

Basophil infiltration in eosinophilic oesophagitis and proton pump inhibitor-responsive oesophageal eosinophilia

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SUMMARY

Background

The features of proton pump inhibitor-responsive oesophageal eosinophilia (PPI-REE) are similar to those of eosinophilic oesophagitis (EoE), but PPI-REE demonstrates symptomatic and histological responses to PPI therapy. Several studies have shown that basophils play a crucial role in the pathogenesis of allergic diseases.

Aim

To identify and compare basophil infiltration in the oesophageal epithelium in patients with EoE, PPI-REE, gastroesophageal reflux disease (GERD) and normal oesophagus (controls).

Methods

Biopsy specimens from 43 patients, including 12 with EoE, 11 with PPI-REE, 10 with GERD and 10 normal oesophagus, were analysed. Immunohistochemistry was performed to quantify the number of basophils and mast cells in the oesophageal epithelium. Double immunofluorescence staining for thymic stromal lymphopoietin (TSLP) and basophils was performed. Patients with EoE were treated with swallowed fluticasone.

Results

There were no differences in clinical, endoscopic or histological features between patients with EoE and PPI-REE. There were more basophils and mast cells in patients with EoE and PPI-REE than in patients with GERD and control subjects. Basophil infiltration of the oesophageal epithelium in patients with EoE was higher than that in patients with PPI-REE (3.6 ± 2.8 per high power field vs. 1.2 ± 0.9 per high power field respectively; $P = 0.02$); however, there was no significant difference in mast cell infiltration between the two groups. TSLP was highly expressed in the oesophageal epithelium in areas infiltrated by basophils. Steroid therapy significantly decreased intraepithelial basophils in patients with EoE.

Conclusion

Basophils may play an important role in the pathogenesis of eosinophilic oesophagitis.

INTRODUCTION

Eosinophilic oesophagitis (EoE) is a chronic, immune-mediated oesophageal disease, which is increasingly well recognised and prevalent in Western countries.^{1, 2} EoE is diagnosed from the presence of typical symptoms such as dysphagia and food impaction, intraepithelial eosinophilic infiltration of ≥ 15 per high power field (hpf), and exclusion of other causes of oesophageal eosinophilia, particularly gastroesophageal reflux disease (GERD).² Proton pump inhibitor-responsive oesophageal eosinophilia (PPI-REE) is a newly recognised condition that has similar clinical features to EoE but demonstrates symptomatic and histological responses to PPI therapy.² Several studies have shown that PPI-REE is involved in 33–74% of adult cases of oesophageal eosinophilia.^{3–9} Whether PPI-REE has the same pathogenic mechanism as that of EoE is unknown.

Basophils are the least abundant granulocyte population in peripheral blood, comprising less than 1% of all leucocytes, and have been considered minor and possibly redundant ‘circulating mast cells,’ because basophils share morphological and functional similarities with tissue-resident mast cells.^{10, 11} However, several studies have emphasised the crucial role of basophils in allergic diseases such as food allergy, bronchial asthma and atopic dermatitis.^{10–12} In IgE-mediated allergies, such as food allergies, interleukin-3 (IL-3)-elicited basophils act as effector cells that depend on cross-linking of high-affinity immunoglobulin (Ig) E receptors (Fc ϵ RI)-bound IgE by allergens, resulting in the release of a number of mediators, including histamine.^{11–13} In contrast, thymic stromal lymphopoietin (TSLP)-elicited basophils are nonresponsive to IgE-antigen complexes but contribute to the development and progression of the Th2 cytokine-mediated inflammation in several allergic conditions such as atopic dermatitis.^{11–13} Noti *et al.* demonstrated that increases in TSLP, but not IgE, promoted exaggerated basophil responses and resulted in the infiltration of the eosinophils into the oesophageal epithelium using a new murine model of EoE-like disease.¹⁴ These results suggest that TSLP-elicited basophils play a role in the pathogenesis of EoE.

