

Eosinophil chemoattractants in the middle ear of patients with eosinophilic otitis media

Y. Iino*, K. Kakizaki*, H. Katano*, H. Saigusa* and S. Kanegasaki†

*Department of Otolaryngology, Teikyo University School of Medicine, Tokyo, Japan and †Effector Cell Institute, Tokyo, Japan

Summary

Background Patients with intractable otitis media associated with bronchial asthma have an extensive accumulation of eosinophils in the effusion and mucosa of the middle ear; this condition is called eosinophilic otitis media (EOM). It remained to be determined how eosinophils accumulate in the middle ear.

Objectives To clarify the pathogenesis of middle ear diseases, we measured the concentration of eosinophil chemoattractants in middle ear effusion (MEE), and carried out immunohistochemical studies of middle ear mucosa specimens to demonstrate the expression of eosinophil chemoattractants.

Methods Middle ear effusion samples were obtained from 15 EOM patients with bronchial asthma and from six controls for the measurement of eosinophil cationic protein (ECP), IL-5, eotaxin and regulated on activation, normal T expressed and secreted concentrations. Middle ear mucosa samples were also taken from 14 EOM patients and 16 controls for immunohistochemical study. In 10 EOM patients, the numbers of immunoreactive cells as well as apoptotic cells were determined before and after the topical application of triamcinolone acetonide into the middle ear.

Results In EOM, significantly higher ECP and IL-5 concentrations were detected in MEE than in serum, and ECP, IL-5 and eotaxin concentrations in MEE were higher in the EOM patients than in the controls. ECP concentration positively correlated with that of IL-5. Immunohistochemically, the numbers of cells positive for EG2 and eaelectin were significantly higher in the EOM patients than in the controls. After the topical application of triamcinolone acetonide, the numbers of infiltrating cells and immunoreactive cells distinctly decreased, whereas the number of apoptotic cells significantly increased.

Conclusion In EOM, locally produced IL-5 may play a crucial role in the accumulation of eosinophils in the middle ear. Chemokines such as eaelectin and eotaxin are also produced in the middle ear, and help activate and enhance the survival of eosinophils to induce the intractable condition in the middle ear. The topical application of triamcinolone acetonide induces the apoptosis of not only eosinophils but also eosinophil chemoattractant-producing cells, thereby improving the middle ear condition.

Keywords bronchial asthma, eosinophil chemoattractants, eosinophilic otitis media, eosinophils, IL-5, apoptosis

Submitted 7 December 2004; revised 25 June 2005; accepted 20 July 2005

Introduction

Recently, there have been some reports on adult patients with intractable otitis media with effusion (OME) or chronic otitis media associated with bronchial asthma and/or allergic rhinitis (AR) [1, 2]. The reported patients generally exhibited the same characteristics of the clinical course and symptoms that were markedly different from those of the common type of OME or chronic otitis media. Derlacki [3] summarized the clinical characteristics of the intractable otitis media as follows: (1) a secretion having a markedly viscous or gelatinous consistency with eosinophils; (2) a middle ear

mucous membrane that is fairly thickened with numerous eosinophils; (3) a protracted healing time; (4) occurrence in allergic disposition such as AR; and (5) a likely good response to anti-allergy treatment. He suggested that the condition resulted from an allergic aetiology.

In the common OME or chronic otitis media, neutrophils, lymphocytes and macrophages predominantly infiltrate into middle ear effusion (MEE) and the middle ear mucosa. In contrast, a marked infiltration of eosinophils in MEE and the middle ear mucosa is a common feature in these patients as Derlacki [3] has described. Tomioka et al. [1] named the specific-type intractable otitis media associated with bronchial asthma eosinophilic otitis media (EOM) because some of the patients do not have type I allergy to a specific allergen by skin testing or RAST. In 2001, we reported seven patients with EOM, and proposed the diagnostic criteria of the disease as follows: (1) the presence of yellow and extremely viscous

Correspondence: Prof. Yukiko Iino, Department of Otolaryngology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan.

E-mail: yiorl@med.teikyo-u.ac.jp

MEE predominantly containing eosinophils and (2) precedence and association with adult bronchial asthma [4]. Although EOM has not yet been widely recognized, it is expected that a sizable population of asthmatic patients will be diagnosed with this disease if it is properly recognized. Recently, a clinical surveillance of EOM was performed in the Otorhinolaryngology department of major hospitals all over Japan, and 341 patients were diagnosed as definite cases of EOM according to the criteria reported. Suzuki et al. [5] summarized the clinical characteristics of all the patients, and showed that generally the age at the onset of EOM is approximately in the fifties, and the female to male ratio is about 2:1. Surprisingly, about one-half the number of the patients showed a deterioration in bone conduction hearing threshold, and among these 6% became completely deaf. The cause of EOM is not fully understood, and the treatment of this disease remains to a major problem.

We previously reported that, in EOM patients, most of the eosinophils in the middle ear mucosa are activated with degranulation, and that the concentration of eosinophil cationic protein (ECP) in MEE is high [6]. Eosinophils are pro-inflammatory cells that normally reside in the circulation, and are induced to extravasate into tissues by a diverse array of chemoattractants and viability-enhancing factors. Therefore, the present study was carried out to determine the presence of eosinophil chemoattractants in MEE and middle ear mucosa that may clarify the mechanism of accumulation of eosinophils in the middle ear. In addition, we analysed whether eosinophilia in the middle ear mucosa is altered by the topical application of a glucocorticoid into the middle ear.

