The expression of thymic stromal lymphopoietin in patients and animal models

with eosinophilic otitis media

(好酸球性中耳炎の症例とモデル動物における TSLP の発現)

申請者 弘前大学大学院医学研究科
感觉統合科学領域 耳鼻咽喉科・頭頸部外科学教育研究分野

氏名 三浦 智也
指導教授 松原 篤
Abstract

Conclusion: The present study indicates that the presence of epithelium-derived thymic stromal lymphopoietin (TSLP) in the eustachian tubes plays an important role in the onset of eosinophilic otitis media (EOM).

Objective: In the present study, we aimed to clarify the expression of TSLP, a key trigger of Th2-type allergic disease, in the middle ear mucosa of EOM.

Methods: An immunohistological study of TSLP was conducted in patients with EOM and in animal models of EOM constructed by intraperitoneal and intratympanic injection of ovalbumin (OVA) for 7 days and 14 days. In addition, the messenger RNA (mRNA) expression of TSLP in the middle ear mucosa of the animal models was analyzed using real-time PCR, and was compared with that of the control animals.

Results: Immunoreactivities for TSLP were observed in the middle ear mucosa around the tympanic ostium of the eustachian tube of patients with EOM. In the animal model, strong immunoreactivity for TSLP was also observed in the eustachian tube epithelium, and mRNA expression of TSLP in the 7-day stimulated animals was significantly higher than that in the controls.

Key words: eosinophil, thymic stromal lymphopoietin, middle ear mucosa, eustachian tube
**Introduction**

Eosinophilic otitis media (EOM) is an intractable otitis media often associated with bronchial asthma and is characterized by eosinophil dominant, highly viscous middle ear effusion (MEE). Tomioka et al. identified cases of this disease as intractable otitis media with bronchial asthma in 1993 [1] and termed the expression EOM [2] based on the critical feature of the accumulation of eosinophils in the MEE.

Over the next two decades, clinical studies were primarily conducted in Japan. Diagnostic criteria for EOM were initially proposed by Nagamine et al. in 2002, and were confirmed by the EOM study group in 2011 [3] based on the clinical characteristics of EOM. In brief, the criteria were as follows: one major criterion, eosinophil dominant MEE, and four minor criteria, highly viscous MEE, resistance to conventional therapy for otitis media, association with bronchial asthma, and association with nasal polyposis.

From the pathological point of view, several reports described the presence of IL-5 in MEE [4, 5] and IL-13 immunoreactivity in middle ear mucosa [6]. These Th2-type cytokines play important roles in the pathological features of EOM in the form of eosinophil infiltration caused by IL-5 and mucosal thickening of middle ear mucosa due to periostin induced by IL-13 [7]. Therefore, a Th2-type immune response is strongly suggested in EOM as well as various allergic diseases.

In recent years, epithelium-derived cytokines, such as thymic stromal lymphopoietin (TSLP), have been shown to induce Th2-type responses that trigger several allergic diseases [8]. TSLP is an IL-7-like cytokine found in
the murine thymic stromal cell line as a growth factor that promotes lymphocyte differentiation [9, 10]. A strong correlation has been reported between TSLP and bronchial asthma, atopic dermatitis, and allergic rhinitis [8, 11]. However, there is no reported evidence of involvement of TSLP in the pathogenesis of EOM. In this study, we investigated the immunohistological localization and mRNA expression of TSLP in the middle ear mucosa of patients with EOM and animal models.

**Materials and methods**

This study was approved by the Ethics Committee of Hirosaki University School of Medicine and written informed consent was obtained from all participants. All animal experiments followed the Guidelines for Animal Experimentation, Hirosaki University.

*Tissue preparation*

Middle ear specimens were obtained from patients diagnosed with EOM, using established diagnostic criteria [2], who attended the Hirosaki University Hospital. Tissue samples of the granulated middle ear mucosa were obtained by the biopsy through the external auditory canal in five outpatients with EOM (No. 1, 2, 3, 4, 5). In one inpatient (No. 6) who underwent radical mastoidectomy at our hospital, samples were taken from several regions including mucosa around the tympanic ostium of the eustachian tube during surgery. The samples were immediately immersed in a 10% formaldehyde solution and paraffin-embedded sections (3 μm) were prepared for immunohistological analysis.
The animal model of EOM was constructed using Hartley guinea pigs (weighing 250–350 g) as previously reported [7]. Animals were intraperitoneally injected with 2000 µg of ovalbumin (OVA) and 100 mg of aluminum hydroxide (alum) on day 0, and with 100 µg of OVA and 100 mg of alum on days 7 and 14, for general sensitization. From day 21, they were topically boosted by daily application of OVA solution: 100 µg of OVA by nasal drip, and 0.1 ml of OVA (1000 µg/ml) by intratympanic injection into both ears. The daily application of OVA was continued for 7 days (OVA 7-day group; n = 5) or 14 days (OVA 14-day group; n = 7). Animals not receiving any sensitization were used as the control group (n = 6). All procedures were carried out under anesthesia with a mixture of medetomidine hydrochlor, midazolam, and butorphanol tartrate (0.23 mg, 3.0 mg, and 3.75 mg/kg, respectively). Twenty four hours after the final OVA injection, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.). The temporal bones on one side were then removed and fixed with 10% formaldehyde, and decalcified with EDTA 2Na in 0.1 M TRIS (pH 7.2). Thereafter, paraffin-embedded sections (3 µm) were prepared for immunostaining. The temporal bones on the other side were cut off around the tympanic ostium of the eustachian tube and immediately put into RNAlater for real-time PCR.