Therefore, we hypothesised that basophils infiltrate the oesophageal epithelium in patients with EoE and/or PPI-REE. The aim of this study was to identify and compare basophil infiltration in the oesophageal epithelium in patients with EoE, PPI-REE, GERD and normal oesophagus (controls) and examine the effects of topical steroid therapy on basophil infiltration in patients with EoE.

MATERIALS AND METHODS

Patients

This study was a sub-analysis of previous collected specimens between June 2008 and October 2013 at the Osaka City and Tohoku university hospitals. Patients were adult (aged >20 years) out-patients who had undergone upper gastrointestinal (GI) endoscopy and were diagnosed with EoE, PPI-REE, GERD or normal oesophagus (control). Written informed consent was obtained from each patient, and procedures conformed to the Declaration of Helsinki.

EoE patients were diagnosed according to the presence of typical symptoms of oesophageal dysfunction, such as dysphagia and food impaction, and intraepithelial eosinophilic infiltration of ≥ 15 per hpf following administration of a standard dose of PPI treatment for at least 2 months.^{1, 2} Patients with oesophageal eosinophilia caused by other diseases, such as achalasia, Crohn disease, bullous pemphigoid and eosinophilic gastroenteritis, were excluded. PPI-REE patients were diagnosed according to the presence of typical symptoms of oesophageal dysfunction, intraepithelial eosinophilic infiltration of ≥ 15 per hpf, and alleviation of symptoms and oesophageal eosinophilia to <15 per hpf following PPI treatment.² Patients were diagnosed for GERD using endoscopy if a mucosal break was present at the oesophagogastric junction and had complaints of GERD symptoms such as heartburn. They were graded according to the Los Angeles classification system.¹⁵ Control specimens were obtained from the normal oesophagi of patients, including those who had undergone endoscopic resection because lesions were indicative of superficial oesophageal cancer or high-grade dysplasia of the oesophagus but pathologically normal (no tumour involvement).

Patients’ clinical data such as age, sex, body mass index, smoking and alcohol drinking habits, allergic conditions and diseases, upper GI symptoms and endoscopic features were extracted from electronic medical record and endoscopic reports.

Histology

A total of 5–6 oesophagus biopsies, including two at the distal oesophagus, two at the middle oesophagus and 1–2 at the proximal oesophagus, were routinely obtained in our institution from patients in whom EoE was suspected. Endoscopists obtained biopsies from areas with abnormal endoscopic findings specific to EoE, such as rings, white plaques and linear furrows, if they were

present. In cases with oesophageal eosinophilia at a previous hospital, the number of biopsies performed was dependent on each physician in each case.

Biopsy specimens were fixed in formalin, embedded in paraffin, cut in 5- μ m sections and stained with haematoxylin-eosin. The number of intraepithelial eosinophils was counted, and the maximum number of eosinophils per hpf (0.1 mm²) was evaluated.

Immunohistochemistry

The polymer peroxidase method was used for immunohistochemical staining of oesophageal biopsies. As the primary antibody, human basophil-specific antibody (BB1, antibasogranulin Ab, mouse IgG2a) at 1:10 dilution was used to detect basophils¹⁶ and antitryptase (mouse IgG2a; Santa Cruz Biotechnology, Inc., TX, USA) at 1:500 dilution were used to detect mast cells. Formalin-fixed, paraffin-embedded sections (5 μ m) were deparaffined with xylene, dehydrated with ethanol, pre-treated with 0.1% trypsin in 50 mM Tris-HCl containing 0.1% CaCl₂ for 37 °C at 30 min, and followed by additional treatment with 0.1% saponin in a phosphate-buffered saline (PBS) buffer for 30 min at room temperature. After immersion of the sections in a blocking agent solution (protein block serum free; Dako Japan, Tokyo, Japan) for 10 min, endogenous peroxidase was inactivated by immersion of sections in 3% hydrogen peroxide for 15 min. They were then incubated with BB1 antibody and a peroxidase-labelled anti-mouse polymer secondary antibody (Histofine Simple Stain MAX-PO (M) kit; Nichirei Biosciences Inc., Tokyo, Japan) for 30 min, stained with a diaminobenzidine chromogen (Nichirei), and counterstained with haematoxylin.¹⁷ Stained slides were dehydrated and coverslipped. Mast cells were stained using the same protocol as described above, but proteinase K (Dako) was used as an antigen retrieval buffer.