Materials and methods

Study subjects

MEE and/or middle ear mucosa samples were obtained from 15 patients (11 females and four males, aged between 35 and 74 years) who participated in this study. All the patients had a long history of bronchial asthma and were under medical treatment with systemic and topical glucocorticoids. Six patients were diagnosed as having atopic asthma, and the remaining nine patients as having non-atopic asthma. Aspirin intolerance was detected in four patients with non-atopic asthma. Some had been treated for OME for a long period, by medication, catheter inflation, myringotomy and insertion of tympanostomy tubes. On their first visit to our hospital, some patients had bilateral or unilateral perforation of the eardrum because of spontaneous rupture or persistent perforation after myringotomy or extrusion of tympanostomy tubes. All the patients except one had bilateral OME or chronic otitis media. The diagnosis of EOM in these patients was based on the criteria proposed by Nagamine et al. [4] as described previously, that is, intractable otitis media with highly viscous MEE, in which many eosinophils were histologically or cytologically detected, in association with atopic or non-atopic bronchial asthma.

MEE samples were collected from the persistent perforation of the eardrum in 11 patients and by myringotomy in four patients for the measurement of the concentrations of ECP and eosinophil chemoattractants. At the same time, peripheral blood samples were also obtained from the

patients to analyse ECP and eosinophil chemoattractant concentrations in the serum. Middle ear mucosa samples were obtained from the persistent perforation in 12 patients and at the time of ear surgery in two patients, and were subjected to histological and immunohistochemical studies, and *in situ* hybridization. In 10 of these patients, middle ear mucosa samples were again obtained one week after the instillation of triamcinolone acetonide into the middle ear through eardrum perforation. In cases without eardrum perforation, myringotomy was carried out followed by the complete removal of viscous MEE, and then triamcinolone acetonide was instilled into the middle ear cavity. In cases with persistent perforation, triamcinolone acetonide was applied via eardrum perforation after the removal of MEE or otorrhoea. Most of the EOM patients were free from MEE 1–3 months after the instillation of triamcinolone acetonide. Triamcinolone acetonide was applied into the middle ear in the same way when MEE or otorrhoea recurred.

As controls, serous or seromucoid MEE samples were obtained by myringotomy in six adult patients with OME but without bronchial asthma or nasal allergy. Samples of the granulated or hypertrophic middle ear mucosa were also obtained from 16 adult patients with chronic perforated otitis media but without bronchial asthma or nasal allergy during the middle ear surgery. All the control patients responded well to the conservative and surgical treatment for OME or chronic otitis media. Informed consent was obtained from all the asthmatic and control patients prior to the study.

Middle ear effusion preparation The middle ear effusion samples were collected using a Jun-Tym-Tap middle ear fluid aspirator/collector (Xomed, Jacksonville, FL, USA). Fifty to 100 mg of the effusion samples was suspended in 1 mL of saline until the fluid became homogeneous by pipetting and vortexing vigorously at room temperature. When the effusion was too viscous to be homogenized, dithioerythritol (DTE, Fluka, Gillingham, Dorset, UK; final concentration, 0.01 M) solution was added to dissolve the effusion according to the method described by Louis et al. [7]. The fluid samples were allowed to stand for 60–120 min, and then centrifuged at 1000–1350 *g* for 10 min. The resultant supernatant was collected and stored at -80°C until measurement.

Measurement of eosinophil cationic protein and eosinophil chemoattractants in middle ear effusion ECP concentration in the samples was measured using a Pharmacia RIA kit (Pharmacia-Upjohn, Uppsala, Sweden). IL-5, eotaxin-1, and regulated on activation, normal T expressed and secreted (RANTES) concentrations in MEE and serum were determined using ELISA IL-5 (Immunotech (MBL), Tokyo, Japan), Quantikine human eotaxin (R&D Systems, Minneapolis, MN, USA) and Quantikine human RANTES (R&D Systems) kits, respectively, according to the manufacturers' recommendations. The sensitivity of the assay was higher than the detection limits of 2 $\mu\text{g/L}$ for ECP, 5 pg/mL for IL-5, 15.6 pg/mL for eotaxin and 31.2 pg/mL for RANTES.

Tissue preparation The middle ear mucosa samples were immediately fixed in 10% formaldehyde solution, and paraffin-embedded sections were prepared by a conventional method.

Immunohistochemical reagents The mouse monoclonal antibodies used were as follows: anti-EG2 antibody (Kabi Pharmacia, Uppsala, Sweden) that recognizes eosinophils; anti-CD3 antibody (DAKO A/S, Glostrup, Denmark); anti-IgE antibody (DAKO A/S); and anti-RANTES antibody (DAKO A/S). The polyclonal antibody against ecalectin was a gift from Dr Akiyama of Effector Cell Institute (Tokyo, Japan). The antibodies were appropriately diluted in 0.01 M phosphate-buffered saline (pH 7.4) containing bovine serum albumin and used for immunohistochemical staining.

