Evidence of an abnormal epithelial barrier in active, untreated and corticosteroid-treated eosinophilic esophagitis

Dagmar Simon¹, Basile Page¹, Monique Vogel², Christian Bussmann³, Carine Blanchard⁴, Alex Straumann⁵, Hans-Uwe Simon⁶

¹Department of Dermatology, Inselspital, Bern University Hospital, University of Bern
²Institute of Immunology, Inselspital, Bern University Hospital, University of Bern
³Division of Pathology, Histopathology Viollier, Basel, Switzerland
⁴Institute of Nutritional Science, Nestlé Research Center, Lausanne, Switzerland
⁵Swiss EoE Research Group, Olten, Switzerland
⁶Institute of Pharmacology, University of Bern, Bern, Switzerland

Correspondence:
Dagmar Simon, MD
Department of Dermatology, Inselspital, Bern University Hospital, 3010 Bern, Switzerland
Tel: +41 31 632 2278; Fax: +41 31 632 2233; e-mail: Dagmar.simon@insel.ch

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Abstract

Background: Eosinophilic esophagitis (EoE) is a chronic, immune/antigen-mediated disease characterized by symptoms related to esophageal dysfunction and an eosinophil-predominant inflammation. This study has aimed to investigate whether the recently observed sensitization to Candida albicans in EoE patients is owing to pre-existing disease and its underlying abnormal epithelial barrier or, alternatively, is linked to corticosteroid (CS) therapy.

Methods: Medical histories, as well as serum and tissue samples of 60 EoE patients (15 CS-naive, 45 with current or previous CS therapy) and 20 controls, stored in the Swiss Eosinophilic Esophagitis Database (SEED) and Biobank, were analyzed. We applied ImmunoCAP to measure IgE levels and immunofluorescence techniques to examine epithelial barrier components.

Results: EoE patients had higher total IgE levels and were more frequently sensitized to Candida albicans than controls. In EoE tissue specimens, increased numbers of eosinophils and mast cells, a higher expression levels of thymic stromal lymphopoietin (TSLP), cathelicidin, proteases, i.e. the kallikreins (KLK)-5 and KLK-7, were observed as compared with controls, while reduced expression of lympho-epithelial Kazal-type-related inhibitor (LEKTI), filagrin, E-cadherin, claudin, occludin, demoglein-1 was found, independent of CS therapy. In CS-treated EoE, significantly lower numbers of CD1a+ cells and cathelicidin expression were noted as compared to CS-naive EoE.

Conclusion: This study provides further evidence that EoE is associated with an abnormal epithelial barrier and postulates that CS therapy, by reducing innate immune mechanisms, may promote Candida albicans colonization and likely subsequent sensitization.

Key words

Candida albicans, cathelicidin, corticosteroids, eosinophilic esophagitis, epithelial barrier
A recent consensus paper on eosinophilic esophagitis (EoE) attached particular significance to antigen-driven immunologic processes involving multiple pathogenic pathways and proposed a conceptual disease definition (1). EoE represents a chronic, immune/antigen-mediated disease characterized by symptoms related to esophageal dysfunction and exhibits an eosinophil-predominant inflammation on histology (1). An association of EoE with atopic diseases such as allergic rhinitis, bronchial asthma and atopic dermatitis has repeatedly been reported (2-4). The observations that IgE sensitization to environmental, including food allergens, is detected in most EoE patients (5, 6) and that EoE improved clinically and histologically upon food elimination diets (7) suggested a food-allergic disease. Recent research, however, has provided evidence that EoE should not be equated with an immunoglobulin (Ig)E-mediated allergy since the pathogenic mechanisms seem to be more complex, involving both innate and adaptive immune responses (8). In addition to food allergens, specific IgE to *Candida albicans* have frequently been observed in pediatric and adult EoE patients (5, 6). Although the pathogenic role of *Candida albicans* in EoE is uncertain, these findings raised the question of what drives the allergic sensitization.

Disturbed epithelial barrier function seems to play a crucial role in the pathogenesis of EoE. In this way, antigens might penetrate the epithelium and initiate inflammatory responses and systemic sensitization. Several components of both tight and adherens junctions, as well as desmosomes, are engaged in constructing the epithelial barrier that is further supplemented by an immunological barrier (9, 10). In EoE patients, an abnormal expression of epithelial cell proteins mainly those involved in adhesion and integrity, such as desmogleins, claudins, cadherins, occludin, filaggrin and keratins as well as antimicrobial peptides and protease inhibitors, has been reported to be associated with esophageal
inflammation (11-15). Esophageal infections with *Candida albicans* have frequently been observed in patients with long-lasting dysphagia (16). Moreover, the use of swallowed corticosteroids (CS) predisposes patients with EoE for infections of the oral cavity with *Candida albicans* (17). So far, it is not clear whether *Candida albicans* colonization and sensitization is the consequence of an epithelial dysfunction associated with the disease and/or is a consequence of the treatment with CS that is the first line therapy for EoE.

In this study, we aimed at investigating the specific IgE response to *Candida albicans* in EoE patients with and without recent CS therapy, and evaluating a variety of components forming the epithelial barrier in order to gain information on its composition as linked to CS therapy in EoE.

