Original Article

Eosinophilic esophagitis and asymptomatic esophageal eosinophilia display similar immunohistological profiles

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Patients with asymptomatic esophageal eosinophilia (aEE) do not exhibit clinical symptoms because of esophageal dysfunction, although they have endoscopic and histological findings similar to those of eosinophilic esophagitis (EoE). The cause of the symptoms and the differences between aEE and EoE are unclear. The aim of this study is to determine whether aEE and EoE are same disease entities by comparing immune-related tissue biomarkers using immunohistological staining. Esophageal biopsy specimens from 61 patients, including 18 with aEE and 43 with EoE, were analyzed. Immunofluorescence staining was performed to quantify the immune-related tissue biomarkers such as major basic protein, eosinophil-derived neurotoxin, eotaxin-3, and immunoglobulin G4. Data are presented as median (interquartile range). There were no significant differences in clinical, endoscopic, or histological features, between patients with aEE and EoE, with the exception of body mass index. There were no significant differences in all immune-related tissue biomarkers between both groups. In conclusions, EoE and aEE displayed similar immunohistological profiles. Hence, they may be similar disease entities with some common pathogenic mechanisms. Our findings suggest that patients with aEE also have histopathological esophageal inflammation.

***Key Words*:** **eosinophilic esophagitis, eosinophils, eosinophil major basic protein, eosinophil-derived neurotoxin, immunoglobulin G**

Eosinophilic esophagitis (EoE) is a chronic immune-related disease characterized by clinical symptoms because of esophageal dysfunction and intraepithelial infiltration of ≥15 eosinophils per high power field (HPF) with multiple biopsies.[1–3] Inflammation and fibrosis of the lamina propria and muscularis propria results in esophageal dysfunction and stricture formation.[4] The prevalence of EoE is higher in Western countries than Asian countries, including Japan. The prevalence of EoE has recently been increasing in both Western and Asian countries.[5,6] We sometimes encounter patients with typical endoscopic findings of EoE, such as exudates, rings, edema, furrows, and significant esophageal eosinophil infiltration during medical health check-ups, even though some are asymptomatic.[7,8]

EoE has an aspect of allergic disease, which is driven by food allergen via Type 2 helper T cells (Th2)-mediated immune reaction.[9,10] Th2 cells produce cytokines, such as interleukin (IL)-4 and 13 that can activate B cells followed by differentiation into plasma cells. Plasma cells produce immunoglobulin (Ig), such as IgE and IgG4. IgE is considered to be involved in the pathophysiology of EoE. However, a recent study reported the involvement of IgG4, rather than IgE, in adults.[11] The levels of food-specific serum IgG4 are increased in EoE compared with controls. Moreover, esophageal IgG4 levels are reportedly related to esophageal eosinophil counts, histological grade, and the expression of Th2 cytokines.[12]

IL-13 stimulates esophageal epithelial cells to produce eotaxin-3, which is a chemoattractant that induces esophageal eosinophilic infiltration.[13] Eosinophils are activated by IL-5 produced by Th2 cells and release intracellular granules, such as eosinophil major basic protein (MBP) and eosinophil-derived neurotoxin (EDN), which induce local inflammation and tissue damage.[14,15] MBP has been used to localize eosinophils and characterize eosinophil degranulation.[16] EDN is a cytotoxic protein that can act as an alarmin to stimulate dendritic cells by the enhancement of Th2 immune responses.[17,18] Marked deposition of extracellular EDN is observed in the majority of EoE patients, and EDN is used as an indicator of eosinophil activation and degradation.[15] In some patients with EoE, marked MBP and EDN depositions have been observed despite small numbers of eosinophils. Eosinophil counts are considered to underestimate disease activity, particularly in individuals with marked eosinophil degranulation. Therefore, the staining of inflammatory markers, such as MBP and EDN, is useful to evaluate eosinophilic activity, rather than eosinophil counts.