Immunofluorescence staining

Double labelling was performed using the immunofluorescence method to examine colocalisation of basophils and TSLP-positive cells in patients with EoE. Formalin-fixed, paraffin-embedded sections were dewaxed, and antigen retrieval was performed as described above. The sections were incubated with 1% bovine serum albumin (BSA) in PBS for 30 min to reduce nonspecific antibody binding. BB1 antibody and anti-TSLP specific antibody (rabbit polyclonal; Merck Millipore, Temecula, CA, USA) at 1:1000 dilution was applied to sections and incubated overnight at 4 °C. The BB1 antibody was allowed to react with a secondary antibody (donkey anti-

mouse IgG) labelled with Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA) at 1:100 dilution. The primary antibody to TSLP was allowed to react with a secondary antibody (donkey anti-rabbit IgG) labelled with Alexa Fluor 488 (Invitrogen) at 1:100 dilution. After washing with PBS, sections were mounted using ProLong Gold antifade reagents with DAPI (Invitrogen). Tissues were examined using a fluorescent microscope (Olympus BX50, Tokyo, Japan).

Quantitative evaluation of inflammatory cell infiltration

The numbers of BB1 and tryptase-positive stained cells present in the epithelium before the PPI treatment were counted in five microscopic fields infiltrated by basophils or mast cells. Data were expressed as mean positive cells per hpf (0.1 mm²) in the oesophageal epithelium.

Treatment of EoE

EoE patients received topical steroid therapy by swallowing fluticasone 400 μ g/day, bid, for 2 months. Upper GI endoscopy was performed to obtain biopsy specimens following steroid therapy. The numbers of eosinophils and basophils in the oesophageal epithelium were re-evaluated.

Statistical analysis

Data were presented as means $\pm$ s.d. or medians with interquartile ranges for continuous variables and numbers and frequencies for categorical variables. Comparison of continuous variables across four groups was performed using the Kruskal-Wallis *H* test. For categorical data, comparisons between two groups were performed using χ^2 tests, whereas continuous data were compared using the Scheffe's test. Changes in BB1-positive cell numbers prior and subsequent to steroid therapy were compared using a Wilcoxon signed-rank test. The overall significance level was set at a *P* value of 0.05. All statistical analyses were performed using SPSS version 16.0 J software (SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Patient characteristics

A total of 43 patients, including 12 with EoE, 11 with PPI-REE, 10 with GERD and 10 with normal oesophagus, were analysed. Table 1 shows the patients' characteristics. The patients with GERD and the control subjects were older than those with EoE and PPI-REE. The proportion of smokers was higher in the control group than in the

