doi: 10.1111/cea.12287

Clinical & Experimental Allergy, **44**, 701–712

ORIGINAL ARTICLE Clinical Mechanisms in Allergic Disease

Local increase in IgE and class switch recombination to IgE in nasal polyps in chronic rhinosinusitis

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Clinical & Experimental Allergy

Summary

Background Chronic rhinosinusitis with nasal polyps is generally characterized by local Th2 inflammation and is categorized into two subtypes in Japan: eosinophilic chronic rhinosinusitis (similar to chronic rhinosinusitis with nasal polyps in western countries) and non-eosinophilic chronic rhinosinusitis (characterized by Th1-dominant inflammation).

Objective To investigate local IgE production and class switch recombination to IgE in these two subtypes of chronic rhinosinusitis with nasal polyps.

Methods The identity of IgE-positive cells was determined using double-immunofluorescent staining for IgE and cell-type-specific molecular markers. To investigate the local class switch recombination to IgE and IgE synthesis in the mucosa, we performed real-time polymerase chain reaction to examine the mRNA expression of Th2 cytokines and class-switch-related molecules, including IL-4, IL-5, IL-13, ε germline gene transcripts, IgE mature transcript, IgG mature transcript, RAG1, RAG2 and activation-induced cytidine deaminase in eosinophilic polyps, non-eosinophilic polyps and controls.

Results The concentrations of total IgE and number of IgE-positive cells were significantly higher in the eosinophilic polyps compared with control and non-eosinophilic polyps. IgE-positive cells were predominantly mast cells in eosinophilic polyps and significantly correlated with the number of FcεR1-positive cells in the subepithelial layer. IL-5 and IL-13 mRNA and ε germline gene transcripts expression levels were significantly higher in eosinophilic polyps compared with control and non-eosinophilic polyps. In contrast, the number of plasma cells and the expression of IgG mature transcripts were increased in non-eosinophilic polyps compared with eosinophilic polyps. RAG2 mRNA was significantly increased in both eosinophilic and non-eosinophilic polyps compared with control mucosa.

Conclusion and Clinical Relevance The current study suggests local class switching to IgE, production of IgE and IgE localization to the surface of mast cells in eosinophilic chronic rhinosinusitis in the Japanese population. The difference in the IgE-related profiles between eosinophilic chronic rhinosinusitis and non-eosinophilic chronic rhinosinusitis suggests heterogeneity in the pathogenesis of chronic rhinosinusitis with nasal polyps.

Keywords class switch, eosinophil, IgE, nasal polyp, sinusitis

Submitted 30 June 2013; revised 09 January 2014; accepted 03 February 2014

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Cite this as: S. Baba, K. Kondo, M. Toma-Hirano, K. Kanaya, K. Suzukawa, M. Ushio, M. Suzukawa, K. Ohta and T. Yamasoba, *Clinical & Experimental Allergy*, 2014 (44) 701–712.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is an inflammatory disease that remains difficult to treat despite advances in medical and surgical therapy. The majority of patients with CRSwNP in the United States and Europe have pronounced infiltration of eosinophils and expression of interleukin-5 (IL-5) in nasal polyps [1]. In contrast, more heterogeneity in CRSwNP has been reported in East Asian countries, such as Japan and China. For example, more than half of CRSwNP cases in Japan do not exhibit eosinophil-dominant inflammation [2, 3]. In Japan, CRSwNP is categorized into two subtypes: eosinophilic chronic rhinosinusitis

(ECRS), which is similar to the CRSwNP in western countries, and non-eosinophilic chronic rhinosinusitis (non-ECRS), which is characterized by Th1-dominant inflammation [2].

Several authors have suggested that, consistent with the increase in IL-5 and other Th2 cytokines, a significant increase in local IgE antibodies can be observed in nasal polyps, independent of allergy skin test results or serum IgE measurements in the patients with CRSwNP [4, 5]. The concentration of total IgE correlated significantly with levels of IL-5, eosinophil cationic protein (ECP) and leukotriene (LT) C4/D4/E4 [6], suggesting an association between increased levels of total IgE and eosinophilic inflammation. Furthermore, anti-IgE treatment is effective in CRSwNP patients with polyclonal IgE formation [7]. These findings suggest that local IgE may contribute to the pathogenesis of CRSwNP.

IgE is characterized by its ε heavy chain. It is produced after heavy-chain switching in B cells from IgM, IgG or IgA to IgE, which proceeds in three stages: germline gene transcription, DNA recombination within the heavy-chain locus producing ε circle transcripts and synthesis of ε-chain mRNA that is translated into protein [8]. The switch to IgE is initiated by the cytokines IL-4 or IL-13, produced principally by Th2 cells, which drive ε germline gene transcription [9, 10]. IL-4 also increases the expression of activation-induced cytidine deaminase (AID) [11], an enzyme required for class switch recombination [12]. The synthesis of IgE by B lymphocytes is under stringent controls, requiring antigens, cytokines and cell–cell contact between B and T lymphocytes. CD40–CD40 ligand contact plus obligatory Th2 cytokine signals are necessary for switching the genetic programme of a B cell from IgM to IgE [13].

The recombinase activating genes (RAG) are essential for editing and revision of the antigen receptors. These processes are initiated by a complex of RAG products, RAG1 and RAG2 [14], which are commonly used as markers for receptor revision. The overall purpose of these processes lies in diversifying the antigen receptor repertoire and in revising autoreactive receptors to prevent autoimmunity. Consequently, these enzymes become promoters of self-tolerance during lymphocyte differentiation [15]. Once T and B cells mature, RAG expression is turned off and the cells are released to the periphery [15]. However, Gevaert et al. [16] very recently provided evidence for local receptor revision in nasal mucosa in CRSwNP.

IgE production, well known to occur in the germinal centres of lymphoid tissue [17], has also been demonstrated within the airway mucosa. Molecular immunopathological comparisons of bronchial biopsies from atopic and non-atopic patients with asthma have demonstrated the following conditions conducive to IgE production in both groups of patients: (i) elevated levels of IL-4 and IL-13, (ii) expression of ε germline gene transcripts (GLTs; εGLTs), (iii) expression of FcεRI mRNA, which is suggestive of IgE synthesis because IgE upregulates its own receptor [8, 18–22]. Furthermore, a similar process has also been demonstrated in the nasal mucosa of patients with allergic rhinitis (AR) [23–26], non-allergic rhinitis [27] and CRSwNP [16]. Allergen binding in the mucosa of some patients with non-allergic rhinitis [27] provides further support to the concept of local IgE production. On the contrary, it is also possible that local IgE production induced by an allergic response might be associated with production of nasal polyps [27].

The aim of this study was to examine the expression and localization of IgE and its receptors in nasal polyps in ECRS and non-ECRS patients using immunohistochemical staining, enzyme-linked immunosorbent assays (ELISA) and real-time polymerase chain reaction (RT-PCR). We also examined the identity of IgE-positive cells in polyps using double immunostaining for IgE and cell-type-specific molecular markers.

We further hypothesized that the nasal mucosa of polyps induces B-cell IgE switching, leading to IgE synthesis in patients with ECRS. To address this issue, we performed real-time PCR to examine the mRNA expression of Th2 cytokines and class-switch-related molecules including IL-4, IL-5, IL-13, εGLTs, IgE mature transcript, IgG mature transcript, RAG1,2 and AID in nasal polyps from ECRS and non-ECRS patients, as well as control mucosa from subjects without chronic sinusitis.

Patients and methods

Patients

The diagnosis of CRSwNP was based on the criteria of the EAACI position paper [28], which defined it as having two or more of the following symptoms: blockage/congestion, discharge, anterior/posterior drip, facial pain/pressure, reduction or loss of smell for at least 3 months and endoscopic signs of nasal polyp(s). Patients with CRSwNP associated with chronic obstructive pulmonary disease, diffuse panbronchiolitis, Churg–Strauss syndrome, congenital mucociliary diseases or cystic fibrosis were excluded from this study. None of the patients included had been treated with systemic corticosteroids or other immune-modulating drugs for at least 1 month prior to surgery, although some patients had received antihistaminic agents and/or macrolide antibiotics.

Patients were classified into two groups: the ECRS group, which was defined as having an eosinophil

count of more than 50 per microscopic field (× 400 magnification) using five fields located in the subepithelial area of polyps [2], and the non-ECRS group, which did not fulfil this criteria. The normal-appearing mucosa of the uncinate processes, which were surgically removed in eight patients without CRS (three with frontal sinus cysts and five with maxillary sinus tumours), served as the non-CRS control group. The study was approved by the ethical committee of The University of Tokyo Hospital (#2656). Informed consent was obtained from each patient before collecting samples.

Sampling of tissue specimens and histological procedures

The nasal polyps and control mucosae were harvested during endoscopic sinus surgery. A part of each sample was fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm-thick, mounted on MAS-coated slides (Matsunami Glass, Osaka, Japan) and used for haematoxylin-eosin staining as well as for immunohistochemistry. The second part was immediately immersed in RNA® (Life Technologies, Carlsbad, CA, USA) later and used for real-time PCR analysis. The rest of the tissue sample was immediately frozen and stored at −80°C until used for ELISA. Unfortunately, we were unable to harvest enough mucosal tissue in some cases and were therefore unable to carry out a number of RT-PCR and ELISA analyses (see Result and Tables 3 and 5).

ELISA for total IgE

The nasal mucosae were homogenized with 10 times as much volume of CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, Tokyo, Japan) with a protease inhibitor cocktail (P8340; Sigma-Aldrich) and benzonase endonuclease (E1014; Sigma-Aldrich). Homogenized samples were centrifuged at 4°C at 15 000 g for 10 min. IgE concentrations in the supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) kit (abcam, Tokyo, Japan) according to the manufacturer's instructions. Absorbance was read at 450 nm on a microplate reader.