Interestingly, in clinical settings, esophageal eosinophilia (EE) does not always produce clinical symptoms. We sometimes encounter patients with asymptomatic EE (aEE) who have similar endoscopic findings as EoE.[19] We previously reported that approximately 26% of EE patients can be asymptomatic at the time of diagnosis during a medical health check-up.[7] Patients with aEE cannot fulfill the diagnostic criteria for EoE because they lack clinical symptoms because of esophageal dysfunction.[1,2]

The natural history of aEE remains unclear and it is not fully clear whether EoE and aEE are the same disease entities. Clinical characteristics, endoscopic findings, and histological findings, such as eosinophil counts, have been reported to be similar between EoE and aEE.[20] However, the difference of immune profile in aEE is still unknown and the causative mechanism(s) of the clinical symptoms have not also been elucidated. In addition, the current clinical guidelines for diagnosis of EoE include symptoms related to esophageal dysfunction, thus, patients with aEE are not followed up. We sometimes encountered patients

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who were diagnosed with aEE the first time and were suffering from severe symptoms the next time. Therefore, we hypothesized that even patients with aEE have esophageal inflammation and untreated chronic eosinophilic inflammation might lead to esophageal dysfunction. The aim of this study is to investigate whether the expressions of immune-related tissue biomarkers, such as MBP, EDN, eotaxin-3, and IgG4, are the same in EoE and aEE using immunohistological staining.

Methods

Study design and participants. We conducted a single center retrospective observational study. Between April 2014 and August 2018, we enrolled aEE and EoE subjects who were diagnosed at medical health check-up in Osaka City University Hospital Advanced Medical Center for Preventive Medicine (MedCity21). EoE was diagnosed according to the guidelines.[1,2] We clinicopathologically diagnosed EoE by the presence of symptoms related to esophageal dysfunction, such as dysphagia and esophageal biopsy, demonstrating ≥15 eosinophils per HPF. The definition of aEE is a patient's esophageal biopsy demonstrating ≥15 eosinophils per HPF without current or previous symptoms because of esophageal dysfunction. In patients with aEE and EoE, mucosal eosinophilia was restricted to the esophagus. We considered typical endoscopic findings as follows: mucosal edema (score 0–2), esophageal rings (score 0–3), white exudates or plaques (score 0–2), longitudinal furrows (score 0–2), and strictures (score 0–1) according to the endoscopic reference score (EREFS) system.[19] Reflux esophagitis (RE) was defined according to the modified Los Angeles classification (Grades M–D).[21] We collected data including age, body mass index (BMI), sex, current cigarette smoking (presence or absence), current alcohol drinking (presence or absence), and concomitant allergic diseases from the medical records. Esophageal symptoms were evaluated by doctors' interview. Concomitant allergic diseases showed the status with or without any allergic diseases (e.g., food allergy, animal allergy, metallic allergy, allergic rhinitis, bronchial asthma, and atopic dermatitis). Patients who received acid suppressive drugs, such as proton-pump inhibitors and histamine H_2 receptor antagonists, and steroid therapy were excluded.

The study protocol was approved by the Ethics Committee of Osaka City University Graduate School of Medicine (protocol number 4141) and was performed in accordance with the principles of the Helsinki Declaration. The need for informed consent was waived by the Ethics Committee of the Osaka City University Graduate School of Medicine. We disclosed the information about this study on our home page on the Internet and the patients had the opportunity to opt out.

Histology and immunofluorescence staining. Endoscopists obtained biopsies from areas with abnormal endoscopic findings specific to EoE, such as edema, rings, exudates, furrows, and strictures, if they were present. Details of the biopsy sites are provided in Table 2. Lower esophagus was the most biopsied site in both EoE and aEE. Biopsy specimens were fixed in formalin, embedded in paraffin, cut in 5-μm sections, and stained with hematoxylin-eosin. The number of mucosal eosinophils was counted, and the maximum number of eosinophils per HPF (0.1 mm^2) was evaluated.

Esophageal biopsy tissue sections were stained for MBP, EDN, eotaxin-3 (C-C motif chemokine ligand 26; CCL26), and IgG4. All sections were performed according to the following protocol. Slides were deparaffinized and steam-treated for antigen retrieval (Histofine; Nichirei biosciences Inc., Tokyo, Japan). After immersion of the sections, they were incubated with 5% donkey serum for 60 min. The primary antibodies were applied to sections and incubated overnight at 4°C. The primary antibodies included anti-MBP antibody at 1:400 dilution (rabbit polyclonal; Abcam, Cambridge, England), anti-EDN antibody at 1:200 dilution (mouse polyclonal; Novus Biologicals, Centennial, CO), anti-CCL26 antibody at 1:400 dilution (rabbit polyclonal; Bioss, Woburn, MA), and anti-IgG4 Fc antibody at 1:200 dilution (mouse monoclonal; Arigo Biolaboratories, Hsinchu, Taiwan). The first antibodies were allowed to react with a secondary antibody (donkey anti-mouse/rabbit IgG) labeled with Alexa Fluor 594/488 (Abcam) at 1:400 dilution. After washing with phosphate buffered saline, sections were mounted using ProLong Gold antifade reagents and nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Tissue were examined using a model BX50 fluorescence microscope (Olympus, Tokyo, Japan).