Table 1 | Characteristics of the study subjects

	EoE (n = 12)	PPI-REE (n = 11)	GERD (n = 10)	Control (n = 10)	P value
Clinical					
Age (years)	42.7 ± 11.5	48.4 ± 11.4	64.6 ± 14.3	61.3 ± 10.1	0.001
Male sex	9 (75)	7 (64)	5 (50)	9 (90)	0.246
Body mass index (kg/m ²)	22.5 ± 5.2	25.3 ± 5.0	23.6 ± 2.9	24.6 ± 2.9	0.288
Smoking	2 (17)	2 (18)	4 (40)	7 (70)	0.041
Alcohol consumption	7 (58)	6 (60)	5 (50)	6 (60)	0.987
Symptom					
Food impaction	12 (100)	6 (55)	3 (30.0)	0 (0)	<0.001
Dysphagia	8 (67)	4 (36)	1 (10)	0 (0)	0.003
Heartburn	2 (17)	4 (36)	10 (100)	1 (10)	<0.001
Chest pain	2 (17)	2 (18)	0 (0)	0 (0)	0.278
Allergy					
None	3 (25)	4 (36)	7 (70)	10 (100)	0.003
Food	3 (25)	3 (27)	3 (30)	0 (0)	0.277
Atopic dermatitis	1 (8)	0 (0)	0 (0)	0 (0)	0.455
Allergic rhinitis	4 (33)	6 (55)	0 (0)	0 (0)	0.006
Endoscopic findings					
Normal	0 (0)	0 (0)	0 (0)	10 (100)	<0.001
Rings	6 (50.0)	7 (64)	0 (0)	0 (0)	<0.001
Linear furrows	10 (83)	8 (73)	0 (0)	0 (0)	<0.001
White plaques	7 (58)	3 (27)	0 (0)	0 (0)	0.002
Stricture	1 (8)	0 (0)	1 (10)	0 (0)	0.604
Erosive oesophagitis	1 (8)	3 (27)	10 (100)	0 (0)	<0.001
Hiatal hernia	2 (17)	3 (27)	6 (60)	3 (30)	0.187
Barrett's oesophagus	0 (0)	0 (0)	0 (0)	0 (0)	
Maximum number of eosinophils (per hpf)	60.5 ± 41.6	50.4 ± 23.8	1.10 ± 1.3	0	<0.001

hpf, high power field.

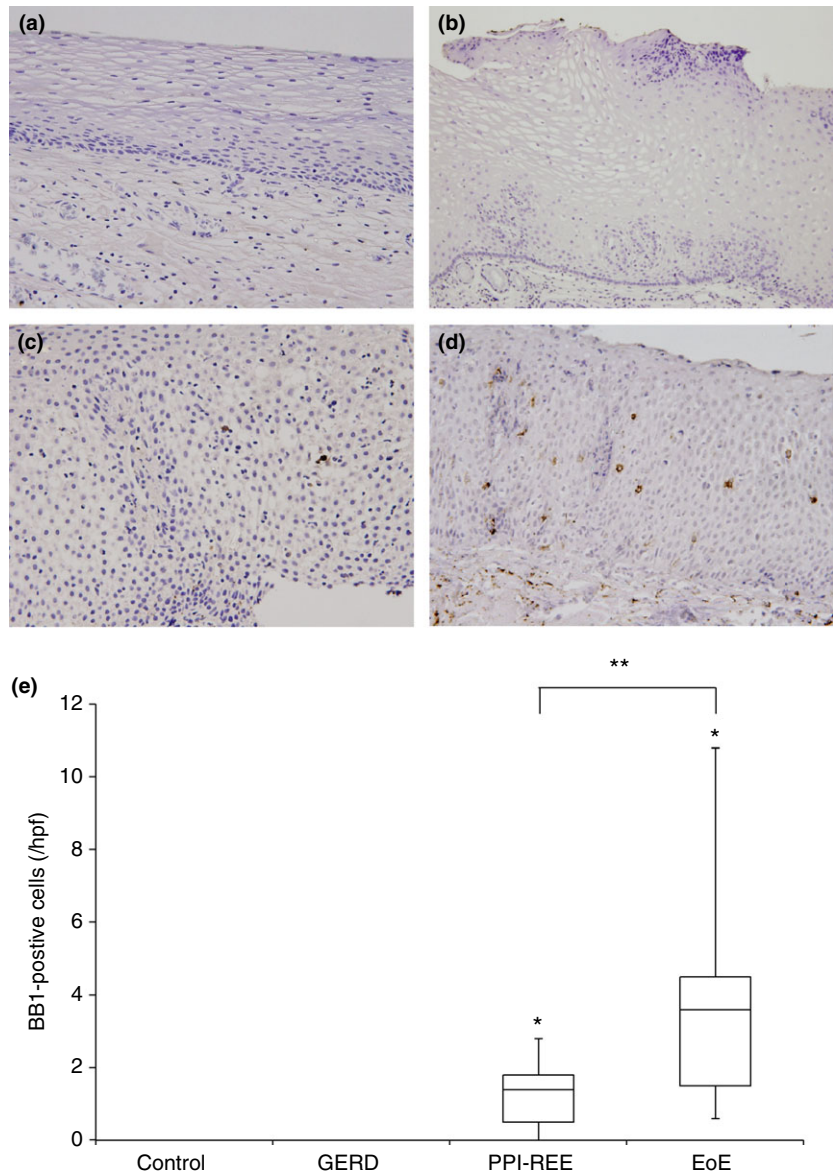
Data were mean ± s.d. or number (frequency).