Immunohistochemistry To examine the distribution of cells that immunoreacted with the above-mentioned antibodies in the middle ear specimens, an immunohistochemical method using a Histofine SAB-PO (G) kit or a Histofine simplestain MAX-PO (M) kit (Nichirei, Tokyo, Japan) was utilized, according to the manufacturer's instruction. The sections were stained with 3,3-diaminobenzidine tetrahydrochloride or alkaline phosphatase.

In situ hybridization (ISH) To detect eotaxin-1, ecalectin and RANTES mRNAs in the section of middle ear mucosa, digoxigenin complementary riboprobes (cRNA antisense) were used. The complementary DNA sequences encoding eotaxin-1, ecalectin and RANTES were inserted into pGEM vectors, amplified in *Escherichia coli* and linearized with the appropriate enzymes. Prior to ISH, *in vitro* transcription to generate sense and antisense probes was performed using a DIG RNA Labelling Mix (Roche, Basel, Switzerland) [8]. There were no significant hybridization signals observed when the sections were treated with RNase prior to hybridization, or when the sections were treated with a sense probe identical to cytokine receptor mRNA. Cells positive for eotaxin-1, ecalectin and RANTES mRNAs were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Mannheim, Germany) followed by staining with nitroblue tetrazolium/BCIP. The sections that hybridized with the sense (negative) probe were examined first to deduce background signals.

In situ detection of apoptotic cells In the sections of middle ear mucosa samples taken before and 1 week after the instillation of triamcinolone acetate, apoptotic cells were detected by the non-radioactive (deoxyuridine triphosphate

(dUTP)-digoxigenin) *in situ* labelling of nuclear DNA fragmentation using Cell Death In-situ Labelling and Detection Systems (DAKO A/S). Bound alkaline phosphatase was detected using nitroblue tetrazolium/BCIP.

Evaluation The sections were coded and evaluated independently by two individuals under a conventional light microscope at low and high magnifications, and the immunohistochemically stained cells in the submucosal layer were observed. We counted immunoreactive cells at $\times 400$ magnification in ten fields where many inflammatory cells were observed. Simultaneously, the sections were stained with haematoxylin and eosin.

Statistical analysis Statistical analysis was carried out using an unpaired *t*-test or a Mann-Whitney's *U*-test. Simple regression analysis was also performed to determine the correlation between two variants. Two-tailed *P*-values less than 0.05 were considered significant.

Results

Concentration of eosinophil cationic protein and eosinophil chemoattractants in middle ear effusion

All the patients in the EOM group had more than 100-fold higher ECP concentration in MEE than in serum, and the difference was statistically significant ($P < 0.0001$) (Table 1). In the control group, MEE from one patient contained ECP at a concentration less than the detection limit ($< 2 \mu\text{g/L}$). The ECP concentration in MEE from the EOM group was significantly higher than that in MEE from the control groups ($P < 0.0001$). When the EOM patients were divided into atopic asthma ($n = 6$) and non-atopic asthma group ($n = 9$), the concentrations of ECP in MEE and serum showed no statistical difference between the two groups. A significantly higher ECP concentration was recovered from MEE from EOM patients even with non-atopic asthma than from MEE from control patients ($P < 0.01$).

An IL-5 concentration less than 5 pg/mL (detection limit) was noted in none of MEE samples and in 10 serum samples from the EOM group, and in two MEE samples from the control group. The concentration of IL-5 in MEE from the EOM patients was significantly higher than that in the serum of the patients as well as that in MEE from the control group.

Table 1. Concentrations of ECP and chemoattractants in MEE and serum

	Patients with EOM ($n = 15$)		Control patients ($n = 6$)
	MEE	Serum	MEE
ECP ($\mu\text{g/L}$)	10 105 (6536, 2610–22 927)	30 (25, 6–83)	193 (179, 2 to > 458)
<i>P</i> value*		< 0.0001	< 0.0001
IL-5 (pg/mL)	2208 (2655, 150–9434)	9 (2, 5 to > 12)	93 (96, 5 to > 231)
<i>P</i> value*		< 0.01	< 0.0001
Eotaxin (pg/mL)	300 (193, 15.6 to > 600)	143 (42, 94–232)	85 (35, 15 to > 114)
<i>P</i> value*		NS	< 0.001
RANTES (pg/mL)	1259 (1046, 31.2 to > 3080)	16 483 (9802, 8970–44 500)	1686 (1742, 260–4039)
<i>P</i> value*		< 0.0001	NS

ECP, eosinophil cationic protein; MEE, middle ear effusion; EOM, eosinophilic otitis media; RANTES, regulated on activation, normal T expressed and secreted; NS, not significant; SD, range.

**P* value vs. MEE of patients with EOM.

An eotaxin concentration less than the detection limit (<15.6 pg/mL) was noted in five MEE samples and none of the serum samples from the EOM group, and in three MEE samples from the control group. Generally, the eotaxin concentration in MEE from the EOM patients was significantly higher than that in MEE from the control group ($P < 0.001$). In contrast, the RANTES concentration in MEE was significantly lower than that in serum from the EOM group ($P < 0.0001$), and five samples of MEE contained RANTES at a concentration less than the detection limit of 31.2 pg/mL. The RANTES concentrations in MEE from the EOM patients were almost the same as those in the controls. There were no statistically significant differences in the concentration of IL-5, eotaxin and RANTES in MEE and serum between the EOM patients with atopic asthma and those with non-atopic asthma.