Immunohistochemistry

The following primary antibodies were used for evaluation of the localization of IgE, Fcε receptor 1 and Fcε receptor 2 (CD23), as well as the identification of inflammatory cells, in the specimens: anti-IgE (rabbit polyclonal, against ε-Heavy Chain, from Nichirei, Tokyo, Japan); anti-Fcε receptor 1 (mouse monoclonal, clone 9E1, from abcam); anti-CD23 (mouse monoclonal, clone 1B12, from Nichirei); antimast cell tryptase (mouse monoclonal, clone AA1, Thermo Fisher Scientific, Fremont, CA, USA); anti-human plasma cells (mouse monoclonal, clone VS38c, Dako Cytomation Japan, Kyoto, Japan); anti-CD20 (rabbit monoclonal, clone L26, Nichirei); and anti-CD3 (rabbit monoclonal, clone SP7, Nichirei).

For single immunostaining for IgE, Fcε receptor 1, CD23, mast cell tryptase, plasma cells, CD20 and CD3, sections rehydrated through a xylene and ethanol series were immersed in 10 mM citrate buffer solution (pH 6.0, Dako Cytomation Japan) and autoclaved at 121°C for 20 min for the retrieval of antigens. Endogenous peroxidase activity was blocked by treatment with 10% hydrogen peroxide in methanol for 15 min at RT. Next, sections were incubated for 30 min in a blocking solution (PBS, pH 7.4, containing 2% bovine serum albumin (Sigma-Aldrich), 0.1% Triton X-100, and 0.1% sodium azide) at RT to reduce non-specific antibody binding, then incubated with: anti-IgE antibody (1 : 750 in blocking solution) over night at RT, anti-Fcε receptor 1 antibody (1 : 100 in blocking solution) overnight at 4°C, anti-CD23 antibody (ready to use) over night at RT, antimast cell tryptase antibody (1 : 2000 in blocking solution) for 1 h at RT, rabbit anti-CD20 antibody (1 : 100 in blocking solution) overnight at 4°C and rabbit anti-CD3 antibody (1 : 400 in blocking solution) overnight at 4°C. These antigen retrieval and incubation conditions were determined based on the results of preliminary experiments (data not shown). After several washes in PBS (pH 7.4), sections were incubated for 30 min at RT with horseradish peroxidase (HRP) conjugated with anti-mouse or rabbit IgG antibodies (Simplestain MAX-PO, (M) and (R), ready to use; Nichirei) corresponding to the primary antibodies. After more washes with PBS (pH 7.4), immunoreactivity was made visible with diaminobenzidine (DAB; Simplestain DAB, ready to use; Nichirei). After washing with distilled water, the sections were counterstained with haematoxylin, then dehydrated and mounted. To ensure that there was no non-specific staining of primary antibodies, the primary antibodies were omitted from the reaction.

Immunofluorescent double staining

Paraffin sections were rehydrated through a xylene and ethanol series before antigen retrieval treatment. Then, the mixture of primary antibodies for IgE and mast cell tryptase was applied overnight at 4°C. The sections were then incubated with a mixture of fluorescence-labelled secondary antibodies: a goat anti-rabbit Alexa 488 antibody for IgE, and a goat anti-mouse Alexa 594 antibody for mast cell tryptase (all purchased from Molecular Probes, Leiden, The Netherlands). Nuclei were then stained using DAPI (Molecular Probes), and the slides were mounted with

Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

To confirm the absence of non-specific staining by secondary antibodies, some sections were incubated with a blocking solution instead of primary antibodies, followed by incubation with the secondary antibodies. The occurrence of potential cross-reactions between antibodies was checked for both IgE and mast cell tryptase antibodies by making cross combinations of primary and secondary antibodies.

Double immunostaining with anti-IgE – anti-plasma cells and anti-IgE – anti-CD20 (B cells) was also carried out in a similar manner to that for double immunostaining with the anti-IgE and antimast cell tryptase antibodies.

Images were acquired with the Radiance 2100 confocal system mounted on a Carl Zeiss Axioskop 2 microscope (with a 60 × plan apo oil immersion objective) and collected with the LSM5Pascal ver.3.2 software (Carl Zeiss Microscopy Ltd, Jena, Germany).

Cell counting

To determine the degree of eosinophil infiltration in the tissues, two of the authors independently manually counted the number of infiltrated cells in five random fields using H-E-stained sections under light microscopy at high magnification (× 400) in a blinded manner. The number of mast cells, T lymphocytes, B cells, IgE-positive cells and FcεR1-positive cells was counted in the same manner using sections immunostained for mast cell tryptase, CD3, CD20, IgE heavy chain and FcεR1, respectively. Initially, we scanned for inflammatory cells in both the subepithelial and submucosal regions in all sections. However, because inflammatory cells were found primarily in the subepithelial region, we only included the number of inflammatory cells in the subepithelial region of each tissue in the analysis.

Real-time quantitative PCR analysis

The sample tissues were lysed in ISOGEN (Nippon Gene, Tokyo, Japan), and the total RNA was extracted according to the manufacturer's instructions. The mRNA expression was analysed using an Applied Biosystems 7500 Real-Time PCR System (PE Applied Biosystems, Foster City, CA, USA). The primers and probes for human β-actin, IL-4, 5, 13, FcεR1α and CD23 were designed by PE Applied Biosystems, and their mRNA expression analysed using the TaqMan detection system. The mRNA of εGLT, AID, RAG1,2 and the mature transcripts of IgE and IgG, were analysed using the SYBR Green detection system as previously described [16, 23, 29]. Forward and reverse primers are listed in Table 1. Nuclease-free water was substituted for cDNA in negative controls. PCR analyses of RAG1 and RAG2 were performed using specific commercial RT2 qPCR Primer Assays (part No. PPH09892A, PPH13159A, SABiosciences, Hilden, Germany) [16].

For relative quantification, data were analysed by the ΔΔCt method and normalized to the average of housekeeping genes: β-actin (ACTB) in the TaqMan detection system and glyceraldehyde-3-phosphate dehygrogenase (GAPDH) [30] in the SYBR Green detection system. For each sample using the TaqMan detection system, the differences in threshold cycles between the cytokine and β-actin or GAPDH genes (ΔCt sample, ΔCt control) were determined, and a calibrated ΔCt value (ΔΔCt, ΔCt sample – ΔCt control) was calculated. Samples using the SYBR Green detection system were normalized to the average of the housekeeping gene GAPDH. Then, the relative quantitation (RQ) values were calculated using the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

Table 1. Real-time PCR primers

Official symbol	Official full name (other name)	Gene ID	References	Primers (5′→3′)
AID	Activation-induced cytidine deaminase	AB040431	[20]	F:GGACTTTGGTTATCTTCGCAATAAG
				R:GTCGGGCACAGTCGTAGCA
				F:ATAGCCATCATGACCTTCAAAGATT
				R:GCCGAAGCTGTCTGGAGAGA
IgE	IgE mature transcripts (IGHE)	X86359	[20]	F:ACCCTGGTCACCGTCTCCTCAG
		J00222		R:CAGAGTCACGGAGGTGGCATT
IgG	IgG mature transcripts (IGHG1)	X86359	[20]	F:ACCCTGGTCACCGTCTCCTCAG
		J00228		R:GTTCCACGACACCGTCACC
εGLT				
IεF2	Human IgE switch region (S epsilon)	X56797	[31]	F:GGCCACACATCCACAGGC
CεR	Immunoglobulin epsilon chain	X95746		R:GGGGTGAAGTCCCTGGAGC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AF261085	[32]	F:CAAGGGCATCCTGGGCTAC
				R:TTGAAGTCAGAGGAGACCACCTG

F, forward primers; R, reverse primers

Statistical analyses

Statistical analyses were carried out using SPSS statistical software (SPSS, Chicago, IL, USA). All data are expressed as mean ± standard error in each group. When comparisons were made between groups, the Kruskal–Wallis test was used to assess significant inter-group variability. The significance of the difference between groups was determined using the Mann–Whitney *U*-test. A difference was considered significant if $P < 0.05$.

Results

Patient profiles

The ECRS group included 18 male patients (age range 31–76 years, mean age 56.8 years), in which the average eosinophil count in the total white cell count in peripheral blood was 7.6% (range 3.1–23.0%) and the average of the number of eosinophils was 483.1/ mm^3 (range 210.8–1817/mm^3). Eight patients in this group had allergic rhinitis, three had asthma and one had aspirin sensitivity, while nine reported no allergy-related diseases. All twelve of the female ECRS patients identified on the hospital database have used topical or oral steroids within a month prior to surgery and therefore had to be excluded from the present study.

The non-ECRS group included 18 patients (five females and 13 males, age range 40–72 years, mean age 57.6 years), in which the average eosinophil/ total white cell count in peripheral blood was 2.1% (range 0.4–5.2%) and the average number of eosinophils was 131.4/mm^3 (range 40.0–252.0/mm^3). Fifteen of these patients did not have any allergy-related diseases, while the other three had allergic rhinitis. No patients in the non-ECRS group had asthma or aspirin sensitivity.

The non-CRS group included eight patients (three females and five males, age range 30–69 years, mean age 52.6 years), in which the average eosinophil count in the total white cell count in peripheral blood was 2.2% (range 1.0–3.7%) and the average eosinophil number was 126.5/mm^3 (range 50.0–210.9/mm^3). Six of these patients did not have any allergy-related diseases, while two had allergic rhinitis. No patients in the non-CRS group had asthma or aspirin sensitivity.

There was no significant difference in age among three groups, whereas the peripheral blood eosinophil count was significantly greater ($P < 0.001$) in the ECRS group compared with the non-ECRS group and non-CRS control group. The patient profiles are shown in Table 2.