other groups. Patients with EoE and PPI-REE experienced food impaction and dysphagia more frequently than patients with GERD or the control subjects. Allergic diseases were more prevalent in patients with EoE and PPI-REE than in those in the control group. The typical endoscopic findings of EoE, such as linear furrows, rings and white plaques, were more frequent in the EoE and PPI-REE groups than in the GERD and control groups. Of 10 patients in the GERD group, 2, 2, 5 and 1 had Grades A, B, C and D reflux oesophagitis respectively. Maximum numbers of eosinophils were higher in the EoE and PPI-REE groups than in the GERD and control groups. However, there were no differences in clinical features, prevalence of symptoms such as food impaction and dysphagia, presence of EoE-like endoscopic findings, or maximum numbers of eosinophils in the oesophageal epithelium before the PPI treatment between the EoE and PPI-REE groups. After the PPI treatment, maximum number of intraepithelial eosinophils decreased to 2.3 ± 3.0 /hpf in PPI-REE group.

BB1- and tryptase-positive staining. Basophil staining for each group is shown in Figure 1a–d. There was no infiltration by BB1-positive cells in patients in the control or GERD groups. BB-1-positive cells were found in the oesophageal epithelium more frequently in the EoE group than in the PPI-REE group. Quantitative analyses revealed that the numbers of basophils that infiltrated the oesophageal epithelium in patients with EoE and PPI-REE before the PPI treatment were significantly higher than that in those in the control and GERD groups (Figure 1e). A comparison of EoE and PPI-REE groups showed that the number of intraepithelial basophils was significantly higher in patients with EoE than in patients with PPI-REE (EoE 3.6 ± 2.8 /hpf vs. PPI-REE 1.2 ± 0.9 /hpf, respectively; $P = 0.02$). Figure 2a–d shows tryptase staining for each group. There were a few tryptase-positive cells in patients in the control and GERD groups, while such cell infiltration was more common in patients in the EoE and PPI-REE groups before the PPI treatment. Figure 2e shows the results of

Figure 1 |

Immunohistochemical staining for basophils (BB1-positive cells) in the oesophageal mucosa and number of basophils (BB1-positive cells) in the oesophageal mucosa. (a) normal oesophagus, (b) gastroesophageal reflux disease, (c) proton pump inhibitor-responsive oesophageal eosinophilia and (d) eosinophilic oesophagitis. (e) Number of basophils (BB1-positive cells) in the oesophageal mucosa. GERD, gastroesophageal reflux disease; PPI-REE, proton pump inhibitor-responsive oesophageal eosinophilia; EoE, eosinophilic oesophagitis. The box represents the interquartile range (25% and 75%) from the median (horizontal line). The bars indicate 10% and 90%. * $P < 0.001$ vs. control and GERD groups. ** $P < 0.01$ between PPI-REE and EoE groups.



quantitative analysis of tryptase-positive cells. The number of mast cells that infiltrated the oesophageal epithelium was significantly higher in the EoE and PPI-REE groups than in the GERD and control groups. (EoE: $12.0 \pm 8.5/\text{hpf}$, PPI-REE: $9.3 \pm 4.5/\text{hpf}$, GERD: $1.8 \pm 1.1/\text{hpf}$ and control: $0.9 \pm 0.8/\text{hpf}$, $P < 0.001$). There was no significant difference in numbers of mast cells between the EoE and PPI-REE groups ($P = 0.74$).