Analysis of specimens. The number of MBP-positive stained cells present in the epithelium were counted in five microscopic fields and eotaxin-3-positive stained cells were counted in three microscopic fields. For each sample, the most stained areas were selected for cell counting. Thus, the area in which cells were counted belonged to all parts of the esophagus. In other words, we compared epithelium and/or lamina propria of the upper, middle, and lower esophagus randomly. We selected this method to evaluate esophageal inflammation equally. Data are expressed as mean positive cell per HPF (0.1 mm^2) in the esophageal epithelium. The extent of extracellular EDN and IgG4 depositions were categorized into five categories according to the percentage of positive depositions in a microscopic field: 0 (none), 1 (<25%), 2 (25–50%), 3 (50–75%), and 4 (>75%).

Statistical analyses. Data are presented as mean ± SD or median with interquartile range for continuous variables and numbers and frequencies for categorical variables. For categorical data, comparisons between two groups were performed using χ^2 tests, whereas continuous data were compared using an unpaired *t* test or Mann-Whitney *U* test. The overall significance level was set at a p value of 0.05. All statistical analyses were performed using EZR ver. 1.38 (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing ver. 3.5.2).[22]

Results

Clinical characteristics. This study targeted 63 subjects. Two were excluded since they received acid suppressive drugs that included proton-pump inhibitors and histamine 2 receptor antagonists. No subject received steroid therapy. Accordingly, a total of 61 subjects, including 43 with EoE, 18 with aEE were analyzed. Table 1 shows the clinical characteristics. There were no significant differences in age, sex, current alcohol drinking, current cigarette smoking, and concomitant allergic diseases including allergic rhinitis, bronchial asthma, and atopic dermatitis. Moreover, there was no significant difference in the proportion of subjects who took medications for allergic diseases, such as histamine H_1 receptor antagonists, between aEE and EoE patients (11.1% vs 14.0%, $p = 1.0$). BMI was significantly higher in EoE than that of aEE (22.4 ± 3.4 vs 25.1 ± 4.7 kg/m^2, $p = 0.032$).

Endoscopic findings and numbers of infiltrated eosinophils. Endoscopic and histological findings are shown in Table 2. Total EREFS score was not significantly different between aEE and EoE. Endoscopic features of edema, rings, exudates, furrows, and strictures did not differ in the two patient groups. Furthermore, there were no significant difference in peak eosinophil counts and the prevalence of RE between both groups. In patients with aEE, the precise grading of RE was Grade M 100%, Grade A 0%, Grade B 0%, Grade C 0%, and Grade D 0%. In patient with EoE, the grading was Grade M 56.3%, Grade A 37.4%, Grade B 0%, Grade C 6.3%, and Grade D 0%.

MBP staining. MBP staining for each group is shown in Fig. 1. MBP was strongly stained in the esophageal epithelial layer, demonstrating scattered eosinophil granules with activated

Table 1. Clinical characteristics of the study subjects

Variable	aEE (n = 18)	EoE (n = 43)	p value
Age (years)	47.1 ± 8.8	46.7 ± 8.3	0.865
Male	10 (56%)	28 (64%)	0.578
BMI (kg/m^2)	22.4 ± 3.4	25.1 ± 4.7	0.032
Current alcohol drinking	4 (22%)	13 (30%)	0.755
Current smoking	3 (17%)	5 (11.6%)	0.683
Symptoms			
Dysphagia	—	34 (79.1%)	—
Heartburn	—	17 (39.5%)	—
Chest pain	—	4 (9.3%)	—
Concomitant allergic diseases	13 (72.2%)	31 (72.1%)	1
Allergic rhinitis	5 (28%)	15 (34.8%)	0.767
Bronchial asthma	6 (33%)	13 (30%)	1
Atopic dermatitis	3 (16.6%)	5 (11.6%)	0.68