Histological observations of the nasal polyps showed that eosinophils were predominant in the infiltrating cells in the ECRS group (Fig. 1a). On the other hand, most of the infiltrating cells were lymphocytes and

Table 2. Patient profiles

Patient group	Number (*n*)	Male: female	Age (year)*	Asthma	Allergic rhinitis	Eosinophil count in the total white cell (%)*	Eosinophil count in nasal tissues†
ECRS	18	18 : 0	56.8 (31–76)	4/18	8/18	7.6 (3.1–23.0)	127.6 (53.2–385.2)
Non-ECRS	18	13 : 5	57.6 (40–72)	0/18	3/18	2.1 (0.4–5.2)	3.3 (0–47.8)
Non-CRS	8	5 : 3	52.6 (30–69)	0/8	2/8	2.2 (1.0–3.79)	0.0 (0–3.8)

*Data are expressed as means and ranges.
†Data are expressed as medians and ranges.

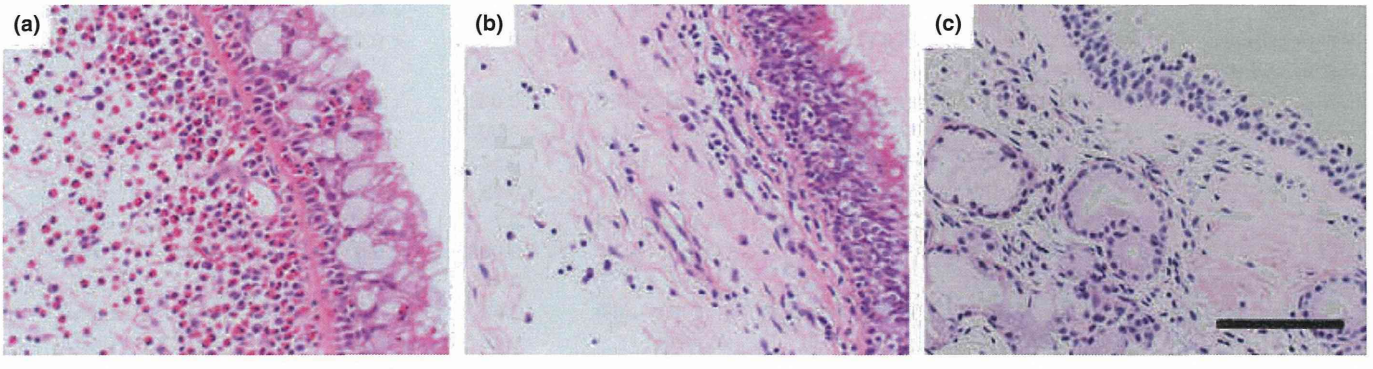

Fig. 1. Representative H-E-stained histological sections of nasal polyps obtained from ECRS (a), non-ECRS (b) and non-CRS (c) groups. In the ECRS group, almost all of the infiltrating cells are eosinophils (a), whereas most of the infiltrating cells are lymphocytes and plasma cells in the non-ECRS group (b). Few inflammatory cells are infiltrating in non-CRS group (c). Scale bar = 100 μm.

plasma cells in the non-ECRS group (Fig. 1b) and few inflammatory cells infiltrated in non-CRS group (Fig. 1c).

Total IgE protein concentrations

IgE concentrations were determined by ELISA. Analysis of total IgE in supernatants prepared from tissue homogenates demonstrated that the concentration was significantly higher in the ECRS polyps (n = 7, median 89.1; range 0.0–269.3) compared with the non-CRS controls (n = 6, median 0.0; range 0.0–0.0; $P < 0.05$) and non-ECRS polyps (n = 8, median 0.0; range 0.0–16.7; $P < 0.05$; Table 3).

Immunohistochemical analysis of inflammatory cells in nasal tissues

The number of eosinophils (H-E) and cells positive for mast cell tryptase (mast cells), VS38c (plasma cells), CD20 (B cells) and CD3 (T cells) were counted using immunohistochemical staining in ERCS polyps, non-ERCS polyps and non-CRS controls. Typical photomicrographs of immunohistochemically stained nasal tissue are shown in Fig. 2. As shown in Table 3, the median and interquartile range counts for eosinophils were significantly higher in ECRS polyps (n = 18, median 127.6; range 53.2–385.2) compared with non-CRS controls (n = 8, median 0.0; range 0.0–3.8; $P < 0.001$) and non-ECRS polyps (n = 18, median 3.3; range 0.0–47.8; $P < 0.001$; Table 3). The median counts for plasma cells and B cells were significantly higher in non-ECRS polyps compared with non-CRS controls and ECRS polyps (Table 3). No significant differences were observed in the median counts for mast cells or T cells among the groups ($P > 0.05$; Table 3).

Immunohistochemical localization of IgE, FcεR1 and CD23

Representative microphotographs of ECRS polyps immunostained for IgE are shown in Fig. 2. IgE immunoreactivity was localized in inflammatory cells in the subepithelial layer of ERCS and non-ECRS polyps (Fig. 2d). The median counts for IgE-positive cells in the subepithelial layer were significantly higher in ECRS polyps (n = 16, median 14.8; range 4.4–28.8) compared with the non-CRS controls (n = 7, median 0.0; range 0.0–3.8; $P < 0.001$) and non-ECRS polyps (n = 18, median 3.8; range 0.0–20.0; $P < 0.001$; Table 3).

FcεR1 was also expressed in inflammatory cells in the subepithelial layer of ERCS and non-ECRS polyps

Table 3. Median (IQR) of protein concentrations, number of inflammatory cells and cytokine and transcript expression in normalized relative quantities (NRQ) in the tissue of ECRS and non-ECRS nasal polyps and normal controls

Nasal tissue analysis	Eosinophilic CRS polyps median (IQR)	Non-eosinophilic CRS polyps median (IQR)	Non-CRS controls median (IQR)	Multiple comparison *P*-value	ECRS vs. NC *P*-value	ECRS vs. non-ECRS *P*-value	Non-ECRS vs. NC *P*-value
Protein concentrations							
IgE (IU/mL)	89.1 (0–153.4)	0 (0–0)	0 (0–0)	**0.028**	**0.036**	**0.046**	0.386
The number of inflammatory cells							
Eosinophils	127.6 (64.4–181.9)	3.3 (0.7–7.9)	0 (0–0.05)	**< 0.001**	**< 0.001**	**< 0.001**	**0.005**
Plasma cells	21.6 (15.2–26.0)	33.1 (20.2–51.2)	6.0 (4.1–6.2)	**< 0.001**	**< 0.001**	**0.038**	**< 0.001**
Mast cells	10.2 (9.2–15.0)	12.6 (11.0–19.5)	11.2 (7.5–13.7)	0.18	0.97	0.08	0.23
T cells (CD3)	45.6 (29.0–93.8)	75.0 (44.4–151.6)	50.4 (33.4–57.0)	0.19	0.75	0.13	0.14
B cells (CD20)	54.0 (45.5–71.5)	94.6 (65.0–173.0)	3.3 (2.5–5.6)	**< 0.001**	**< 0.001**	**0.02**	**< 0.001**
IgE+ cells	14.8 (11.6–21.8)	3.8 (1.3–5.4)	0 (0–0.19)	**< 0.001**	**< 0.001**	**< 0.001**	**0.004**
FcεR1+ cells	7.0 (5.3–10.2)	5.8 (3.0–8.8)	1.0 (0.7–4.4)	**< 0.001**	**< 0.001**	0.21	**0.002**
Cytokine and transcript expression							
IL-5	68.1 (4.9–207.6)	0.11 (0–1.1)	0.2 (0.02–0.4)	**< 0.001**	**< 0.001**	**< 0.001**	0.65
IL-13	11.4 (2.6–19.4)	0.3 (0.002–5.0)	0.2 (0–4.6)	**0.002**	**0.025**	**0.002**	0.96
IL-4	0.43 (0.19–1.1)	0.3 (0.13–0.95)	0.78 (0.22–1.6)	0.62	0.83	0.42	0.42
FcεR1	0.78 (0.09–1.3)	0.3 (0.2–0.5)	0.6 (0.4–0.9)	0.59	0.88	0.51	0.22
CD23	0.19 (0.07–1.2)	0.1 (0.05–0.19)	0.4 (0.02–0.9)	0.7	0.95	0.43	0.53
εGLT	0.17 (0–1.4)	0 (0–0)	0 (0–0)	**0.0055**	**0.017**	**0.013**	0.46
IgE mature transcript	0.22 (0.013–0.77)	0.055 (0–0.57)	0 (0–0.22)	0.54	0.28	0.54	0.57
IgG mature transcript	6.1 (2.0–29.7)	103.0 (13.8–239.4)	12.6 (6.0–14.8)	**0.03**	0.71	**0.015**	0.06
AID	0 (0–0.75)	0 (0–1.7)	0 (0–0)	0.8	0.55	0.86	0.55
RAG1	1.28 (0.33–3.12)	2.3 (0.97–10.9)	2.0 (0.9–3.5)	0.59	0.77	0.33	0.51
RAG2	0.83 (0.50–1.62)	1.35 (0.90–3.3)	0.28 (0.19–0.86)	**0.049**	**0.039**	**0.36**	**0.031**

$P < 0.05$ were considered to indicate statistical significance (bold values).