Localisation of TSLP and basophils in EoE. Figure 3 shows the double staining of BB1 and TSLP in the control group and patients with EoE. TSLP (green) was strongly stained in the oesophageal epithelial cells, and colocalisation with BB1-positive cells (red) was observed in patients with EoE. Control specimens revealed no positive BB1 or TSLP cells.

Effects of topical steroid treatment on basophil infiltration in patients with EoE. Eight patients with EoE received fluticasone swallowing therapy. Steroid therapy improved symptoms such as food impaction and dysphagia in addition to reducing intraepithelial eosinophil infiltration from $46.0/\text{hpf}$ ($31.5\text{--}52.5$) to $0/\text{hpf}$ ($0\text{--}1.3$; $P = 0.01$). Steroid therapy led to a significant reduction in numbers of intraepithelial basophils from 2.6 ($1.4\text{--}4.6$) to $0.1/\text{hpf}$ ($0\text{--}0.6$; Figure 4).

DISCUSSION

This study emphasised three findings of note. First, both basophils and mast cells infiltrated the oesophageal epithelium of patients with EoE and PPI-REE to a greater extent than that of patients in the GERD and control groups. Second, intraepithelial basophil infiltration was

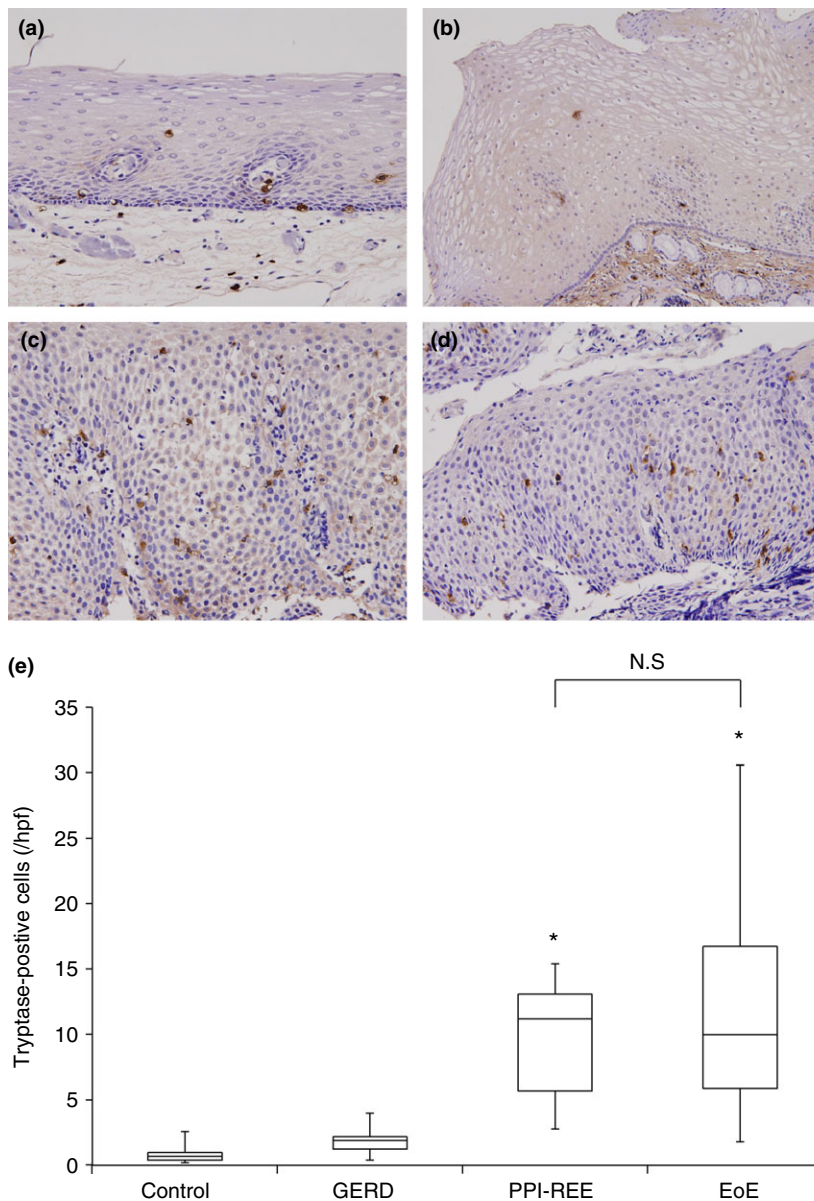


Figure 2 | Immunohistochemical staining for mast cells (tryptase-positive cells) in the oesophageal mucosa and number of mast cells (tryptase-positive cells) in the oesophageal mucosa. (a) normal oesophagus, (b) gastroesophageal reflux disease, (c) proton pump inhibitor-responsive oesophageal eosinophilia and (d) eosinophilic oesophagitis. (e) Number of mast cells (tryptase-positive cells) in the oesophageal mucosa. GERD, gastroesophageal reflux disease; PPI-REE, proton pump inhibitor-responsive oesophageal eosinophilia; EoE, eosinophilic oesophagitis. The box represents the interquartile range (25% and 75%) from the median (horizontal line). The bars indicate 10% and 90%. * $P < 0.001$ vs. control and GERD groups. N.S., no significance between PPI-REE and EoE groups.

higher in the EoE group than in the PPI-REE group; however, there was no significant difference in mast cell infiltration between the two groups. Third, there was a significant reduction in intraepithelial basophils following topical steroid therapy in patients with EoE. These results suggest that basophils may play a crucial role in the pathogenesis of EoE.

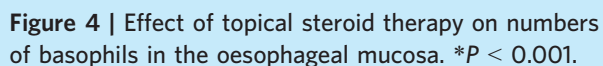
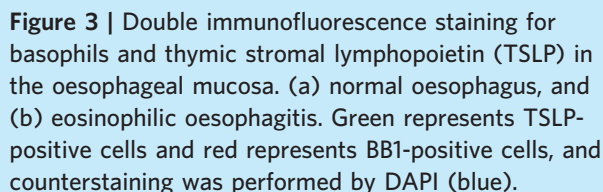