When correlation was studied between the concentration of ECP and that of each eosinophil chemoattractant in MEE from the EOM and control patients, a significantly positive correlation was found between ECP and IL-5 concentrations (Fig. 1: $Y = 151.758 + 0.199X$, $R^2 = 0.341$, $P < 0.01$). However, no correlation was noted between ECP and eotaxin concentrations ($Y = 99.211 + 0.009X$, $R^2 = 0.103$),

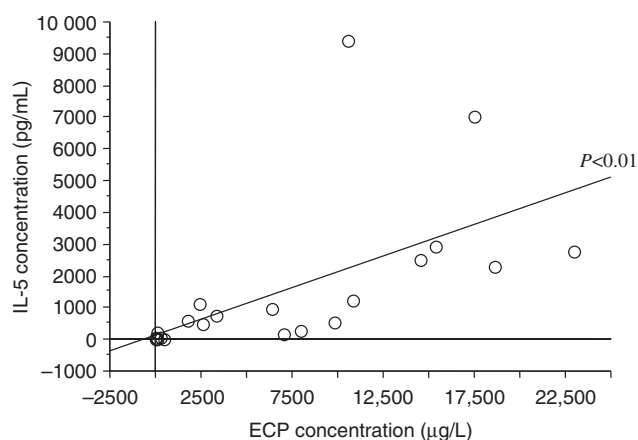


Fig. 1. Correlation between eosinophil cationic protein (ECP) and IL-5 concentrations in middle ear effusion of the eosinophilic otitis media patients and control patients. A significantly positive correlation between ECP and IL-5 concentrations was noted.

or ECP and RANTES concentrations ($Y = 1515.451 - 0.959X$, $R^2 = 0.107$).

Immunohistochemically stained cells in middle ear mucosa Numerous eosinophils labelled with the antibody against EG2 were observed in the submucosal layer in the middle ear mucosa of the EOM patients (Fig. 2a). On the other hand, in the control patients, few EG2⁺ cells were distributed in the submucosal area. The number of EG2⁺ cells was significantly higher in the EOM patients than in the control patients (Table 2). Positive staining for CD3 was observed on the surface of small, round cells in the submucosa of the middle ear mucosa, and the numbers of CD3⁺ cells were almost the same for the EOM and control patients. Ecalectin-positive cells were observed in the submucosal layers in the middle ear mucosa. Two types of ecalectin-positive cell were identified in the submucosa, namely, spindle-shaped, presumably fibroblasts, and large and irregularly shaped, presumably macrophages. Each cell showed many granules in the cytoplasm, which was positively immunostained. In addition, some endothelial cells were also positive for ecalectin. Cells positive for ecalectin were observed in the submucosa, particularly in the area where many EG2⁺ cells accumulated (Fig. 2b). Compared with control patients, a significantly large number of ecalectin-positive cells were present in the EOM patients. Many RANTES⁺ cells were also found in the submucosa not only in the EOM patients but also in the controls. These cells were spindle-shaped, presumably fibroblasts, and large and round, presumably macrophages. Some endothelial cells and epithelial cells were also RANTES⁺ (Fig. 2c). Positive correlations of the number of EG2⁺ cells with the numbers of cells positive for ecalectin ($Y = 8.294 + 0.399X$, $R^2 = 0.412$, $P < 0.001$) and RANTES ($Y = 50.477 + 1.03X$, $R^2 = 0.58$, $P < 0.0001$) were found, but not with that of CD3⁺ cells ($Y = 102.559 + 0.15X$, $R^2 = 0.014$).

After the topical application of triamcinolone acetonide, the number of infiltrating cells markedly decreased in the submucosa of the middle ear in the EOM patients. Immunohistochemically, the numbers of EG2⁺, CD3⁺ and ecalectin⁺ cells significantly decreased after the application ($89 \pm 46 - 24 \pm 16$, $P < 0.01$ for EG2; $115 \pm 48 - 43 \pm 29$, $P < 0.01$ for CD3; $49 \pm 35 - 13 \pm 14$, $P < 0.05$ for ecalectin).