Data are expressed as mean ± SD or median (IQR) for continuous variables and as numbers (percentage) for categorical variables. Concomitant allergic diseases show the status with some allergic diseases (e.g., food allergy, animal allergy, metallic allergy, allergic rhinitis, bronchial asthma, and atopic dermatitis). aEE, asymptomatic esophageal eosinophilia; BMI, body mass index; EoE, eosinophilic esophagitis; IQR, interquartile range.

Table 2. Endoscopic findings and the numbers of infiltrating eosinophils

Variable	aEE (n = 18)	EoE (n = 43)	p value
Total EREFS score	2 (1, 2)	2 (1, 3)	0.153
Edema			0.701
Grade 0	10 (55.6%)	19 (44.2%)	
Grade 1	8 (44.4%)	23 (53.5%)	
Grade 2	0 (0%)	1 (2.3%)	
Rings			0.559
Grade 0	14 (77.8%)	27 (62.8%)	
Grade 1	4 (22.2%)	15 (34.9%)	
Grade 2	0 (0%)	1 (2.3%)	
Grade 3	0 (0%)	0 (0%)	
Exudates			0.298
Grade 0	7 (38.9%)	21 (48.8%)	
Grade 1	11 (61.1%)	18 (41.9%)	
Grade 2	0 (0%)	4 (9.3%)	
Furrows			0.0991
Grade 0	9 (50%)	14 (32.6%)	
Grade 1	8 (44.4%)	29 (67.4%)	
Grade 2	1 (5.6%)	0 (0%)	
Stricture			1
Grade 0	18 (100%)	43 (100%)	
Grade 1	0 (0%)	0 (0%)	
Reflux esophagitis	4 (22.2%)	16 (37.2%)	0.368
No gastric atrophy	13 (72.2 %)	29 (67.4 %)	0.771
Biopsy sites			0.53
Upper esophagus	12 (21.4%)	47 (25.1%)	
Middle esophagus	16 (28.6%)	63 (33.7%)	
Lower esophagus	28 (50%)	77 (41.2%)	
Peak eosinophilic counts (per HPF)	65 (31.3, 94.8)	54.5 (24.3, 80.0)	0.23

Data are expressed as median (IQR) for continuous variables and as numbers (percentage) for categorical variables. aEE, asymptomatic esophageal eosinophilia; EoE, eosinophilic esophagitis; EREFS, eosinophilic esophagitis endoscopic reference score; HPF, high power field; IQR, interquartile range.

eosinophils (Fig. 1A). Eosinophil degranulation occurred in aEE and aEE. Quantitative analysis revealed no significant difference in the numbers of MBP-positive cells between aEE and EoE [50 (32.6, 70.8) vs 60 (40.6, 84.0) cells/HPF, p = 0.376].

EDN staining. EDN staining for each group is shown in Fig. 2. EDN was strongly stained in the esophageal epithelial surface layer and extracellular space of epithelial cells reflecting eosinophil granule protein deposition (Fig. 2A–E). There were no significant differences in EDN deposition scores between aEE and EoE [1.9 (1.4, 3.2) vs 1.6 (1.1, 2.2), p = 0.191].

Eotaxin-3 staining. Eotaxin-3 staining for each group is shown in Fig. 3. Eotaxin-3 was strongly stained in the esophageal epithelial cells (Fig. 3A). The number of eotaxin-3-positive cells was not significantly different between aEE and EoE [231.5