**Immunohistology**

The sections were deparaffinized and rehydrated through a graded series of ethanol to phosphate-buffered saline (PBS). Then the specimens were incubated in the following solutions: (1) 0.3% H₂O₂ for 30 min; (2) Protein
Block Serum-Free (Code X0909, DAKO, Carpinteria, CA, USA) for 30 min; (3) anti-TSLP polyclonal antibody (ab47943, Abcam, Cambridge, UK) and 1% bovine serum albumin in PBS at 4°C overnight; (4) reagents of Envision/HRP (rabbit) for 30 min; and (5) diaminobenzene/H$_2$O$_2$ for 5 min. Sections were rinsed well with PBS between each step. Samples were counterstained with hematoxylin and examined under a light microscope. As control experiments, not only the absorption test using TSLP protein (ab202263, Abcam) but substitutional control test using normal rabbit serum IgG (I5006-10mg, Sigma-Aldrich, St. Louis, MO, USA) instead of primary antibody, were performed. No specific staining was observed in both experiments.

**Real-time PCR**

Total cellular RNA was extracted from the middle-ear mucosa and purified using a RNeasy mini kit (Qiagen GmbH, Strasse, Germany). Cloned DNA (cDNA) was prepared from 500 ng of total RNA by reverse transcription at 37°C for 60 min and 95°C for 5 min using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s protocols. Quantitative real-time PCR was carried out using the TaqMan assay and GeneAmp PCR system (Applied Biosystems). The quantitative real-time PCR assay was based on primers that specifically amplify TSLP. The primers for TSLP (AIRSB0F) and β-actin (Cq03755212_g1) were purchased from Applied Biosystems. The amplification efficiency of the specific primers and β-actin were validated in a preliminary PCR analysis. Each sample was run in duplicate in separate tubes to permit quantification of the TSLP gene normalized to β-actin. The
PCR conditioning consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 60 sec.

Data analysis
Data analysis was performed using Bio-Rad CFX Manager 3.1-CFX connect (Applied Biosystems). For quantitative analysis, we used the comparative threshold cycle (Ct) method according to the manufacturer's instructions (Applied Biosystems). TSLP mRNA levels were normalized to the β-actin mRNA level.

Unpaired t-test was performed using Stat Mate ver. 4.01 (ATMS, Tokyo, Japan) to compare the data obtained from the animal model and control. P < 0.05 was considered statistically significant.

Results
Six patients with EOM were included in this study. Gender, age, and complications are listed in Table 1.

TSLP immunoreactivities were detected in the epithelial cells of the middle ear mucosa around the tympanic ostium of the eustachian tube in one patient (No. 6; Fig.1A), and in the inflammatory cells in three patients (No. 3, 5, 6; Fig. 1B). TSLP-positive cells were also observed in the vascular endothelial cells in the sample from No. 6 (data not shown). No immunoreactivity for TSLP was shown in other three cases (No. 1, 2, 4). Strong immunoreactivity for TSLP was observed in the middle ear tissue near the eustachian tube in
the specimen collected during surgery (No. 6).

In the middle ear mucosa from the OVA-stimulated animal models, a strong immunoreactivity for TSLP was observed in many eustachian tube epithelial cells (Fig. 2: A-1, B-1) in both the OVA 7-day and OVA 14-day groups. In the middle ear mucosa, TSLP-positive cells were detected around the tympanic ostium of the eustachian tube (Fig. 2: A-2, B-2). However, fewer cells showed TSLP immunoreactivity in sites farther from the tympanic ostium of eustachian tube (Fig. 2: A-3, B-3). When comparing the topical stimulation days, the TSLP immunoreactivity in the OVA 14-day group appeared stronger than that in the OVA 7-day group.

No immunoreactivity for TSLP was observed in the control group (Fig. 2 C). In real-time PCR, the expression of TSLP mRNA was increased in the stimulated animal model OVA 7-day and OVA 14-day groups compared with the control group (Fig. 3). There is no significant difference between the OVA 7-day and OVA 14-day groups (Fig. 3).