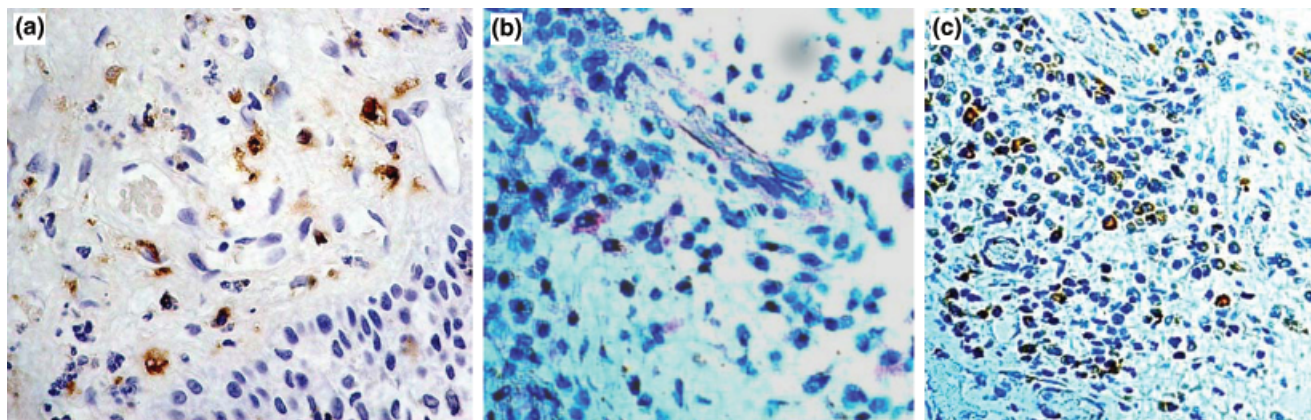


Fig. 2. Immunohistochemical staining of middle ear mucosa of an eosinophilic otitis media patient. (a) Many EG2⁺ cells were observed in the subepithelial layer of the middle ear mucosa. (b) Red-stained ecalectin-positive cells were spindle-shaped, presumably fibroblasts, and large and irregular, presumably macrophages. Some of the endothelial cells were also ecalectin-positive. (c) Many regulated on activation, normal T expressed and secreted-positive cells were observed in the submucosa ($\times 400$).

Table 2. Number of immunopositive cells in middle ear mucosa ($\times 400$, 10 fields)

	Patients with EOM ($n = 14$)	Control patients ($n = 14$)	<i>P</i> -value
EG2	116 $\pm$ 80	15 $\pm$ 17	<0.05
CD3	100 $\pm$ 48	117 $\pm$ 77	NS
Ecalectin	65 $\pm$ 54	7 $\pm$ 7	<0.01
RANTES	146 $\pm$ 131	80 $\pm$ 45	NS

EOM, eosinophilic otitis media; RANTES, regulated on activation, normal T expressed and secreted; NS, not significant.

Although the number of RANTES⁺ cells also decreased after the application (120 $\pm$ 49–46 $\pm$ 35), the difference between before and after the application was not statistically significant.

Gene expression for eotaxin, ecalectin and regulated on activation, normal T expressed and secreted of middle ear mucosa

Some of the cells in the submucosa in the EOM patients were positive for eotaxin-1, ecalectin and RANTES mRNAs (Figs 3a–c). In particular, ecalectin mRNA⁺ cells were observed in the areas where the eosinophils accumulated.

Apoptotic cells in middle ear mucosa

The number of apoptotic cells was very small in the middle ear mucosa before the application of triamcinolone acetonide in the EOM patients. However, blue-stained, apoptotic cells were among the infiltrating cells in the submucosa as well as in the epithelium of the middle ear mucosa after the application. The infiltrating cells included eosinophils, macrophages, fibroblasts and epithelial cells (Fig. 4). The number of apoptotic cells after the application of triamcinolone acetonide significantly increased in each patient compared with that before the application (Fig. 5).

Discussion

Tissue eosinophilia is a characteristic feature of chronic inflammatory airway diseases including nasal polyposis with chronic rhinosinusitis, AR and bronchial asthma. Sensitive immunoassays for ECP indicate eosinophil activation in asthma and allergic diseases [9–11]. Hurst and Venge [12] reported that elevated levels of ECP are detected in MEE from atopic children with OME, suggesting that allergic response is one of the main causes of OME. The present study also clearly demonstrated that in EOM patients, a higher ECP concentration was detected in MEE than in serum. This ECP concentration was also higher than that in MEE from the control patients. The detected levels of ECP in the EOM patients in our study was 10–100 times higher than the levels of ECP in the atopic children reported by Hurst and Venge [12]. These results may have been dependent on the severity of eosinophilic inflammation in the middle ear.

It is still controversial as to how eosinophils are recruited and accumulate in the airway mucosa including the middle ear. We have recently reported that, in EOM patients, most eosinophils in the middle ear mucosa and MEE are activated,