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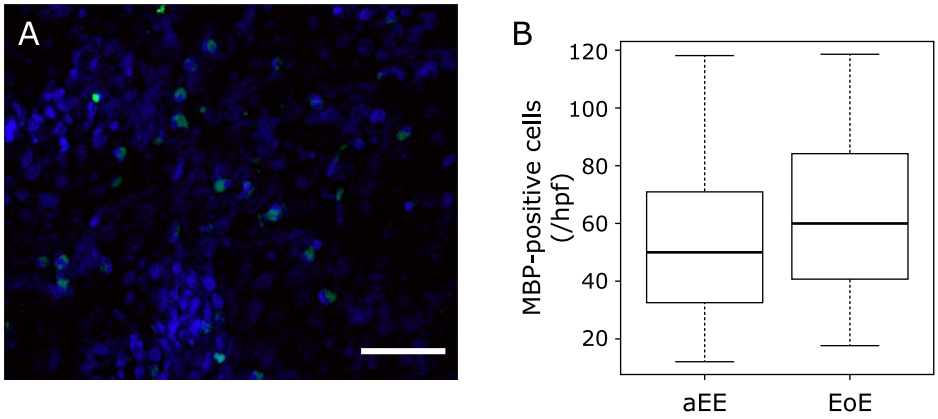

Fig. 1. Immunofluorescence staining of major basic protein (MBP) in an esophageal biopsy specimen. (A) Localization of MBP. MBP was strongly stained green in the esophageal epithelial layer, demonstrating eosinophil granules. The blue is nuclear counterstain. Scale bar denotes 50 μm. (B) Number of MBP-positive cells in the esophageal mucosa. Data were analysed with Mann-Whitney *U* test. The box presents the interquartile range (25% and 75%) from the median (horizontal line). The number of MBP-positive cells did not differ significantly between asymptomatic esophageal eosinophilia (aEE) and eosinophilic esophagitis (EoE) tissue. See color figure in the on-line version.

