition, the expression of IL-6 was 2.127±2.320 ng/ul in CSOM patients and 0.005±0.006 ng/ul in normal ear skin (p=0.010).

Conclusion: The expression of TNF-α and IL-6 in CSOM patients with cholesteatoma are significantly different from patients with normal ear skin.

Keywords: Chronic suppurative otitis media, cholesteatoma, interleukin-6, real-time polymerase chain reaction, tumor necrosis factor-alpha

ÖZ

Giriş: Kolestetomlu Kronik süpüratif otitis media (KSOM), orta kulakla keratin epitel invazyonu ve kulak kemikleri ile birlikte temporal kemikte erime (osteoliz) ile tanımlanmış bir hastaluktur. Tümör Nekroz Edici Faktör-alfa (TNF-α) ve İnterlökin -6 (IL-6) kemik rezorpsiyonundan sorumlu enfamatuar faktörler olarak tanımlanmıştır. Bu çalışmanın amacı TNF-α ve IL-6 ifadelerinin kolestetomlu KSOM’ünün rolünü araştırmaktır.

Gereç ve Yöntemler: Çalışma, 16 yanıtlı ya da kolestetomlu hastanın normal kulak cildinden alınan örnekler ile gerçekleştirilmişdir.

Bulgular: Kolestetomlu KSOM hastalarında TNF-α ve IL-6 ifadeleri, sağlıklı kulak cildi ile karşılaştırıldığında daha yüksek olarak bulundu. KSOM hastalarında TNF-α ifadesi 0.1835±0.322 iken normal ciltte bu ifade 0.005±0.006 (p=0.043) olarak saptandı. IL-6 ifadesi ise, hastalarda 2.127±2.320 iken normal kulak cildinde bu 0.005±0.006 olarak bulundu (p=0.01).

Sonuç: Kolestetomlu KSOM hastalarında TNF-α ve IL-6 düzeyleri normal cilde göre daha yüksektir.

Anahtar kelimeler: Kronik süpüratif otitis media, kolestetom, interleukin-6, gerçek zamanlı-polimeraz zincir reaksiyonu, tümör nekroz edici faktör-alfa

Introduction

Chronic suppurative otitis media (CSOM) is middle ear infection, which is characterized by fluid secretion through tympanic membrane perforation for more than 2 months either intermittent or persistent.[1–3] The number of patients CSOM in the worldwide is estimated to be 65–330 million cases, mainly found in developing countries.[4–9]
The main causes are the low level of hygiene, nutritional problems, upper respiratory tract disease, and air pollution.\[^{10,11}\] The prevalence of CSOM in Southeast Asia is 5.2%, whereas in 3.9% of the Indonesian population in general.\[^{12–15}\] Between January 2015 and September 2016 there were 228 CSOM patients found in Dr. M. Djamil General Hospital, where 43 of them suffered from cholesteatoma.

Chronic suppurative otitis media with cholesteatoma is characterized by epithelial invasion of keratin in the middle ear, and osteolysis in hearing and temporal bones.\[^{16,17}\] The prevalence of cholesteatoma is estimated to occur in 3 out of 100,000 CSOM cases in children, and 9 out of 100,000 in adults.\[^{7,18–20}\] This disease tends to be dominant in male patients compared to women (1.4:1).\[^8\] Some inflammation factors involved in bone resorption are interleukin (IL) IL-1, IL-6, IL-8, Tumor necrosis factor alpha (TNF-\(\alpha\)), neurotransmitter, prostaglandin, interferon-\(\alpha\), parathyroid hormone-related protein, and receptor activator for nuclear factor-kB (RANKL).\[^{16,20–22}\] TNF-\(\alpha\) is a multipotent cytokine that has a variety of biological effects. TNF-\(\alpha\) stimulates bone resorption by inducing proliferation and differentiation of osteoclast progenitors and indirectly activating osteoclasts.\[^{23}\] Vitalle\[^{23}\] quotes Amar et al. who found an increased TNF-\(\alpha\) level in cholesteatoma. Kuczkowski\[^{16}\] with western blot analysis on cholesteatoma, granulation tissue, and normal ear skin, found high amounts of TNF-\(\alpha\), IL-1 and IL-6 in cholesteatoma compared to granulation tissue and normal ear skin.