Two studies have examined differences in features between EoE and PPI-REE. Moawad *et al.* reviewed two databases from Walter Reed and Swiss and found no differences in symptoms including dysphagia, food impaction and heartburn, endoscopic features such as furrows, rings, plaques and strictures or numbers of proximal or distal eosinophils between patients with EoE and those with PPI-REE.¹⁸ Dellon *et al.* prospectively examined

223 patients in whom EoE was suspected and found that none of the clinical or endoscopic features distinguished PPI-REE from EoE.⁹ Similarly, our results confirmed no significant differences in clinical and endoscopic features or numbers of intraepithelial eosinophils between the EoE and PPI-REE groups.

Does PPI-REE have the same pathogenesis as that of EoE? Several studies have tried to answer this question. Molina-Infante examined 53 patients, including 23 with PPI-REE and 30 with EoE, and found no differences in mRNA expression of eotaxin-3, IL-5 or IL-13 at the proximal and distal oesophagus between groups.¹⁹ Dellon *et al.* performed a prospective study of 196 consecutive cases, including 50 patients with EoE, 23 patients with PPI-REE and 123 control subjects. They found higher

Two theories are suggested to explain the reasons why PPI is effective in patients with PPI-REE. Acid reflux could be the first event, followed by the disturbance of oesophageal epithelial barrier function by acid exposure, resulting in antigen entry from the oesophageal lumen.²² This is supported by a study conducted by Rhijn and colleagues, which demonstrated that electrical tissue impedance and transepithelial electrical resistance of the oesophagus were reduced in patients with EoE and PPI-REE prior to treatment, and PPI therapy led to partial a reduction in oesophageal integrity in patients with PPI-REE but not in those with EoE.²³ Another study demonstrated that flaggrin, ZO-3 and claudin-1 expressions were reduced in patients with EoE,²⁴ but expression of these tight junction proteins in patients with PPI-REE and differences between EoE and PPI-REE are unclear. Another hypothesis is that of anti-inflammatory action of proton pump inhibitors (PPIs). Two basic studies using oesophageal epithelial cultured cells showed that eotaxin-3 increased in oesophageal squamous cells stimulated by IL-4 or IL-13, and PPIs significantly reduced such increased expression of eotaxin-3 by blocking STAT6 binding to the eotaxin-3 promoter.^{25, 26} These hypotheses are supported by results indicating that PPI therapy significantly downregulated the expression of eotaxin-3 mRNA in the oesophageal specimens of patients with PPI-REE but not in those with EoE.¹⁹