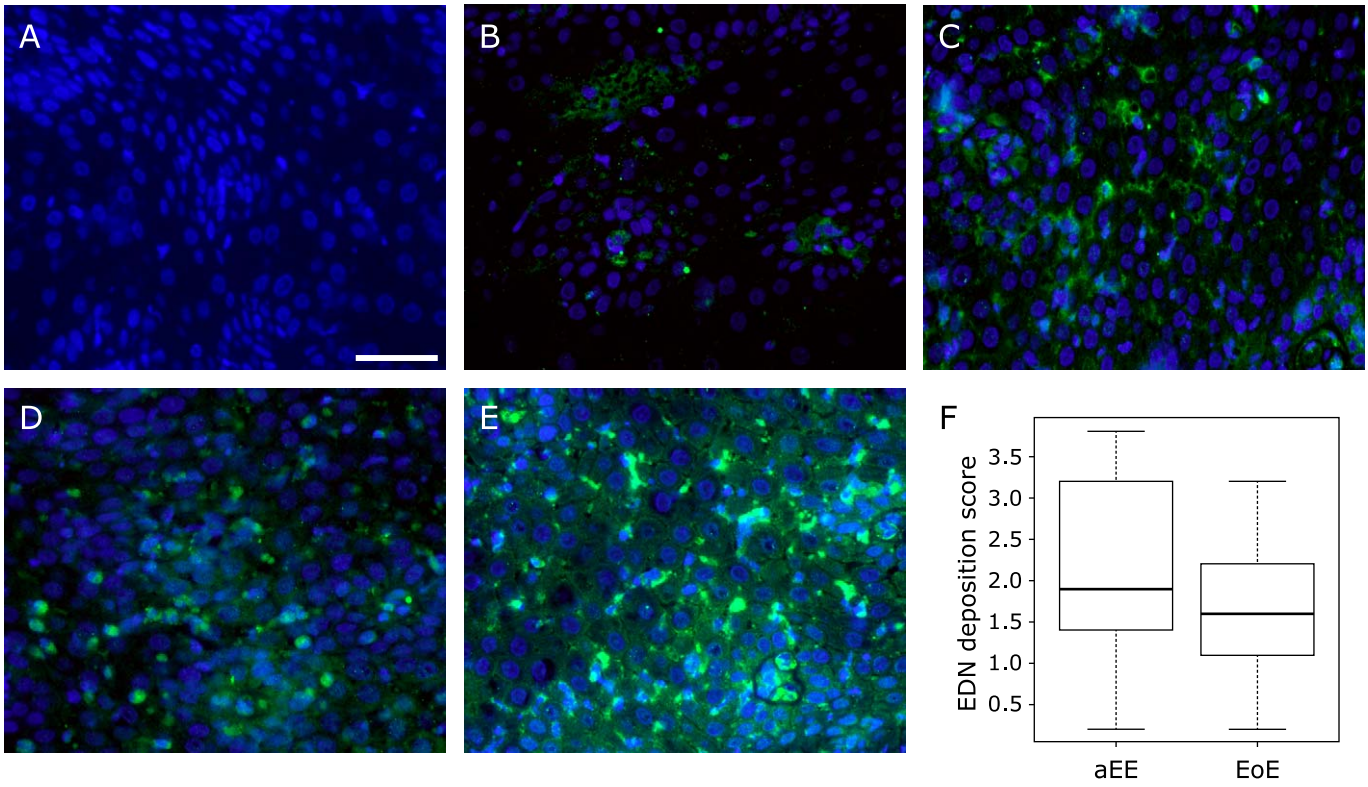

Fig. 2. Immunofluorescence staining for eosinophil-derived neurotoxin (EDN) deposition in the esophageal mucosa and scoring of extracellular EDN deposition. Green shows the localization of EDN with blue nuclear counterstain. (A–E) EDN deposition was categorized into five categories according to the percentage of positive depositions in a microscopic field: (A) grade 0 (none), (B) grade 1 (<25%), (C) grade 2 (25–50%), (D) grade 3 (50–75%), and (E) grade 4 (>75%). Scale bar denotes 50 μm. (F) EDN deposition score in the esophageal mucosa. Data were analysed with Mann-Whitney *U* test. The box presents the interquartile range (25% and 75%) from the median (horizontal line). There were no significant differences on EDN deposition score between asymptomatic esophageal eosinophilia (aEE) and eosinophilic esophagitis (EoE) tissue. See color figure in the on-line version.

(168.6, 274.0) vs 192.3 (164.8, 228.5) cells/HPF, $p = 0.146$].

IgG4 staining. IgG4 staining for each group is shown in Fig. 4. IgG4 was strongly stained in the esophageal epithelial middle layer and extracellular space of epithelial cells (Fig. 4A–E). There were no significant differences in IgG4 deposition score between aEE and EoE [1.0 (0.6, 1.4) vs 1.4 (0.7, 1.7), $p = 0.364$].

Discussion

We found that aEE showed a similar immune profile compared to that of EoE as assessed by immunohistological staining. The expressions of the MBP, EDN, eotaxin-3, and IgG4 inflammatory markers, which were selected based on their roles in the

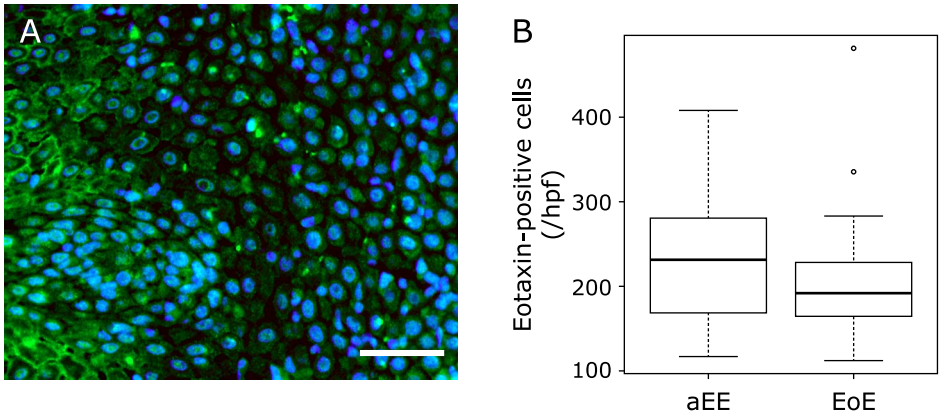

Fig. 3. Immunofluorescence staining for eotaxin-3 in an esophageal biopsy specimen. (A) Localization of eotaxin-3. Eotaxin-3 was stained green in the esophageal epithelial layer. Blue is nuclear counterstain. Scale bar denotes 50 μm. (B) Number of eotaxin-3-positive cells in the esophageal mucosa. Data were analysed with Mann-Whitney *U* test. The box presents the interquartile range (25% and 75%) from the median (horizontal line). The number of eotaxin-3-positive cells between asymptomatic esophageal eosinophilia (aEE) and eosinophilic esophagitis (EoE) tissue was not significant. See color figure in the on-line version.