IL-6 is a cytokine that plays a role in a variety of cellular roles including inflammatory reactions, immune responses and cell proliferation. IL-6 is very important in the pathogenesis of cholesteatoma such as epithelial hyperproliferation and bone destruction, although the mechanism of IL-6 as a pathogenesis of cholesteatoma is still not clear.\[^{23}\] Kuczkowski's study\[^{16}\] showed that IL-6 in cholesteatoma is 3.5 times higher than normal ear skin. Marenda SA et al., quoted by Liu\[^{24}\], found 100% IL-6 expression in the cholesteatoma epithelium, and 25% in the outer ear skin. Nason et al., quoted by Liu\[^{24}\], conducted a study by inhibiting IL-6 to prevent osteoclastogenesis that causes bone destruction. While in Liu's own study there was no association between IL-6 and degree of bone destruction in cholesteatoma.

### Material and Methods

This analytical study is based on the cross-sectional comparative approach by Biomedical Laboratory of Medical Faculty, Andalas University, West Sumatra, Indonesia. There were 16 CSOM patients with cholesteatoma with normal ear skin that are treated with tympanomastoidectomy in Dr. M. Djamil Padang General Hospital. The procedure was conducted on the same patients. The patients had tympanomastoidectomy; besides we removed the cholesteatoma, and we also removed some of the ear skin. This study had been approved by ethical committee, and patients had been signed the inform consent papers.

Inclusion criteria of the samples referred to cholesteatoma skin tissue collection procedure of Anatomical Pathology Laboratory of Dr. M. Djamil Padang General Hospital. The samples may have been rejected if they were not equipped with complete medical record status, as congenital cholesteatoma is also unacceptable as a sample. In addition, the expression of TNF-\(\alpha\) and IL-6 were analyzed using real-time PCR by comparative CT method (\(\Delta\Delta C_t\)).

RNA extraction is taken from the cholesteatoma and the ear skin. RNA isolation using TRIzol® reagent. Initial stages were precipitated RNA from sample size (106 cells or <10 mg tissue), plus 5-10 \(\mu\)g RNase-free glycogen as carrier for aqueous phase. For homogenization 0.5 mL of 100% isopropanol is added to the aqueous phase per 1 mL of TRIzol® reagent, incubating at room temperature for 10 min. Centrifuged at 12,000 \(\times\) g for 10 minutes at 4°C, and this is followed by RNA washing. RNA washing is done by removing the supernatant from the tube, leaving only RNA pellets. Pellets are washed with 1 mL of 75% ethanol per 1 mL of TRIzol® reagent, incubating at room temperature for 10 min. Centrifuged at 12,000 \(\times\) g for 10 minutes at 4°C, and this is followed by RNA washing. RNA washing is done by removing the supernatant from the tube, leaving only RNA pellets. Pellets are washed with 1 mL of 75% ethanol per 1 mL of TRIzol® reagent, incubating at room temperature for 10 min. Centrifuged at 7500 \(\times\) g for 5 minutes at 4°C, the washing liquid removed. RNA pellets are vacuum dried for 5–10 minutes (do not dry the pellet with a vacuum centrifuge).

Making cDNA using the iScript TM cDNA Synthesis Kit: iScript is the optimum modification of Moloney murine leukemia virus (MMLV) reverse transcriptase for the synthesis of extensive and dynamic RNA inputs. As a control for the formation of cDNA, GAPDH was used.

**TNF-\(\alpha\) Forward primer:** 5’-GGC TGA TTA GAG AGG TTA TTA GAG AGA GGT CC-3’, **Reverse primer** 5’-CAC TGA AAG AGA GGT CC-3’.
GAT CCG GG-3'. IL-6 Forward primer 5'-GGA ACA TCC TCG ACG GCA TCT-3', Reverse primer 5'-GGT TCC TCG ACG GCA TCT-3'. The study data were processed with SPSS program using T test to see the difference of TNF-α and interleukin-6 expression between cholesteatoma with normal ear skin.