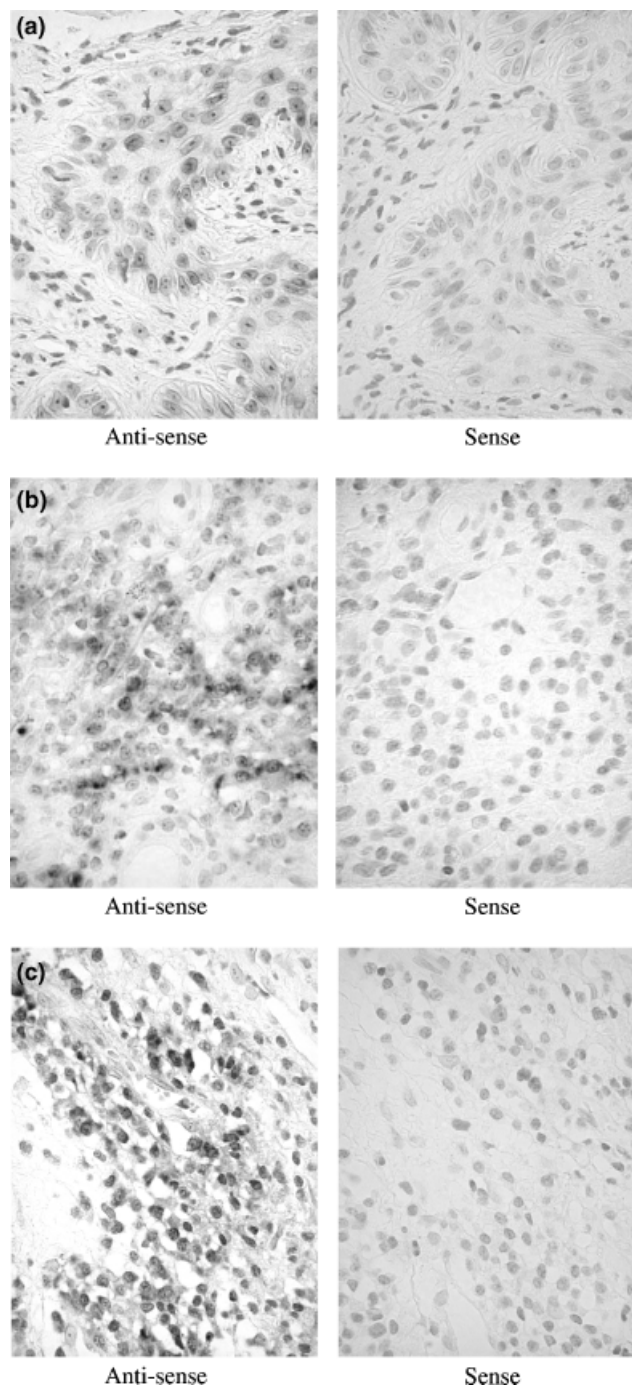


Fig. 3. *In situ* hybridization staining of mRNA of each eosinophil chemoattractant in the middle ear mucosa of an eosinophilic otitis media patient. Cells positive for mRNA of each eosinophil chemoattractant were observed adjacent to the area where many eosinophils accumulated in the submucosa of the middle ear. (a) eotaxin, (b) ecalectin, (c) regulated on activation, normal T expressed and secreted ($\times 400$).

resulting in the degranulation and release of ECP, and that local IgE production occurs in the middle ear mucosa [6]. The nasal polyps of patients who were designated as non-atopic by skin testing and RAST exhibited local specific IgE production against *Staphylococcus aureus* enterotoxins A and B [13]. At the same time, in nasal polyp tissues, the concentrations of eosinophil chemoattractants such as IL-5,

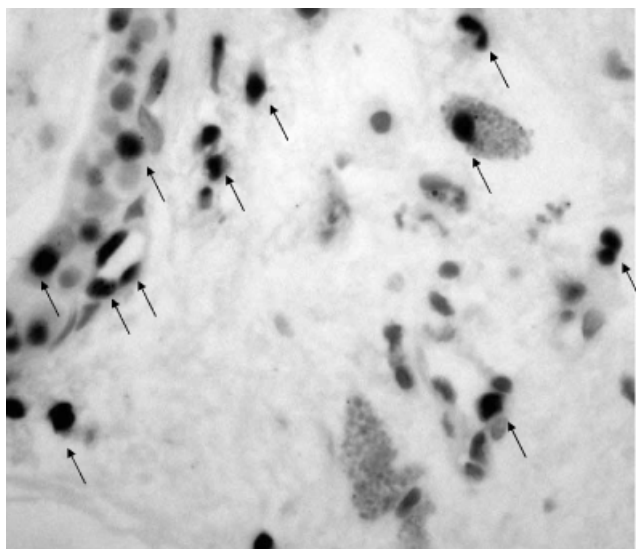


Fig. 4. *In situ* detection of apoptosis in the middle ear mucosa. Many apoptotic cells with a dark-stained nucleus (arrows) were observed in the submucosa and the epithelium of the middle ear mucosa after the instillation of triamcinolone acetonide into the middle ear cavity ($\times 400$).

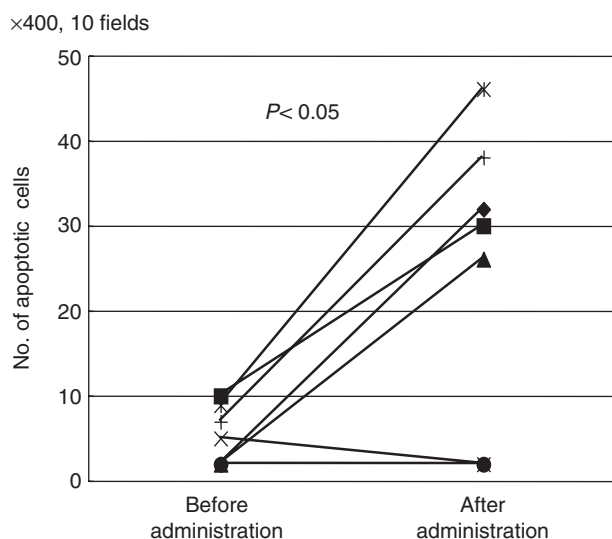


Fig. 5. Numbers of apoptotic cells before and after the instillation of triamcinolone acetonide into the middle ear cavity in 10 patients with eosinophilic otitis media. The number of apoptotic cells significantly increased after the instillation.

eotaxin and RANTES are significantly higher than those in the normal turbinate mucosa [13–16]. It was suggested that these cytokines and chemokines are also responsible for eosinophilia in the nasal tissues. Therefore, the present study was conducted to clarify whether such eosinophil chemoattractants contribute to eosinophilia in the middle ear.