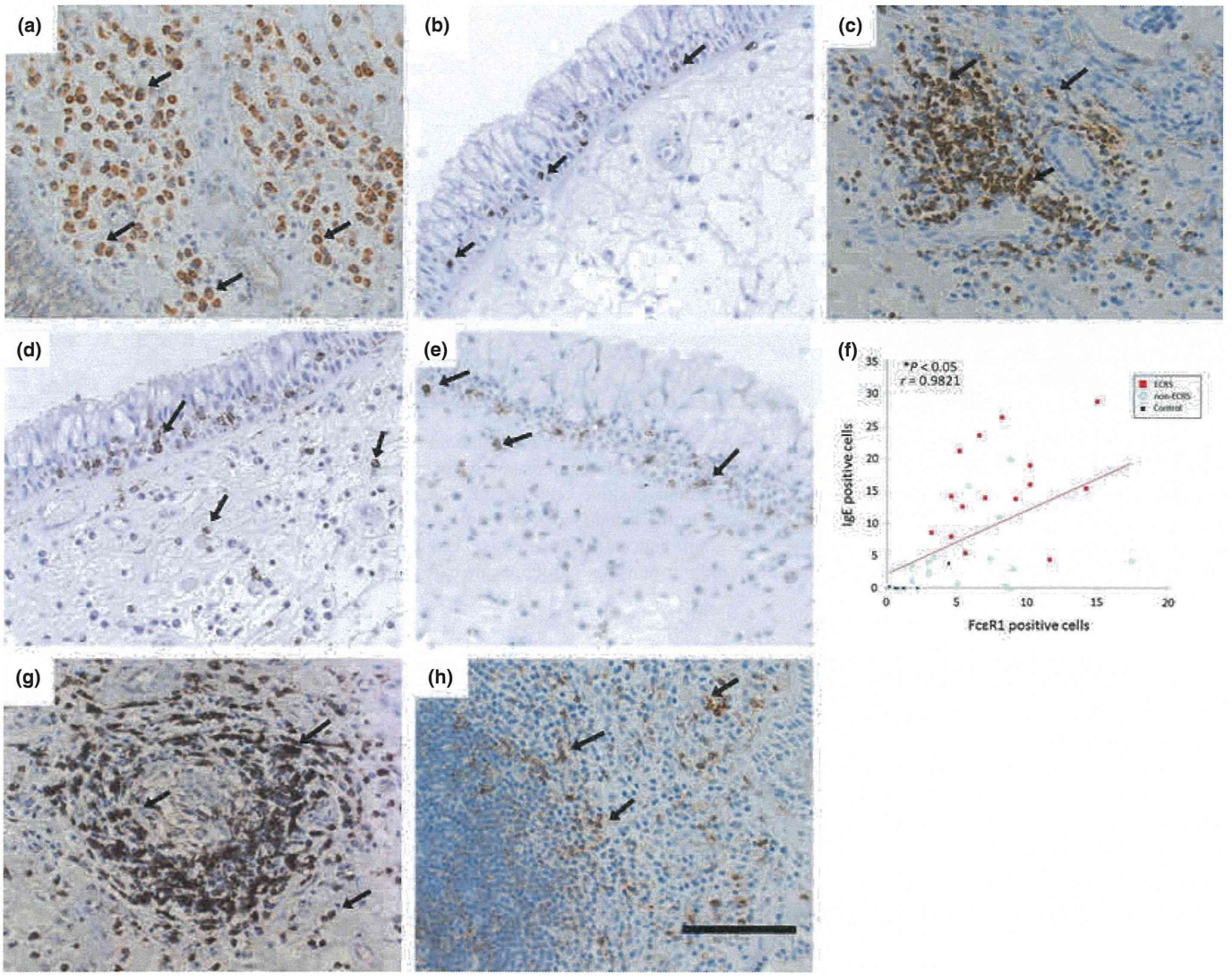

Fig. 2. Photomicrographs showing immunohistochemical staining for VS38c (plasma cells, arrows; a), mast cell tryptase (mast cells, arrows; b), CD3 (T cells, arrows; c), IgE (arrows; d), FcεR1 (arrows; e), CD20 (B cells, arrows; g) and CD23 (arrows; h). Scale bar = 100 μm. (f) Correlation between the number of FcεR1-positive cells and the number of IgE-positive cells in the ECRS group (red squares), non-ECRS group (green diamonds) and non-CRS group (black squares).

(Fig. 2e). The median counts for FcεR1-positive cells in the subepithelial layer were significantly higher in ECRS polyps (n = 15, median 7.0; range 3.2–15.0) compared with non-CRS controls (n = 7, median 1.0; range 0.2–4.4; $P < 0.001$), and the counts of positive cells in non-ECRS groups (n = 17, median 5.8; range 1.0–17.4; $P < 0.005$) were also significantly higher than those of non-CRS controls (Table 3). The number of FcεR1-positive cells inside the subepithelial layer had a significant correlation with the number of IgE-positive cells in both ECRS and non-ECRS polyps (n = 39, r = 0.9821, $P < 0.005$; Fig. 2f).

CD23 was expressed in cells around the capillary vessels, but in low numbers in both ECRS and non-ECRS polyps (Fig. 2h).

Identity of IgE-positive cells

The identity of IgE-positive cells in the subepithelial layer was examined using double-immunofluorescent staining (Fig. 3). IgE in mast cells (mast cell tryptase positive) was localized to the membranous rim, suggesting IgE receptor expression (IgE-bound) at the cell surface (Fig. 3a). The fraction of double-positive cells for IgE and mast cell tryptase in mast-cell-tryptase-positive cells ranged from 39.7% to 72.0% (median 56.8%, n = 7) in ECRS polyps, whereas the fraction of double-positive cells in non-ECRS polyps was 0–45.0% (median 9.1%, n = 6; Table 4). The fraction of double-positive cells in mast-cell-tryptase-positive cells was significantly higher in the ECRS group compared with the

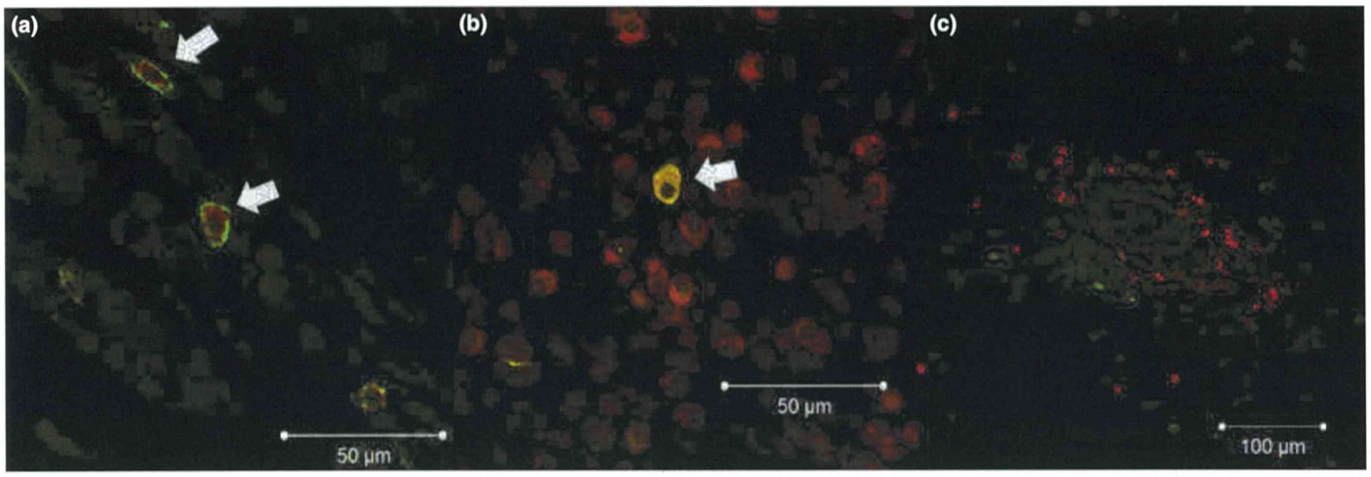

Fig. 3. Double-immunohistochemical staining for IgE-mast cell tryptase (a), IgE-VS38c (plasma cells; b) and IgE-CD20 (B cells; c) in nasal polyps from ECRS patients. Immunoreactivity for IgE is visualized in green and immunoreactivity for mast cell tryptase, and VS38c is visualized in red. Double-positive cells are coloured yellow. (a) A number of double-positive cells for IgE and mast cell tryptase were observed in the mucosa (arrows). IgE immunoreactivity in the mast cells was localized to the membranous rim. Scale bar = 50 μm. (b) A few of the VS38c-positive cells (plasma cells) were also positive for IgE in both the ECRS group and non-ECRS groups. Scale bar = 50 μm. (c) No CD20-positive cells (B cells) were positive for IgE immunoreactivity in either the ECRS group or non-ECRS group. Scale bar = 100 μm.

Table 4. Median percentages (IQR) of mast cells, plasma cells and CD20+ B lymphocytes expressing IgE (*$P < 0.005$)

Nasal tissue analysis	Eosinophilic CRS polyps median (IQR)	Non-eosinophilic CRS polyps median (IQR)	ECRS vs. Non-ECRS *P*-value
% IgE-expressing cells			
Mast cells	56.8 (51.7–65.7)	9.1 (5.9–25.2)	**0.0043**
Plasma cells	8.2 (7.6–11.3)	2.5 (0.7–3.1)	**0.0039**
CD20	0 (0–0)	0 (0–0)	–
Total count (cells / field)			
IgE-expressing plasma cells	2.1 (1.5–2.35)	0.4 (0.233–0.9)	**0.00042**

*$P < 0.05$ were considered to indicate statistical significance (bold values).

non-ECRS group ($P < 0.005$). The fraction of double-positive cells for IgE and mast cell tryptase in IgE-positive cells ranged from 54.0% to 95.0% (median 75.9%, $n = 7$) in ECRS polyps, whereas the fraction of double-positive cells in non-ECRS polyps was 57.5–100% (median 97.8%, $n = 6$). The fraction of double-positive cells in IgE-positive cells was not significantly different between the groups ($P = 0.12$).

IgE in plasma cells was localized in the cytoplasm, suggesting IgE production in the cells (Fig. 3b). The fraction of double-positive cells for IgE and plasma cell in plasma cells ranged from 5.6% to 16.4% (median 8.3%, $n = 10$) in ECRS polyps, whereas the fraction of double-positive cells in non-ECRS polyps was 0–4.4% (median 2.5%, $n = 10$; Table 4). The fraction and median number of double-positive cells in plasma cells were significantly higher in the ECRS group compared with the non-ECRS group ($P < 0.005$).

There were no double-positive cells for CD20 and IgE in either ECRS polyps or non-ECRS polyps (Fig. 3c, Table 4).