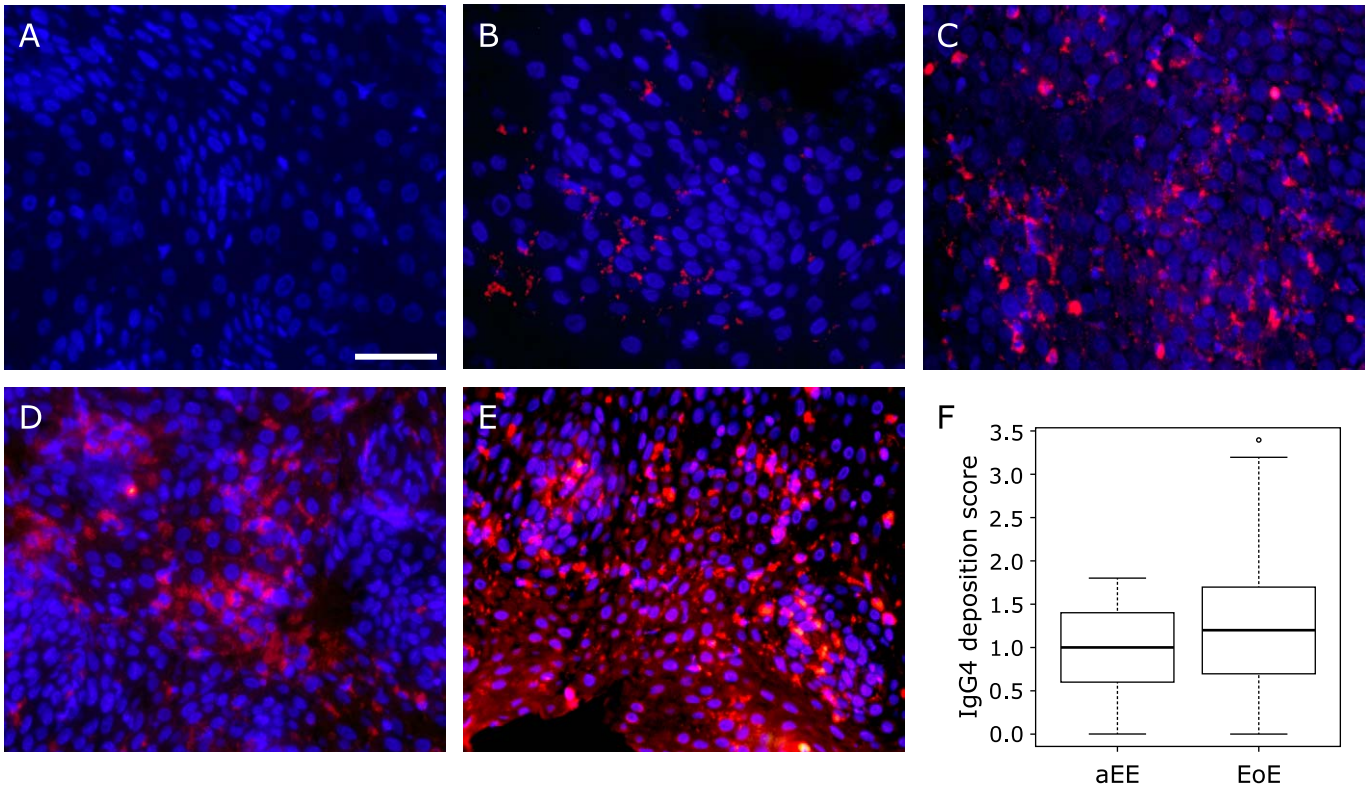

Fig. 4. Immunofluorescence staining for IgG4 deposition in the esophageal mucosa and scoring of extracellular IgG4 deposition. (A–E) Red denotes IgG4 and blue is nuclear counterstain. IgG4 deposition was categorized into five categories according to the percentage of positive depositions in a microscopic field; (A) grade 0 (none), (B) grade 1 (<25%), (C) grade 2 (25–50%), (D) grade 3 (50–75%), and (E) grade 4 (>75%). Scale bar denotes 50 μm. (F) IgG4 deposition score in the esophageal mucosa. Data were analysed with Mann-Whitney *U* test. The box presents the interquartile range (25% and 75%) from the median (horizontal line). There were no significant differences in the IgG4 deposition score between asymptomatic esophageal eosinophilia (aEE) and eosinophilic esophagitis (EoE) tissue. See color figure in the on-line version.

pathogenesis of EoE, were similar between aEE and EoE patients. Thus, it was difficult to distinguish aEE from EoE based on clinical, endoscopic, and immunohistological features. These markers were not associated with clinical symptoms. These results provide evidence of ongoing immunological disease activity in EoE and aEE. EoE and aEE might share some common pathogenic basis. To our knowledge, this is the first study to provide immunohistological data of inflammatory markers for a group of aEE patients compared to that of EoE patients.