The role of IL-5 in eosinophil accumulation has been well documented. Bronchoalveolar lavage fluid from atopic and non-atopic patients with asthma contains a high IL-5 concentration [17]. In the bronchial mucosa of asthmatic patients, the level of IL-5 mRNA expression correlates well with the severity of eosinophilic infiltration. IL-5 has been highly implicated in the local accumulation and activation of

eosinophils and also in the survival of eosinophils. In addition, Wright et al. [18] reported that in the middle ear mucosa of atopic children with OME, a significant level of IL-5 expression was detected, and they concluded that the middle ear may serve as a target organ for allergic inflammation. In our study, a significantly higher IL-5 concentration was detected in MEE from the EOM patients regardless of the presence of atopy than in that from the control group. In addition, compared with those in the serum, IL-5 concentration in MEE was significantly high and showed a positive correlation with ECP concentration. Nonaka et al. [19] also reported that in MEE from OME patients with asthma, there was a significant correlation between percentage of eosinophils and IL-5 concentration. Therefore, IL-5 is highly likely to be responsible for eosinophilia in the middle ear.

Eotaxin, a C–C chemokine, is a strong chemoattractant of eosinophils, and RANTES is another C–C chemokine that acts as a chemoattractant and an activator of most inflammatory cells except neutrophils. Although the eotaxin concentration was slightly higher in MEE than in serum from the EOM patients, there was no statistically significant difference. Interestingly, the RANTES concentration was significantly higher in serum than in MEE from the EOM patients. The expression of eotaxin and RANTES mRNAs was determined in the middle ear mucosa of the EOM patients, which supported the local production of eotaxin and RANTES. However, there is minimal effect of these chemokines on eosinophilia compared with IL-5.

Ecalectin is a novel chemoattractant of eosinophils. Hirashima et al. [20] first reported that concanavalin A-activated or antigen-activated CD4⁺ T cells from patients infected with *Schistosoma mansoni* produce an apparently novel 30–40 kDa eosinophil chemoattractant with a relatively neutral isoelectric point. Later, Matsumoto et al. [21] purified ecalectin and clarified that it is a 36 kDa protein consisting of 323 amino acids and is not related to any known cytokines or chemokines but is rather a variant of human galectin-9. Recombinant ecalectin exhibited a potent eosinophil chemoattractant activity and attracted eosinophils, *in vitro* and *in vivo*, in a dose-dependent manner, but not neutrophils, lymphocytes or monocytes. The eosinophil chemoattractant activity of ecalectin was similar to that obtained using an optimal dose of RANTES and IL-5, and was approximately one-half that obtained using an optimal dose of eotaxin-1. In our study, ecalectin was immunohistochemically detected in the cytoplasm of fibroblasts, macrophages and endothelial cells. Many ecalectin mRNA⁺ cells were also detected in the submucosa of the middle ear of the EOM patients particularly adjacent to the area where many eosinophils were found, suggesting that ecalectin is expressed at the protein level as well as the mRNA level, contributing to eosinophil accumulation.

It is a very important finding that endothelial cells in the middle ear mucosa express ecalectin and RANTES in EOM. These endothelial cells may be activated presumably by pro-inflammatory cytokines under some conditions, to produce ecalectin, resulting in the recruitment and adhesion of eosinophils in local tissues. Consistent with the hypothesis mentioned above, we clearly demonstrated that the number of EG2⁺ cells correlates positively with that of ecalectin-positive cells, and ecalectin-positive cells are usually located in

the region where eosinophils accumulate. Activated eosinophils can release various cytokines and chemokines, such as IL-3, granulocyte macrophage colony-stimulating factor, IL-5, TGFs and RANTES, which may perpetuate the inflammatory process and enhance fibroblast function. The present study demonstrated that many fibroblasts in the middle ear mucosa can produce eosinophil chemoattractants such as eaelectin, RANTES and eotaxin. These chemokines play a supportive role in autocrine activation and survival of eosinophils.

Most EOM patients showed a patulous eustachian tube rather than tubal stenosis [4]. In a patient with T helper type 2-dominant predisposition such as bronchial asthma, an antigenic material may easily invade via a patulous eustachian tube to stimulate IL-5 production in Th2 cells, resulting in the accumulation of eosinophils and further inducing fibroblasts or endothelial cells to produce eosinophil chemoattractants. The accumulated eosinophils, in turn, stimulate the mucus production of goblet cells or glandular cells [22] around the eustachian tube. Mucus containing activated eosinophils, degranulating eosinophils and cell debris forms extremely viscous yellowish MEE, which can disturb the mucociliary function, damage the middle ear epithelium and cause intractable middle ear inflammation.

At present, the systemic administration or topical application of glucocorticoids is believed to be the most effective treatment for EOM. The present study also demonstrated decreases in the numbers of cells positive for EG2, CD3 and eaelectin, and an increase in the number of apoptotic cells, after the topical application of triamcinolone acetate. These results suggest that a decrease in the number of EG2⁺ cells, that is, an improvement of eosinophilia, is in part because of the induction of the apoptosis of eosinophils, and also the induction of apoptosis of eosinophil chemoattractant-producing cells such as T cells, macrophages, fibroblasts and endothelial cells.