Double immunostaining with anti-IgE and antieosinophil major basic protein (MBP) antibodies was carried out in a similar manner to double immunostaining with the anti-IgE and antimast cell tryptase antibodies. However, it did not go well because the incubation of sections in proteinase for the retrieval of MBP antigens disturbed the staining for IgE. We observed IgE-positive cells that were morphologically recognizable as eosinophils, but there were very few such cells even in the area of massive eosinophil infiltration in ECRS polyps.

Real-time quantitative PCR analysis for cytokines and IgE receptors

Differences in the expression of IgE-related Th2 cytokine and IgE receptors were assessed at the mRNA level in ECRS, non-ECRS and non-CRS controls (Table 3). In the ECRS group ($n = 14$), we found a significantly higher mRNA expression of IL-5 compared with non-CRS controls ($n = 6$, $P < 0.001$) and the non-ECRS

group ($n = 13$, $P < 0.001$). Also, the mRNA expression of IL-13 was significantly higher in the ECRS group ($n = 17$) compared with non-CRS controls ($n = 8$, $P < 0.005$) and the non-ECRS group ($n = 18$, $P < 0.05$; Table 3). No significant difference was observed in mRNA expression of IL-4 among controls ($n = 8$), ECRS ($n = 17$) and non-ECRS ($n = 18$) subjects (Table 3). Also, the mRNA expression of FcεR1α and CD23 was not significantly different among groups (Table 3).

εGLT expression in the nasal mucosa

The expression of εGLT marks the first step in the commitment of B cells to the synthesis of IgE. We analysed mRNA expression of εGLT in samples from 14 patients in the ECRS group, 13 patients in the non-ECRS group and seven patients in the non-CRS group. As shown in Tables 3 and 5, εGLT expression was detected in eight of 14 ECRS samples and one of 13 non-ECRS samples. In contrast, no εGLT mRNA expression was detected in any samples from the non-CRS subjects. We also found a significantly higher mRNA expression of εGLT in ECRS polyps compared with non-CRS controls ($P < 0.05$) and non-ECRS polyps ($P < 0.05$; Table 3).

IgE and IgG mature transcript mRNA expression in the nasal mucosa

IgE synthesis by B cells is the third and final step in the production of IgE. We detected IgE mature transcript mRNA in samples from 10 of 14 ECRS patients, six of 13 non-ECRS patients and three of seven non-CRS controls (Table 5). The mRNA expression level of IgE mature transcript was not significantly different among the groups (Table 3) although IgG total mRNA levels were significantly increased in the non-ECRS group compared with the ECRS group ($P < 0.05$; Table 3).

Table 5. Distribution of εGLT, IgE mature transcript mRNA and AID mRNA in the bronchial mucosa

	Patient no.														
Markers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
ECRS															
εGLT	•	•						•	•	•	•	•		•	8/14
IgE	•	•	•				•		•	•	•	•	•	•	10/14
AID					•				•	•	•				4/14
non-ECRS															
εGLT		•													1/13
IgE	•	•				•		•		•			•		6/13
AID		•	•					•							3/13
non-CRS															
εGLT															0/7
IgE		•			•	•									3/7
AID				•											1/7

AID, RAG1 and RAG2 mRNA expression in the nasal mucosa

We detected AID mRNA in samples from four of 14 ECRS patients, three of 13 non-ECRS patients and one of seven non-CRS patients, as shown in Tables 3 and 5. The mRNA expression of AID was not significantly different among the groups.

RAG2 mRNA was also significantly increased in ECRS and non-ECRS polyps compared with non-CRS controls, while the amount of RAG1 mRNA was not different among the groups (Table 3).

Discussion

The current study demonstrated higher IgE protein concentration and significant upregulation of IL-5 mRNA in ECRS polyps compared with non-ERCS polyps and non-CRS controls in the Japanese population. Mature IgE transcript mRNA was detected more frequently in ECRS polyps compared with non-ECRS polyps and non-CRS controls. Furthermore, mRNA expressions of IL-13 and εGLT were significantly higher in ECRS polyps compared with non-ECRS polyps and non-CRS controls. These data suggest that in the nasal polyps in ECRS, B cells undergo class switch recombination to IgE *in situ* and that an ongoing, elevated synthesis of mature IgE occurs.

Accumulating evidence suggests that CRSwNP is at least partly linked to local IgE production. Bachert et al. [6] reported that the concentration of total IgE is significantly elevated in nasal polyps compared with levels in serum or non-polyp mucosa and that IgE concentration is significantly correlated with IL-5, ECP and LTC4/D4/E4, suggesting an association between increased levels of total IgE and eosinophilic inflammation [6]. Bachert et al. [6] also reported that in marked contrast to allergic rhinitis, specific IgE in the nasal polyps of patients with CRSwNP is unrelated to skin prick test positivity, which supports the concept of local IgE production. Sejima et al. [31] and Shi J et al. [32] also reported that total IgE concentration was significantly higher in the ethmoid mucosa of patients with CRSwNP than that of CRS without nasal polyps (CRSsNP) and non-CRS control in Japanese and Chinese populations. Sheahan et al. [33] reported that, although the total IgE levels did not differ in sinus or turbinate tissue between CRSwNP, CRSsNP or non-CRS controls, sinus tissue in CRSwNP patients had higher levels of IgE specific to multiple common inhalant allergens compared with other groups regardless of the evidence of systemic atopy. Very recently, Gevaert et al. [16] reported that local class switch recombination to IgE was increased in CRSwNP in the European population, evidenced by the enhanced expression of key markers of the process, including IL-4, εGLT, ε-mRNA and IgE protein.

The results of the current study are basically in line with the above-mentioned findings, showing a higher IgE concentration in the sinus mucosa of CRSwNP patients. To the best of our knowledge, this report is the first regarding class switch recombination to IgE in nasal polyps in an Asian population. Our data further suggest that the concentration of IgE in the nasal polyps of Japanese patients is heterogeneous. The IgE concentration appears to be correlated with the magnitude of eosinophil infiltration in polyps: IgE concentration is elevated in the polyps of ECRS patients, but not in non-ECRS patients. In contrast to the ECRS polyps, we found an increased number of plasma cells and increased expression of IgG mature transcripts in non-ECRS polyps. These data suggest a difference in pathogenesis of ERCS and non-ERCS: ECRS is characterized by local increase in IgE, which is similar to the nasal polyposis in Western patients, while non-ECRS is characterized by an increase in IgG locally.

We found that although the number of IgE-positive plasma cells was small in both ECRS polyps and non-ECRS polyps, it was significantly higher in the ECRS group compared with the non-ECRS group. Furthermore, there were no double-positive cells for CD20 (B cells) and IgE in either ECRS polyps or non-ECRS polyps. Therefore, it would be reasonable to conclude that the source of IgE synthesis in the ECRS polyp tissues is plasma cells.

Some of the data in our results appear to be discrepant and therefore require some explanation. First, our data revealed that although the number of IgE-positive cells is higher in ECRS than non-ECRS polyps and that most of the IgE-positive cells were mast cells, which usually express FcεRI, there was no difference in the number of FcεRI-positive cells between the groups. This suggests that some FcεRI-positive cells did not bind detectable amounts of IgE to their surface. This discrepancy could be interpreted if cells from ECRS polyps express a higher level of FcεRI, possibly by positive feedback due to the higher IgE concentration. Therefore, a larger amount of IgE could bind to each individual FcεRI-positive cell, leading to easier IgE immunohistochemical detection in the ECRS group.

Second, although we reported a higher number of IgE-positive cells and larger amount of IgE protein, we did not detect higher mRNA expression of IgE mature transcript in ECRS polyps. We speculate that mRNA translation of IgE and IgE protein synthesis are increased in ECRS polyps, but IgE mRNA is at the same level in ECRS and non-ECRS groups as well as non-CRS controls.

Third, we detected IgE mature transcript mRNA in samples from all groups: from 10 of 14 ECRS patients, seven of 14 non-ECRS patients and three of seven non-CRS controls (Table 5). The mRNA expression level of IgE mature transcript was not significantly different among the groups (Table 3). The underlying mechanism for this finding is unclear, but it may be that a small amount of IgE, which is under the detectable level in ELISA, is produced even in the non-ECRS polyps and non-CRS mucosa.

Several recent studies have demonstrated that the concentration of IgG, in addition to IgE, is increased in nasal polyps [34–36]. However, the association of IgG concentration with eosinophilic inflammation has not been investigated. We demonstrated an increased expression of IgG mature transcripts in non-ECRS polyps. Increased IgG concentration in tissues is often observed in other Th1-dominant diseases, such as rheumatoid arthritis [37]and sclerosing sialadenitis [38], and our findings appear to agree with this idea.

Local receptor revision is suggested by the upregulation of RAG1 and RAG2 in B cells, plasma cells and T cells [14]. In our experiments, mRNA expression of RAG2 was significantly higher in ECRS and non-ECRS polyps compared with non-CRS controls, suggesting that local receptor revision occurs within the polyps. It is speculated that increased RAG may be associated with a class switch to IgE in ECRS polyps and associated with the class switch revision to IgG in non-ECRS polyps.

No direct evidence has indicated how the increased IgE could perpetuate eosinophilic inflammation in nasal polyps. We showed that the number of IgE-positive cells in ECRS polyps increased significantly compared with non-ECRS polyps and non-CRS controls and that the localization of IgE expression in inflammatory cells was predominantly confined to mast cells in ECRS polyps. Consistent with the latter finding, the number of FcεR1-positive cells in the subepithelial layer had a significant correlation with the number of IgE-positive cells. These data indicate that IgE formed in the polyp may contribute to the development of chronic inflammation by IgE-combined mast cell degranulation in patients with ECRS, as has been hypothesized previously [6]. The role of IgE and mast cells needs to be further addressed in future studies.

It is important to recognize the limitations of our study. In spite of repeated trials with various experimental conditions, we failed to detect Iε-Cμ and Iε-Cγ Circular Transcript, which would have provided the most direct evidence for local class switch recombination and have been previously detected in CRSwNP [16]. Furthermore, we were not able to obtain reliable results in the immunohistochemistry of RAG1 and 2 proteins. We need to address these issues again in future analyses.