In this study, basophils infiltration was significantly higher in areas of the oesophageal epithelium in which TSLP was highly expressed in patients with EoE.²⁷ Unlike general food allergies, EoE is not mediated with IgE, as anti-IgE therapy with omalizumab did not alter symptoms or oesophageal eosinophils in patients with EoE.^{28, 29} Therefore, the oesophageal basophils found in EoE are TSLP-elicited basophils. Several studies have shown that increases in functional polymorphisms of *TSLP* are associated with the development of EoE. Noti *et al.* showed that TSLP promoted an IgE-independent murine model of EoE-like disease through TLSP-elicited basophils and Th2 cytokine production.¹⁴ These findings suggest the presence of a TSLP-basophil axis in EoE. The exact reasons for reduced basophil infiltration in PPI-REE relative to that observed in EoE are unknown



levels of major basic protein, eotaxin-3 and tryptase, on the basis of immunohistochemical analysis, in both EoE and PPI-REE groups but no differences in expression of

but may include different allergic reactions, lesser TLSP production and lower responses to TSLP.

This study had some potential limitations. First, the small sample size is a major limitation. Recognition of EoE is recent in Japan, and the prevalence of EoE remains low.^{4, 6, 30} Exact reasons of differences in prevalence of EoE between Japan and Western countries are unknown, but might be due to genetics, dietary differences or environmental exposures. Especially, higher incidence of *Helicobacter pylori* infection in Japan³¹ might be associated with lower prevalence of EoE because Dellon *et al.* reported that *H. pylori* infection was inversely associated with oesophageal eosinophilia.³² Second, some of the observed differences in the present study were small possibly due to small number of samples. Therefore, our results have a limit to generalisability. Third, as the size of specimens was small due to biopsy, only the epithelial layer of the oesophagus could be evaluated, and the lamina propria or submucosa could not. Fourth, we did not assess acid reflux via oesophageal pH monitoring. However, examination via pH monitoring would not predict PPI responses in oesophageal eosinophilia⁵ or distinguish between EoE and PPI-REE.¹⁶ Finally, as the BB1 antibody detects a basophil granule-associated protein (basogranulin),²³ degranulated basophils may not have been detected, resulting in the underestimation of infiltrated basophils.

In conclusion, this study showed that patients with EoE and PPI-REE displayed higher levels of basophil and mast cell infiltration in the oesophageal epithelium than patients with GERD and controls. Furthermore, basophils infiltration in the oesophageal epithelium of

patients with EoE was significantly higher than that in those with PPI-REE, and this infiltration decreased following fluticasone swallowing therapy. These results suggest that basophils may play an important role in the pathogenesis of EoE and are candidates for consideration as novel markers to distinguish EoE from PPI-REE. Since markers to distinguish EoE from PPI-REE have not been reported,^{19, 20} a large prospective study is required to elucidate this.

AUTHORSHIP

Guarantor of the article: Yasuhiro Fujiwara, MD.

Author contributions: NI: study design, immunohistochemistry, data collection and analysis and manuscript writing; YF: study design, data collection and analysis and manuscript writing; FT, TT, HY, MS, KT, TW, KI and TK: data collection and critical revision of the manuscript for important intellectual content; AW: supply of BB1 antibody, technical suggestions for immunohistochemistry, and critical revision of the manuscript for important intellectual content; TA: study supervision. All authors approved the final draft of the manuscript.

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