Acknowledgements

The authors thank Prof. Kazuoki Kodera, the chairman of the Department of Otolaryngology, Teikyo University School of Medicine for valuable comments on this manuscript, and Misses Emi Saito and Chie Ishikawa for their technical assistance in the preparation of immunohistological sections.

References

- 1 Tomioka S, Kobayashi T, Takasaka T. Intractable otitis media in patients with bronchial asthma (eosinophilic otitis media). In: Sanna M, ed. *Cholesteatoma and Mastoid Surgery*. Rome: CIC Edizioni Internazionali, 1997; 851–3.
- 2 Morinaka S. Allergic otitis media with gelatinous mucoid fluid containing eosinophils. *Otolaryngol Head Neck Surg* 1996; 114:665–8.
- 3 Derlacki EL. Aural manifestations of allergy. *Ann Otol* 1952; 6:179–88.
- 4 Nagamine H, Iino Y, Kojima C, Miyazawa T, Iida T. Clinical characteristics of so-called eosinophilic otitis media. *Auris Nasus Laryx* 2002; 29:19–28.
- 5 Suzuki H, Matsutani S, Kawase T, Iino Y, Kawauchi H, Gyo K et al. Epidemiologic surveillance of 'eosinophilic otitis media' in Japan. *Otol Jpn* 2004; 14:112–7.
- 6 Iino Y, Nagamine H, Yabe T. Eosinophils are activated in middle ear mucosa and middle ear effusion of patients with intractable otitis media associated with bronchial asthma. *Clin Exp Allergy* 2001; 31:1135–43.
- 7 Louis R, Shute J, Goldring K et al. The effect of processing on inflammatory markers in induced sputum. *Eur Respir J* 1999; 13:660–7.
- 8 Hamid Q, Wharton J, Terenghi G et al. Localization of atrial natriuretic peptide mRNA and immunoreactivity in the rat heart and human atrial appendage. *Proc Natl Acad Sci USA* 1987; 84:6760–4.
- 9 Bousquet J, Chané P, Lacoste JY et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990; 323:1033–9.
- 10 Venge P, Bergstrand H, Hakansson L. Neutrophils and eosinophils. In: Kelley W, Harris Jr E Jr, Ruddy S, Sledge C, eds. *Textbook of Rheumatology*, 4th Edn. Philadelphia: WB Saunders Co., 1992; 269–82.
- 11 Rasp G, Thomas PS, Bujia J. Eosinophil inflammation of the nasal mucosa in allergic and non-allergic rhinitis measured by eosinophil cationic protein levels in native nasal fluid and serum. *Clin Exp Allergy* 1994; 24:1151–6.
- 12 Hurst DS, Venge P. The presence of eosinophil cationic protein in middle ear effusion. *Otolaryngol Head Neck Surg* 1993; 108: 711–22.
- 13 Bachert C, Gavaert P, Holtappels G, Johansson SGO, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. *J Allergy Clin Immunol* 2001; 107:607–14.
- 14 Beck LA, Stellato C, Beall LD et al. Allergens, IgE, mediators, inflammatory mechanisms: detection of the chemokine RANTES and endothelial adhesion molecules in nasal polyps. *J Allergy Clin Immunol* 1996; 98:766–80.
- 15 Hamilos DL, Leung DY, Wood T, Bean DK, Schotman E, Hamid Q. Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF- α . *Am J Respir Cell Mol Biol* 1996; 15:443–50.
- 16 Shin S-H, Park J-Y, Jeon C-H, Choi J-K, Lee S-H. Quantitative analysis of eotaxin and RANTES messenger RNA in nasal polyps: association of tissue and nasal eosinophils. *Laryngoscope* 2000; 110:1353–7.
- 17 Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow JC. Allergic and non-allergic asthmatics have distinct patterns of T cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992; 146: 109–15.
- 18 Wright ED, Hurst DS, Miotto D, Giguere C, Hamid Q. Increased expression of major basic protein (MBP) and interleukin-5 (IL-5) in middle ear biopsy specimens from atopic patients with persistent otitis media with effusion. *Otolaryngol Head Neck Surg* 2000; 123:533–8.
- 19 Nonaka M, Fukumoto A, Ozu C et al. IL-5 and eotaxin levels in middle ear effusion and blood from asthmatics with otitis media with effusion. *Acta Otolaryngol* 2003; 123:383–7.
- 20 Hirashima M, Ueno M, Kamiya K-I, Higuchi S, Matsumoto R. Functional heterogeneity of human eosinophil chemotactic lymphokines. *Lymphokine Cytokine Res* 1991; 10:481–6.
- 21 Matsumoto R, Matsumoto H, Seki M et al. Human eaelectin, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes. *J Biol Chem* 1998; 273: 16976–84.
- 22 Borge PR, Lazarus SC, Tam DC et al. Human eosinophils induce mucin production in airway epithelial cells via epidermal growth factor receptor activation. *J Immunol* 2001; 167:5948–54.