Conclusion

The current study suggests local class switching to IgE, local production of IgE and IgE localization to the surface of mast cells in the nasal polyps of ECRS patients. In contrast, in non-ECRS polyps, local IgG mature transcripts production was increased. This heterogeneity of immunological profiles in Japanese CRS patients suggests that the pathogenesis of nasal polyps in the Japanese population is not identical to that of nasal polyposis in western patients. IgE formed in the polyp may contribute to the development of chronic inflammation by IgE-combined mast cell degranulation in patients with ECRS.

Acknowledgements

We thank Sayaka Igarashi, Yukari Kurasawa, Atsuko Tsuyuzaki and Kimiko Miwa for technical assistance. We also thank Professor Shigeharu Fujieda for many valuable comments of this work. This work was supported by Grants-in-Aid for Young Scientists (B), Health Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan, Environmental Restoration and Conservation Agency of Japan and Japan Society for the Promotion of Science.

Conflict of interest

The authors declare no conflict of interest.

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Tumour necrosis factor inhibitor-associated sinusitis*

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Rhinology 52: 246-251, 2014
DOI:10.4193/Rhino13.074

***Received for publication:**
May 26, 2013
Accepted: January 12, 2014

Abstract

Aim: To describe the features of chronic sinusitis associated with the use of tumour necrosis factor (TNF) inhibitors.

Methodology: A retrospective review of the medical records between 2003 and 2011 revealed that five patients had developed chronic sinusitis after the start of TNF inhibitor administration and required rhinological evaluation and treatment.

Results: The incidence of refractory sinusitis associated with TNF inhibitors was approximately 2%. Of the five patients identified, four patients were medicated with etanercept and one with infliximab. The maxillary sinus was most commonly involved and cultures of the sinus discharge revealed *Pseudomonas aeruginosa* in three cases. Two patients showed improvement of sinusitis with antibiotic medication, despite the continuous use of TNF inhibitor, while in two other patients, sinusitis was resistant to antibiotic medication. Another patient who had developed recurrence of sinusitis after complete remission of previous chronic sinusitis by endoscopic sinus surgery showed remission only after cessation of TNF inhibitor.

Conclusion: Chronic sinusitis associated with TNF inhibitors is considered to be a new disease entity, and it will become more common due to the increasing use of TNF inhibitors.

Key words: TNF inhibitor, sinusitis, etanercept, infliximab

Introduction

Tumour necrosis factor (TNF) inhibitors are recently developed biological drug products for chronic inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease and psoriasis. Because TNF inhibitors have been proven to be clinically effective, they have been used in a rapidly increasing number of patients in recent years, and are recommended for the treatment of medication-resistant RA in the guidelines for RA management [1,2].

On the other hand, as TNF-α, the target of these drugs, plays an important role in the immune system [3,4], the use of TNF inhibitors potentially induces increased susceptibility to infection, particularly in the airway, where the TNF-α mediated immune response is important in mucosal host defense against constantly invading pathogens. TNF-α is required for induction of inflammatory cytokines and subsequent leukocyte recruitment, and to increase the potential for phagocytosis by macrophages to prevent the progress of bacterial infection. When TNF-α knockout mice are infected by aerosol challenge with *Mycobacterium tuberculosis*, bacterial growth is markedly elevated and mice show poorer survival compared to infected wild-type mice [5]. Previous clinical studies have revealed that TNF inhibitors sometimes cause serious infective adverse events, such as tuberculosis and pneumocystis pneumonia [4,6-8]. With regard to the upper respiratory tract, sinusitis is reported to be one such complication. Clinical trials have shown an increase in sinusitis in patients with RA treated with TNF inhibitors [9], and a longitudinal study of rheumatic disease outcome revealed that etanercept, a TNF inhibitor, significantly increases the risk of sinus problems [10]. However, there have been few reports [11] focusing on sinusitis associated with the use of TNF inhibitors.

In the present study, we report the clinical features of chronic

Table 1. Clinical features of patients.

Patient No.	Sex	Underlying disease (Age*)	TNF inhibitors (Age**)	Other medications before and at TNF inhibitor onset	Other adverse events
1	F	RA (53)	Etanercept 50 mg/week (56)	MTX (2~6 mg/week)	None
2	F	RA (30)	Etanercept 50 mg/week (50) and up to 100 mg/week (52)	PSL (5~30 mg/day) and MTX (4~10 mg/week)	Pneumonia and OME
3	F	RA (54)	Infliximab 3.7 mg/kg/2 months (57)	PSL (2.5~5 mg/day) and MTX (10~14 mg/week)	Pneumonia and cellulitis
4	F	RA (45)	Etanercept 50 mg/week (50) (short-term infliximab and adalimumab)	PSL (5~15 mg/day), MTX (8~13 mg/week) abatacept for short time	Septic shock due to pneumonia and OME
5	F	RA (50)	Etanercept 50 mg/week (73)	salazosulfapyridine (2000 mg/day)	None

Age* = age at diagnosis; Age** = age when TNF inhibitors were started; F = female; RA = rheumatoid arthritis; PSL = prednisolone; MTX = methotrexate; OME = otitis media with effusion

Table 2. Clinical features of sinusitis.

No.	Interval	Sinuses involved	History of sinusitis	Bronchictasis as a comorbidity	Treatment for sinusitis	Culture of sinus discharge	Outcome
1	3 months	Bilateral maxillary sinuses	(-)	(-)	Roxithromycin for 4 months and nasal irrigation	Normal flora	Improvement
2	2 years (1 month after increase of etanercept)	Bilateral maxillary and frontal sinuses	(+)	(-)	Levofloxacin, garenoxacin, cefditoren-pivoxil and nasal irrigation	*Pseudomonas aeruginosa* (not MDR), Methicillin -resistant *Staphylococcus aureus*	Remission after cease of etanercept
3	1 month	Bilateral maxillary sinuses	(-)	(+)	Cefcapene -pivoxil, clarithromycin and nasal irrigation	*Pseudomonas aeruginosa* (not MDR)	Refractory
4	1 month	Bilateral maxillary sinuses	(-)	(+)	Ampicillin, clarithromycin and nasal irrigation	*Pseudomonas aeruginosa* (not MDR)	Refractory
5	3 months	Right maxillary sinus	(-)	(-)	Clarithromycin for 2 months, Intranasal steroid spray and nasal irrigation	Data not available	Improvement

Interval = Interval between the start of TNF inhibitors and onset of sinus symptoms; MDR = multidrug-resistant

sinusitis in five patients that occurred after the start of TNF inhibitor administration.

Materials and methods

After approval by the Research Ethics Committee of The University of Tokyo Hospital (protocol #2487), we retrospectively reviewed the medical records at the Department of Allergy and Rheumatology and at the Department of Otorhinolaryngology, between 2003 and 2011. This period was selected because TNF inhibitors have been used for RA in Japan since July 2003. During this period, a total of 188 patients, including 154 suffering from RA, underwent treatment with TNF inhibitors at

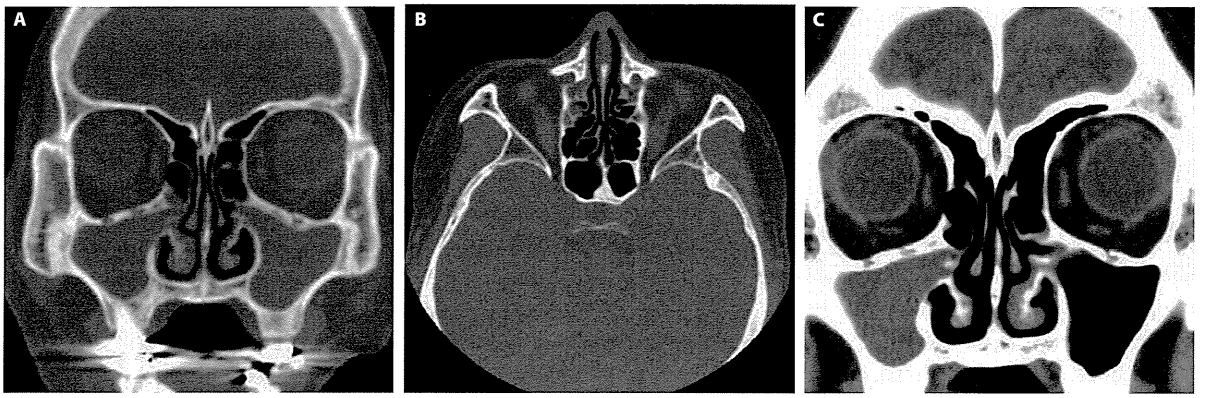

Figure 1. Computed tomography (CT) images of the paranasal sinuses in patient 1 before (A, B) and four months after (C) treatment of sinusitis. A. Coronal sinus CT before treatment showing total bilateral maxillary sinus opacification. B. Axial sinus CT before treatment showing no involvement of ethmoid and sphenoid sinuses. C. Coronal sinus CT four months after treatment with roxithromycin (150 mg/day) showing complete remission of left maxillary sinusitis and partial improvement of aeration in right maxillary sinus.

the Department of Allergy and Rheumatology. Four of these patients (2.1%) developed chronic sinusitis after the use of TNF inhibitor and were referred to the Department of Otorhinolaryngology for rhinological evaluation and treatment. The records at the Department of Otorhinolaryngology revealed one additional patient who developed chronic sinusitis after the use of a TNF inhibitor, and who was referred from another hospital for treatment at our clinic. These five patients were included in the present detailed analysis.