There were no significant differences in the clinical characteristics between aEE and EoE patients, except for BMI, which was higher in EoE patients. We previously reported that higher BMI is

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a non-allergic risk factor for EE.[7] BMI is also a well-known risk factor for gastroesophageal reflux disease (GERD). Presently, RE prevalence was higher in EoE than in aEE, though the difference was not statistically significant. As a cause of EE, EoE, and GERD are not mutually exclusive.[1,2] On other hand, another report showed that BMI was not different between EoE and aEE, and the prevalence of concomitant allergic diseases was higher in patients with EoE than that of aEE.[20] Presently, the prevalence of concomitant allergic diseases was not statistically different between the aEE and EoE patients. Because both studies were conducted in a single center, the difference might be based on the study population, rather than the difference of pathophysiology. To clarify this limitation, a multicenter study is warranted. Furthermore, in our results, there were no significant differences on endoscopic findings between EoE and aEE, consistent with a previous study.[20] These results indicated that it was difficult to distinguish EoE and aEE in terms of endoscopic findings as well as clinical characteristics.

Previous reports showed that some patients with EoE have marked depositions of extracellular eosinophil granule proteins, such as MBP and EDN, despite small numbers of eosinophil infiltration.[14,23] However, presently there were no significant differences in MBP and EDN depositions between EoE and aEE patients, which indicated that eosinophil activity was not involved in the perception of esophageal symptoms even if the deposition was widely observed. The expressions of eotaxin-3 were not also different, which indicated that chemoattractant activity for eosinophils was similar between both groups. Furthermore, the similarity of IgG4 expressions might indicate that the activity of plasma cells was the same as the results of eosinophils. Intercellular IgG4 deposition was compatible with previous reports, although it was difficult to observe IgG4-positive plasma cells because these cells were mainly in the deep lamia propria.[11] Taken together, both EoE and aEE share a common pathogenesis.

There are some limitations in this study. First, the assessment of clinical symptoms in patients with aEE was performed by the medical interviewer without using a validated questionnaire because the assessment was done in the setting of a medical health check-up. Second, sampling error could lead to the underestimation of immune depositions. Because the study was done in a medical health check-up setting, there was a limitation on taking multiple biopsy specimens, even though multiple biopsies (at least four biopsies) should be required to evaluate the histological state.[3] Of course, we obtained specimens from areas with the most abnormal endoscopic findings, which were compatible with EE, and sufficient eosinophilic infiltration were observed in all evaluated specimens. Third, we did not evaluate cytokines, such as IL-4 and IL-13, directly. Another study reported that the anti-IL-4 antibody dupilumab reduces dysphagia in adults with EoE;[24] hence, IL-4 might be related to the pathology of aEE. In addition, we evaluated only esophageal mucosa by biopsies, but not muscularis propria. A previous study hypothesized that eosinophils infiltrating into the esophageal muscle might degranulate and release toxic proteins, such as EDN, which will destroy neurons and these events might cause esophageal motility abnormalities that present as obstructive symptoms.[25] Future study is warranted to evaluate immune response including eosinophil activity in muscularis propria.

In conclusion, EoE and aEE had similar immune profile using immunohistological staining. Thus, they may be similar disease entities underlying some common pathogenic mechanisms. We suggest that patients with aEE also have histopathological esophageal inflammation.

Author Contributions

HK: data collection, data analysis, and manuscript writing; FT: data analysis, study design, and manuscript writing; YNadatani, KO, SH, NKamata, KT, YNagami, TT, SF, TW, NKawada, and YF: critical revision of the manuscript for important intellectual content. All authors read and approved of the final manuscript.

Conflict of Interest

No potential conflicts of interest were disclosed.

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