**Discussion**

EOM is well known as a high-risk disease for hearing loss. The EOM study group reported that 81 out of 138 cases of EOM showed bone conductive hearing loss (BCHL), and 8 cases experienced complete hearing loss [2]. High-tone BCHL was also progressive, worsening as the period to appropriate treatment of the EOM became longer [12, 13]. These results indicate that it is important to understand the pathology of EOM and to promptly start appropriate treatment. Previous studies have shown that the presence of Th2
cytokines, such as IL-5 and IL13 [4, 5, 6], and the pathology of EOM are most likely to result from a Th2-type immune response. The presence of epithelium-derived cytokines such as TSLP is a key trigger for a Th2-type immune response; however, the presence of TSLP in EOM had not been reported.

In this study, we found the immunoreactivity for TSLP in inflammatory cells of the middle ear mucosa in three cases of EOM patients. With regard to the epithelial layer, no positive staining was found in the biopsy specimens through the external auditory canal, but positive staining was detected in the specimens around eustachian tube who underwent radical mastoidectomy. It is difficult to clarify the correlation between TSLP expression and clinical features of EOM patients from the present results, because the number of EOM patients is small. Therefore, basic experiments using model animals are important to find out the pathogenesis of EOM.

In our model animals, TSLP immunoreactivities were also detected around the tympanic ostium of the eustachian tube, and the molecular biological study also supported these immunohistological results in the animal model. As we described in the previous reports of our animal model of EOM, there is a significant difference of eosinophils infiltrating in the middle ear mucosa between the control group and the OVA 14-day group (the number of eosinophils /0.01mm²; mean ± SD: 0.28 ± 0.23 and 1.88 ± 0.78 respectively), but not the OVA 7-day group (mean ± SD: 0.44 ± 0.41) [7, 14], and immunoreactivity of eotaxin has also been found in the OVA 7-day group [15]. In the present study, we detected the immunoreactivity and gene
expression of TSLP in the epithelial cells of the eustachian tube and around the tympanic ostium of the eustachian tube in the OVA 7-day group. These results suggest that, in the OVA stimulation model, TSLP produced by the epithelial cells of the eustachian tube may play an important role in allergic inflammation of the middle-ear mucosa prior to eosinophil infiltration, as eosinophil infiltration occurs in the lower layer of the Th2-type immune response cascade. For instance, both mast cells and dendritic cells are reported to release high levels of IL-5, which is key to eosinophil activation and infiltration through TSLP stimulation [16].

Research of middle ear allergy using animal models was conducted about three decades ago. At that time, the eustachian tube was reported to act as a “gatekeeper,” limiting the entry of most substances into the tympanic cavity, although type-I allergic reactions in the middle ear were reported [17]. However, Iino et al. identified more cases of patulous eustachian tube, which allows the entry of antigenic substances into the tympanic cavity, in patients with EOM compared with the controls [18]. Indeed, a recent report described the presence of antigen-specific IgE for house dust mite, fungi, and staphylococcus enterotoxins in the MEE [19]. Morphologically, the epithelium cilia is present in the eustachian tube and around the tympanic ostium, similar to airway epithelium, but the tympanic epithelium loses the cilia and takes a different form farther away from the eustachian tube. In the present study, TSLP immunoreactivity was found in the epithelium around the eustachian tube, indicating that EOM is a kind of airway allergy and supporting the concept of “one airway, one disease.”
We used OVA stimulation as an allergen in the present study and identified TSLP in the epithelial cells around the eustachian tube. Since TSLP is released from epithelial cells in response to not only allergens but also microbial products [16], and pathogens in MEE are risk factors for BCHL and EOM severity [13, 19], the eustachian tube is deeply involved in the pathology of EOM from a functional and immunological point of view.

For the treatment of EOM, we use a combination of anti-allergic drugs, such as Cys-LTs receptor antagonists, phosphodiesterase inhibitors, and systemic or topical steroids. If TSLP acts as a trigger to eosinophil infiltration and the activation of a Th2-type immune response, the development of anti-TSLP modalities can be expected as a target for new treatments. Further study is required to identify more effective treatments for EOM.

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**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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**Figure legends**

Table 1. Clinical features, allergic background, and immunohistological findings in six patients with EOM. Note that TSLP positive immunostaining in the inflammatory cells was observed in three cases, and that in the epithelial cells was detected in only one case whose specimens were taken during mastoidectomy.

AR: allergic rhinitis, BA: bronchial asthma.

Figure 1. Immunoreactivity for TSLP in patients with EOM
TSLP was detected in epithelial cells (arrows, case No. 6 in Table 1) and inflammatory cells (arrow heads, case No. 3 in Table 1).

Figure 2. Immunoreactivity for TSLP in animal models of EOM
OVA 14-day group (A), OVA 7-day group (B), and control group (C).
Eustachian tube (-1), around the tympanic ostium (-2), and distant site (-3).

Figure 3. Comparison of mRNA level of TSLP expression in the animal model
$t$-test, *$P < 0.05$
References


Matsubara A, Nishizawa H, Kurose A, Nakagawa T, Takahata J, Sasaki


Table 1

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Figure 1
Figure 2
Figure 3

![Bar graph showing relative TSLP mRNA levels in control, OVA7-day, and OVA14-day groups.](Image)