Of the five patients, four patients had been treated with etanercept, a soluble TNF-α receptor. The other had been treated with infliximab, which is a chimeric anti-TNF-α monoclonal antibody. All patients underwent head and neck examination, including nasal endoscopy and radiological evaluation by sinus computed tomography (CT). Diagnosis of chronic sinusitis was performed based on the definition reported in the European position paper for rhinosinusitis [12], i.e., two or more of the following symptoms for 12 weeks or longer: mucopurulent drainage, nasal obstruction, facial pain-pressure-fullness, or sense of smell; and findings of purulent mucus or oedema in the middle meatus or ethmoid region, polyps in the nasal cavity or the middle meatus, and/or radiographical imaging showing shadow of the paranasal sinuses. The following data were collected from the medical records: age; sex; diagnosis; type of TNF inhibitor used; other medications; interval between start of TNF inhibitor and onset of sinus symptoms; physical examination findings; endoscopic findings; sinus computed tomography (CT) findings; culture results; and co-morbidities, their treatment and outcome.

Results

Summaries of the clinical features of the five patients and their sinusitis are presented in Tables 1 and 2, respectively. All of the patients were Japanese females who had been treated with TNF inhibitors for their RA symptoms. The average age of patients when they started using TNF inhibitors was 57.2 years (50 – 73). All of the patients were also treated with other immunosuppressive drugs such as methotrexate (MTX), glucocorticoid and salazosulfapyridine. None of the five patients had neutropenia at the time of sinusitis diagnosis.

One patient (patient 2) was previously diagnosed as having sinusitis and had undergone endoscopic sinus surgery before the use of TNF inhibitor (see case report). A review of the medical records of the other four patients revealed no subjective or objective symptoms indicative of sinus problems before the use of TNF inhibitors, and none of them underwent radiological evaluation of the sinus condition before the use of TNF inhibitors. The interval from the start or increase in dose of TNF inhibitors until onset of sinus symptoms was one month (patients 2, 3 and 4), and three months (patients 1 and 5).

CT examination revealed that the maxillary sinus was most commonly involved. Four patients (patients 1, 2, 3 and 4) showed bilateral maxillary lesions and the other patient (patient 5) showed unilateral maxillary lesions. In one patient (patient 2), bilateral frontal sinus was also involved. Other local and systemic complications included otitis media with effusion in two patients (patients 2 and 4), bronchiectasis and pneumonia in three patients (patients 2, 3 and 4), and cellulitis in one patient (patient 3). Neither eosinophilia nor asthma was present in these five patients. Culture of nasal swabs yielded *Pseudomonas aeruginosa* in three patients (patients 2, 3 and 4), and methicillin-resistant *Staphylococcus aureus* (MRSA) was also identified in

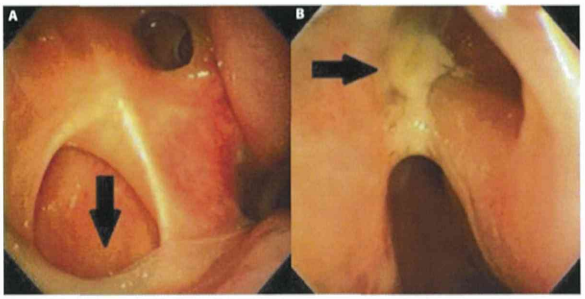

Figure 2. Endoscopic image of the patient 2 before cessation of etanercept. A. Purulent discharge from wide opening of the right maxillary sinus (arrow). B. Purulent discharge from frontal sinus (an arrow).

one of these (patient 2).

All of the patients were treated with antibiotics and nasal irrigation using saline. Two patients (patients 1 and 5) showed improvement with antibiotic medication despite the continuous use of TNF inhibitors. Sinusitis of two other patients (patients 3 and 4) were resistant to antibiotic medication, but these patients had been followed with the continuous use of TNF inhibitors without surgical treatment because they refused surgery. Sinusitis in patient 2 who had previously undergone endoscopic sinus surgery was also resistant to antibiotic medication and showed remission only after cessation of etanercept.

Case Reports

Patient 1

A 53-year-old female was diagnosed as having RA in 2005 and had received MTX (2~6 mg/week) for three years. With disease progression, she started taking 50 mg of etanercept every week, with MTX (2 mg/week) from August 2008. RA symptoms became well controlled thereafter, but postnasal purulent discharge developed in November 2008. When she was referred to our department in September 2011, we observed purulent rhinorrhea draining from bilateral hiatus semilunaris. CT revealed total bilateral maxillary sinus opacification (Figure 1A) and other paranasal sinuses were clear (Figure 1B). Culture of the sinus discharge revealed normal flora. The patient was treated with roxithromycin administration (150 mg/day) for four months and nasal irrigation. She noticed improvement of postnasal discharge thereafter, and follow-up CT four months later showed that her left maxillary sinus had become clear, and the right side was partially aerated (Figure 1C).

Patient 2

A 30-year-old female was diagnosed as having RA in 1987 and was treated with prednisolone (5~30 mg/day) and MTX (4~10 mg/week). She suffered from chronic sinusitis from 1998 and underwent ESS in 2006. Her sinusitis had been in complete remission after ESS.

To control RA symptoms, she started using 50 mg of etanercept a week with MTX (6 mg/week) from April 2007 at the age of 50 years. The dose was raised to 100 mg/week in April 2009. A month later, purulent rhinorrhea increased and nasal endoscopy revealed purulent discharge draining from the widely opened bilateral maxillary sinus and also from the bilateral frontal sinus (Figure 2A, B). Culture of the nasal swab revealed *Pseudomonas aeruginosa* and MRSA. Her sinusitis was resistant to antibiotic treatment using cefditoren pivoxil, levofloxacin and garenoxacin, and she also developed pneumonia. She stopped using etanercept, and her sinusitis has since been well controlled with daily nasal irrigation.

Discussion

We herein described five cases with chronic sinusitis that developed after the use of TNF inhibitors. The incidence of sinusitis with the use of TNF inhibitors is reported to be 7-15% [13,14], which is higher than that in general adult populations (5.7% in women and 3.4% in men) [15]. Our study further suggests that the incidence of refractory sinusitis which requires rhinological evaluation and treatment was approximately 2%.

Although there is no direct evidence that repeated administration of TNF inhibitors caused chronic sinusitis in our patients, the time lapse between the two events suggests a strong relationship. As all of the patients also used immunosuppressive medication in addition to TNF inhibitors, however, we cannot exclude the possibility that sinusitis was induced by synergistic immunosuppressive effects of TNF inhibitors and such concomitant drugs rather than TNF inhibitor alone. In our case series, two patients developed symptoms associated with sinusitis after the start of etanercept at 50 mg/week, while other patients developed sinusitis only after increasing the dose of TNF inhibitors to 100 mg/week. The dose that induces sinus pathology may thus vary with each patient.

Our data also demonstrated that there was a time lag between TNF inhibitor administration and the onset of sinus symptoms. All of our patients developed sinus symptoms within a few months of starting or after an increase in the dose of TNF inhibitors. Similar time lags were documented by Haroon et al. [11], sinusitis occurred within a few weeks of starting adalimumab, which is a fully human anti-TNF-α monoclonal antibody. The reasons underlying this time lag are unclear, but it may reflect the differences in short-term and long-term effects of TNF inhibitors on the airway mucosa. As TNF-α is a proinflammatory cytokine, the use of TNF inhibitors should lead to inhibition of inflammation in the short-term. In fact, experimentally acute sinusitis induced by lipopolysaccharide in rats was improved by admi-

nistration of TNF inhibitor [16]. On the other hand, as described in the Introduction, the inflammation mediated through TNF-α is an essential element of mucosal immunity against pathogens [3], and thus long-term suppression of TNF signaling may lead to chronic infection.

In the present cases, the maxillary sinus was the most commonly involved site. Cultures of sinus discharge showed *Pseudomonas aeruginosa* in three of the five patients. These characteristics have been found in patients with infective sinusitis of other systemic etiology, such as those with cystic fibrosis [17,18] and immunocompromised patients with haematological diseases, HIV infection or organ transplantation [19,20]. This is in contrast to the sinusitis associated with eosinophil infiltration, which usually shows ethmoid sinus-dominant involvement [21]. The reason why the maxillary sinus was most commonly involved in our patients remains unknown, but it may reflect yet unspecified differences in immune response of the mucosa between maxillary and ethmoid sinuses. Interestingly, two patients also had bronchiectasis as a comorbidity. It remains unclear whether the sinusitis and bronchiectasis were coincident in our patients or whether they are closely associated [22].

One limitation of this retrospective study is that histological examination of the sinus mucosa in TNF inhibitor-associated sinusitis was not performed in any of the five patients, because of the lack of specimen availability. It is necessary to address this issue in a future study.

Clinical management of chronic sinusitis associated with TNF inhibitors has not been fully discussed due to the paucity of reports. In patient 2, purulent discharge continued to drain from the ostium of the maxillary sinus after opening by endoscopic sinus surgery. This phenomenon suggests that sinusitis is induced by the pathological alteration of the mucosa itself and not by the obstruction of the ostium, thus, cessation of TNF inhibitors is considered necessary in such cases.

It is interesting that sinusitis in patient 1 responded well to long-term macrolide therapy [23]. The effectiveness of macrolide therapy should therefore be further examined.

Although not common, chronic sinusitis associated with TNF inhibitors is considered to be a new disease entity. Because TNF inhibitors are now becoming more commonly used, this entity should be kept in mind.

Authorship contribution

All authors were involved in the design and conduct of this study. Data collection and analysis was performed by S, KKo, KKa, KS, SB, MT-H, SK, YI, KF. SY and KKo wrote the manuscript. SY, KKo and TY take overall responsibility for the integrity of the study.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

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The Laryngoscope

Expression of IL-33 and its Receptor ST2 in Chronic Rhinosinusitis With Nasal Polyps

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Objectives/Hypothesis: Interleukin (IL)−33 is a novel member of the IL-1 cytokine family and a ligand for the orphan IL-1 family receptor ST2. IL-33 induces T helper 2-type inflammatory responses and is considered to play a crucial role in allergic inflammatory reactions such as asthma and atopic dermatitis. However, the role of IL-33 and its receptor ST2 in chronic rhinosinusitis remains unclear.