**Results**

All CSOM patients with cholesteatoma complained of ear discharge and hearing loss (100%). The other symptoms are reported dizziness (12.5%), asymmetric face (6.25%), retroauricular swelling (43.75%), severe headache (12.5%), seizures (6.25%) and consciousness loss (6.25%) (Table 1). Based on the comparative analysis method, the expression of IL-6 in cholesteatoma of patients with SOCm was 2.127±2.320, but it is 0.507±1.530 (p=0.010) in the normal ear (Table 2). Furthermore, the expression of TNF-α in cholesteatoma patients with CSOM was 0.1835±0.322, while it was 0.005±0.006 in the normal ear (p=0.043) (Table 3).

**Discussion**

The number of female responders was slightly higher than male responders, which were 9 (56.25%) and 7 (43.75%) respectively. The comparison of responders was similar to Mustafa et al. (2014) stated that the number of female cholesteatoma patients was 85 (58.6%), or slightly higher than male patients consisted of 60 men (41.4%).[25] Similar results were also observed by Sinha et al. (2004) shows the incidence of CSOM was insignificantly higher in a female with a number of 26 women (56.5%) than in male with a number of 20 men (43.5%).[26] In addition, it has been reported that the incidence of CSOM was mostly found in the ages between 21 and 30 among 64 patients who were diagnosed (40%).[31] However, the tendency of each country may be different due to the variation of malnutrition cases, hygiene, and economic growth of the countries.

The most frequent symptoms found in the responders are ear discharge and hearing loss (100%). Sinha et al. (2004) also described that 46 CSOM patients (100%) reported that they suffered from watery ears which is the effect of fluid discharge, and they also suffered from hearing loss. [26] In accordance, Benson and Mwarni (2012) reported that the most common symptoms of CSOM with cholesteatoma were odorless fluid secretions and hearing problems.[27] Yousef et al. (2011) added that 100% of CSOM patients experienced the same.[28]

Based on clinical examination, it is found that 6 responders (37.5%) experienced fistula retroauricula, 5 (31.25%) had granulation tissue, and 3 (18.75%) had cholesteatoma. These findings are similar to Asroel et al. (2013) who found fistula retroauricula in 28 people (23.53%), granulation tissue in 29 (24.37%), and cholesteatoma in 3 (2.52%).[29]

The expression of TNF-α in patients with cholesteatoma was 0.1835±0.322, whereas it was 0.005±0.006 in patients with normal ear skin. In other words, TNF-α expression increased in patients with cholesteatoma. Western Blot Analysis’ results show an increase of TNF-α expression in cholesteatoma epithel by 3.8 times of normal ear.[27] as found in biomolecular studies performed by Maniu et al. (2014).[30] Vitale et al. (2007), through immunohistochemical analysis, found an increase of

---

**Table 1. General characteristics (n=16)**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>F</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>43.75</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>56.25</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear discharge</td>
<td>16</td>
<td>100.00</td>
</tr>
<tr>
<td>Hearing Loss</td>
<td>16</td>
<td>100.00</td>
</tr>
<tr>
<td>Dizziness</td>
<td>2</td>
<td>12.50</td>
</tr>
<tr>
<td>Asymmetric face</td>
<td>1</td>
<td>6.25</td>
</tr>
<tr>
<td>Retroauricular Swelling</td>
<td>7</td>
<td>43.75</td>
</tr>
<tr>
<td>Severe headache</td>
<td>2</td>
<td>12.50</td>
</tr>
<tr>
<td>Seizures</td>
<td>1</td>
<td>6.25</td>
</tr>
<tr>
<td>Consciousness loss</td>
<td>1</td>
<td>6.20</td>
</tr>
<tr>
<td><strong>Indications</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fistula retroauricula</td>
<td>6</td>
<td>37.50</td>
</tr>
<tr>
<td>Granulation tissues</td>
<td>5</td>
<td>31.25</td>
</tr>
<tr>
<td>Cholesteatoma</td>
<td>3</td>
<td>18.75</td>
</tr>
</tbody>
</table>