Study Design: In vitro study.

Methods: The expression patterns of IL-33 and ST2 at both mRNA and protein levels in nasal polyps from eosinophilic chronic rhinosinusitis (ECRS) patients (n = 10) and non-ECRS patients (n = 13), as well as in seemingly normal mucosa of the uncinate processes in patients without sinusitis (control; n = 5), were compared using immunohistochemical staining, enzyme-linked immunosorbent assay, and real-time polymerase chain reactions.

Results: ST2-positive cells in the inflammatory cells in the subepithelial layer were significantly higher in the ECRS group than other groups. The expression of ST2 mRNA in polyps of the ECRS group was significantly increased compared with controls. Many ST2-positive eosinophils were observed in the mucosa of ECRS but not in the mucosa of non-ECRS patients. The expression level of IL-33 mRNA was not significantly different among the three groups.

Conclusions: The current study suggests that IL-33 and its receptor ST2 may play important roles in the pathogenesis of chronic rhinosinusitis, especially in ECRS, through the increased expression of ST2 in eosinophils.

Key Words: Cytokine, expression, nasal polyp, eosinophil, rhinosinusitis, IL-33, ST2, inflammatory cells.

Level of Evidence: N/A.

***Laryngoscope*, 124:E115–E122, 2014**

INTRODUCTION

Chronic rhinosinusitis with nasal polyps (CRSwNP) is an inflammatory disease that remains difficult to treat despite advances in medical and surgical therapy. Recent studies have shown that the majority of patients with CRSwNP in the United States and Europe have pronounced infiltration of eosinophils and expression of interleukin-5 (IL-5) in the nasal polyps.[1] In contrast, more heterogeneity in CRSwNP has been reported in East Asian countries such as Japan, Korea, and China. For example, more than half of CRSwNP cases in Japan do not exhibit eosinophil-dominant inflammation.[2,3] In Japan, CRSwNP is categorized into two subtypes: eosinophilic chronic rhinosinusitis (ECRS), which is similar to the CRSwNP in Western countries; and noneosinophilic chronic rhinosinusitis (non-ECRS), which is characterized by Th1-dominant inflammation.[2] Although a number of hypotheses have been proposed regarding the pathogenesis of CRSwNP,[4,5] the precise molecular mechanisms underlying the development of CRSwNP are still largely unclear.

IL-33 is a recently described cytokine that has been identified as a ligand for the orphan IL-1 family receptor ST2.[6] IL-33 is produced by airway epithelial cells, fibroblasts, and smooth muscle cells. ST2 is expressed in monocytes, mast cells, eosinophils, Th2 lymphocytes,[7] and innate lymphoid cells.[8–10] IL-33 drives the production of Th2 cytokines such as IL-4, IL-5, and IL-13 by Th2 cells, mast cells, basophils, eosinophils, NKT cells, NK cells,[6,11–13] and innate lymphoid cells.[8–10] Recent studies have shown that IL-33 may play an important role in Th2-mediated eosinophilic inflammation,[14] and that polymorphisms within the IL-33 receptor gene are associated with the severity of asthma.[15] In an experimental mouse model of allergic rhinitis, IL-33 is promptly released from nasal epithelial cells in response

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Editor's Note: This Manuscript was accepted for publication October 7, 2013.

This work was supported by Grants-in-Aid for Young Scientists (B) (Shintaro Baba, MD; Kenji Kondo, MD, PhD; Maho Suzukawa, MD, PhD), a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan (Ken Ohta, MD, PhD), and a grant from the Environmental Restoration and Conservation Agency of Japan (Ken Ohta, MD, PhD). Maho Suzukawa, MD, PhD, is supported by a Postdoctoral Fellowship for Research Abroad from the Japan Society for the Promotion of Science. The authors have no additional funding, financial relationships, or conflicts of interest to disclose.

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DOI: 10.1002/lary.24462

to exposure to the allergen, and is essential for sneezing and the accumulation of eosinophils and basophils in the nasal mucosa by increasing histamine release from the mast cells and inducing production of chemoattractants from the basophils.[16] IL-33 expression is also reported to be increased in cultures of sinonasal epithelial cells in recalcitrant CRSwNP, and it is further enhanced by a bacteria-associated molecular pattern.[17] Very recently, Shaw et al.[18] reported that the expression of ST2 was elevated in inflamed ethmoid sinus mucosa from patients with CRSwNP compared with CRS without nasal polyps (CRSsNP); and controls and innate lymphoid cells within diseased mucosa in CRSwNP produce IL-13 in response to stimulation with recombinant IL-2 and IL-33 within diseased mucosa in CRSwNP.[18] However, the information regarding the in vivo expression of IL-33 and ST2 in the nasal polyps and the normal, non-CRS mucosa is still limited.

Therefore, the aim of the present study was to examine the expression and localization of IL-33 and ST2 in sinonasal polyps in ECRS and non-ECRS patients using immunohistochemical staining, enzyme-linked immunosorbent assay (ELISA), and real time-polymerase chain reaction (RT-PCR). We also examined the identity of ST2 positive cells in the polyps using double-immunostaining for ST2 and cell type-specific molecular markers.

MATERIALS AND METHODS

Patients

CRSwNP was diagnosed based on the criteria of the EAACI position article,[19] in which this disease entity was defined as having two or more of the following symptoms: blockage/congestion, discharge, anterior/posterior drip, facial pain/pressure, reduction or loss of smell for at least 3 months, and endoscopic signs of nasal polyp(s). Patients with CRSwNP associated with chronic obstructive pulmonary disease, diffuse panbronchiolitis, Churg-Strauss syndrome, congenital mucociliary diseases, or cystic fibrosis were excluded from this study. None of the patients included had been treated with systemic corticosteroids or other immune-modulating drugs for at least 1 month prior to surgery, although some patients had received antihistaminic agents and/or macrolide antibiotics.

The patients were classified into two groups: the ECRS group, which was defined as having the eosinophil count of more than 50 per microscopic field (×400 magnification) using five fields located in the subepithelial area of the polyps,[2] and the non-ECRS group, which did not fulfill this criteria. The normal-appearing mucosa of the uncinate processes, which were surgically removed in six patients without CRS (two with frontal sinus cysts, four with maxillary sinus tumors) served as controls. The study was approved by the local ethical committee of The University of Tokyo Hospital (#2656). Informed consent was obtained from each patient before collecting the samples.

Sampling of Tissue Specimens and Histological Procedures

The nasal polyps and control mucosae were harvested during endoscopic sinus surgery. A part of each sample was fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm-thick, mounted on MAS-coated slides (Matsunami Glass, Osaka Japan), and used for hematoxylin-eosin staining as well as the following immunohistochemistry. Another part was immediately immersed in RNA later for RT-PCR Analysis. The rest was immediately frozen and kept at −80°C until use for ELISA.

Immunohistochemistry

The following primary antibodies were used for evaluation of the expression of IL-33 and ST2, as well as the identification of inflammatory cells in the specimens: anti-IL-33 (mouse monoclonal, clone Nessy-1; Alexis Biochemicals, CA); anti-ST2 (mouse monoclonal, clone HB12;Medical & Biological Laboratories, Nagoya, Japan); anti-eosinophil major basic protein (MBP) (mouse monoclonal, clone BMK-13; Millipore, CA), anti-mast cell tryptase (mouse monoclonal, clone AA1, Thermo Fisher Scientific; CA), anti-human plasma cells (mouse monoclonal, clone VS38c; Dako Cytomation Japan, Kyoto, Japan) and anti-CD3 (rabbit monoclonal, clone SP7; Nichirei, Tokyo, Japan).

For single immunostaining for IL-33, ST2, MBP, mast cell tryptase, plasma cells, and CD3, immunoreactivity was made visible by diaminobenzidine (DAB) (Simplestain DAB, ready-to-use; Nichirei). To ensure that there was no nonspecific staining of secondary antibodies, the primary antibodies were omitted from the reaction.

For double-immunostaining for MBP and ST2, we chose enzymatic visualization of the immunoreactivity because the reliable primary antibodies for MBP and ST2 were both raised in mouse; thus, double immunofluorescence staining was impossible. MBP immunoreactivity was made visible by the DAB reaction (Simplestain DAB, Nichirei). After MBP staining by DAB, the sections were placed in citrate buffer solution (Dako Cytomation, Japan) and autoclaved at 121°C for 20 minutes to abolish the antigenicity of the anti-MBP antibody to the secondary antibody[19] and to retrieve ST2 antigenicity. The sections were then incubated with mouse anti-ST2 antibody, and immunoreactivity was made visible by the Vector Red kit (Vector Labs, Burlingame, CA).

Double-immunostaining for mast cell tryptase-ST2 and CD3-ST2 were also performed, using the enzymatic visualization described above for MBP and ST2 double immunostaining.

The details of immunohistochemical procedures are provided in Supplementary file 1.

ELISA for IL-33 and ST2

The nasal mucosae were homogenized with 10 times as much volume of CelLytic MT Cell Lysis Reagent (Sigma-Aldrich, Tokyo, Japan), and with a protease inhibitor cocktail (P8340 Sigma-Aldrich, Tokyo, Japan) and benzonase endonuclease (E1014 Sigma-Aldrich, Tokyo, Japan). Homogenized samples were centrifuged at 4°C at 15,000 g for 10 minutes. IL-33 and ST2 protein concentrations in the supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Tokyo, Japan) according to the manufacturer's instructions. Absorbance was read at 450 nm on a microplate reader.

Real-Time Quantitative PCR Analysis

The sample tissues were lysed in ISOGEN (Nippon Gene, Tokyo, Japan), and the total RNA was extracted according to the manufacturer's instructions. The mRNA expression was analyzed using an Applied Biosystems 7500 Real Time PCR System (PE Applied Biosystems, Foster City, CA). The primers and the probes for human β-actin, IL-33, and ST2 were designed by PE Applied Biosystems. For each sample, the differences in threshold cycles between the cytokine and β-actin