**Table 2. Expression of TNF-α in normal cholesteatoma and ear skin**

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-α Expression (Mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteatoma</td>
<td>0.1835±0.322</td>
<td>0.043</td>
</tr>
<tr>
<td>Normal ear skin</td>
<td>0.005±0.006</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. The IL-6 in cholesteatoma and normal ear skin**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Expression IL-6 (Mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteatoma</td>
<td>2.127±2.320</td>
<td>0.01</td>
</tr>
<tr>
<td>Normal ear skin</td>
<td>0.507±1.530</td>
<td></td>
</tr>
</tbody>
</table>
TNF-α expression in cholesteatoma affecting bone destruction. Vitale et al. (2007) mentioned that in the previous study, the similar result is observed in congenital cholesteatoma through PCR, ELISA (Enzyme Linked Immuno Sorbent Assay), and immunohistochemical examinations. There was an increase of TNF-α expression in CSOM patients with cholesteatoma.\[23\]

The expression of IL-6 in cholesteatoma was 2.127±2.320, which is 4 times higher than in normal ear 0.507±1.530. The expression of IL-6 is higher because osteoclastogenetic cytokines such as TNF-α and IL-1 are produced by macrophages during inflammation, and play their role in the pathogenesis of bone resorption by cholesteatoma. IL-1 stimulates fibroblasts and macrophages to induce collagen and prostaglandins (PGE2) that cause bone destruction. Macrophages also produce TNF-α and IL-1 as the body’s response to antigens from bacteria.\[21\]

Western Blot Analysis found an increase of IL-6 in cholesteatoma by 3.5 times compared to the normal ear.\[27\] Immunohistochemical research conducted by Liu et al. (2014) found IL-6 expression by 72% in cholesteatoma that is significantly higher than its expression in the normal ear by 20%.\[24\] In a immunohistochemical examination, it is reported that the expression of IL-6 in the cholesteatoma epithelium was 100%, while in the normal ear it was 25%. The elevated levels of IL-6 in cholesteatoma ear have been examined through both immunohistochemical and ELISA analysis.\[28,29\]

Cytokine examination of cholesteatoma patients obtained the low IL-6 expression through immunohistochemical, ELISA, and Western Blot Analysis.\[29\] Endotoxin is a part of the bacterial wall that plays a role in the inflammation of the middle ear.\[22\] Besides, lipopolysaccharide (LPS) as the part of the bacterial membrane will also trigger inflammation. The bacteria elements and cholesteatoma debris will potentially provide a growth medium for bacteria.\[4,5,17\] Taking antibiotics during the infection process can inhibit induction of macrophages by endotoxin that leads to the down-regulation of proinflammatory cytokines, such as IL-6.\[29\]

There was a limitation in this study regarding antibiotic usage, such as: the type of antibiotics that were taken by responders were not the same. Furthermore, the usage periods may vary to each patient. However, each patient is treated equally before the operative action by the administration of antibiotic-containing ear drops, such as ofloxacin. Patients were administered with first-line oral antibiotics from ampicillin group or erythromycin (if the patient is allergic to penicillin) before the resistance test results are obtained. If the patient is suspected or proven to be resistant, the second-line antibiotics or cultures and sensitivity tests, such as amoxicillin-clavulanic acid, ciprofloxacin, levofloxacin, cefixime, clindamycin, and cefadroxil are prescribed. In addition, other biomolecular studies suggest that anti-inflammatory administration significantly reduces the expression of IL-6.\[22,29\]

Based on the results of several previous studies, there are differences in the expression of TNF-α and IL-6; although most of them show an increase in gene expression of both precursors in all outcomes. This may be due to the difference examination methods that have been done by each researcher. In this study, the examination was done through real-time polymerase chain reaction (RT-PCR) as a thorough examination of every cell part of mutation or genetic evolution. This method detects each reaction process during the reaction in details. The process of amplification, mutation, and deletion of genes can be detected up to the levels of DNA transcription (deoxyribonucleic acid) and RNA (ribonucleic acid).\[31\]

Conclusion

The expression of TNF-α was 0.1835±0.322 in CSOM patients, while it was 0.005±0.006 in the normal ear (p=0.043). In addition, the expression of IL-6 was 2.127±2.320 in CSOM patients and 0.005±0.006 in the normal ear (p=0.010). In the other words, it can be concluded expressions of TNF-α and IL-6 were significantly higher in CSOM patients than normal.
References