**Methods**

**Patients**

Medical histories, as well as serum and tissue samples of 60 patients with EoE verified according to international criteria (1) and 20 controls without esophageal disease who were undergoing diagnostic endoscopy were obtained from the Swiss Eosinophilic Esophagitis Database (SEED) and Biobank. The patients' characteristics are given in table 1. Patients with EoE were assigned to two groups: 1. Active EoE without previous topical or systemic CS therapy (EoE naive; n=15), 2. EoE with current or previous CS therapy (EoE CS-treated; n=45). For evaluating epithelial barrier markers, we further stratified according to patients currently under CS therapy (EoE on steroids) and patients following CS withdrawal (EoE off steroids). The study has been approved by the Cantonal Ethics Committee of Aargau/Solothurn. Written informed consent was obtained from all patients prior to enrollment in the study.

**IgE measurements**

Serum samples stored at -20°C were available from 15 EoE naive, 33 CS-treated and 19 control patients. Serum levels of total IgE and specific IgE to *Candida albicans* were measured using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacture’s protocol.
Esophageal specimens
Biopsies from 21 patients taken for diagnosis or follow-up of EoE, were analyzed (EoE naive: n=9; EoE on steroids: n=6; EoE off steroids (for mean 14 months, range 1-33 months): n=6). Because of the patchy nature of EoE, eight biopsies from the proximal and distal esophagus (each n=4) were analyzed. Normal tissue was obtained from nine patients without esophageal diseases undergoing diagnostic endoscopy.

Immunofluorecence staining
Tissues were fixed in 4% formaldehyde and paraffin-embedded. Cellular infiltration and cytokine expression were analyzed by immunofluorescence techniques as described previously (13, 18) using antibodies directed against eosinophil peroxidase (EPX; Lee Laboratory, Mayo Clinic, AZ, USA), mast cell tryptase (MCT), CD1a (Langerhans cells) (both Dako, Glostrup, Denmark), lympho-epithelial Kazal-type-related inhibitor (LEKTI), cathelicidin LL-37, human beta-defensin (HBD)-3 (all from Novus Biologicals, Littleton, CO, USA), thymic stromal lymphopoietin (TSLP), kallikrein (KLK) 7 (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), KLK5 (Sigma Life Science, St.Louis, MO, USA), filaggrin, claudin, E-cadherin, and desmoglein-1 (all from Abcam, Cambridge, UK). Appropriate secondary antibodies labelled with Alexa Fluor 488 (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) were applied. DNA was visualized with propidium iodide (PI: Molecular probes, Eugene, OR, USA). Evaluation was performed using a confocal laser scanning microscope (LSM 510; Carl Zeiss Jena GmbH, Jena, Germany). Cells were counted in five fields of highest inflammatory activity per biopsy corresponding to a total of 40 fields per patient at a magnification x630. To quantify the intensity of LEKTI, TSLP, KLK5, KLK7, LL-37, HBD-3, filaggrin, claudin-1, desmoglein and cadherin expression, Imaris software version 8.2.0 (Bitplane, Zurich, Switzerland) was used.

Statistics
Mean values with ranges for demographic variables and ±SEM for measured parameters are given. For comparisons between groups, t test (two groups) and one-way ANOVA or Kruskal-Wallis test were applied. P values < 0.05 were
Results
Increased specific IgE to Candida albicans in CS-treated patients
The patient demographics are given in table 1. Noteworthy are the predominance of males, the younger age of EoE patients as compared with control patients (p=0.0175) and the slightly shorter disease duration in EoE patients without any CS therapy as compared to CS-treated patients. All patients with EoE and the majority of patients of the control group had been treated with PPI. EoE patients more frequently had concomitant atopic diseases. Accordingly, the total IgE serum levels were higher in EoE patients than in controls (p=0.0059) (Figure 1). Specific IgE to Candida at serum levels >0.35 kU/l were found in 7/33 EoE patients treated with CS, but not in patients of the CS-naïve EoE or control groups (Supplementary table S1). When we analyzed the absolute levels of IgE to Candida that were detectable in 46/48 EoE patients and 15/19 controls, we found significantly higher levels in EoE patients treated with CS (p=0.0046), suggesting an increased risk of Candida albicans sensitization upon CS therapy (Figure 1).

Reduced inflammation in CS-treated EoE
Eosinophil infiltration is the characteristic histologic feature of EoE (1) and thus high numbers of eosinophils were found in patients with active, CS-naive EoE (Figure 2). In patients under CS therapy, eosinophil numbers were significantly reduced. Mast cells were also significantly elevated in CS-naive EoE as compared with CS-treated EoE and controls (Figure 2). We noted a significant decrease of CD1a+ cell numbers toward levels below that of controls in EoE patients treated with CS. Whereas CD1a+ cells were observed in basal and suprabasal layers of the esophageal epithelium in control specimens, in CS-naive EoE they were found in all layers conglomerated in clusters (Figures 2 and 4B).

TSLP is produced by epithelial cells on surfaces exposed to the environment upon stimulation via protease-activated receptor (PAR)-2 and toll-like receptors (TLR), as well as after mechanical injury; this is believed to drive T-helper 2 type inflammation (19). The expression of TSLP was significantly increased in CS-naive
EoE patients. The low TSLP expression in CS-treated patients suggested direct or indirect effects of CS on TSLP production via suppression of inflammation.

**Reduced expression of cathelicidin and HBD in CS-treated EoE**

Next, we were interested in the expression of antimicrobial peptides such as cathelicidin LL-37 and human beta-defensin (HBD)-3 that have been shown to exert candidacidal and immunostimulatory effects (20-22). In patients with active, CS-naïve EoE, the expression of cathelicidin was significantly increased when compared to CS-treated EoE and controls (p=0.0003) (Figure 3). Noteworthy is the observation that EoE patients on CS therapy had significantly less cathelicidin expression than CS-naïve patients. Furthermore, we observed a significantly reduced HBD-3 expression by epithelial cells in EoE as compared to controls (p<0.0001) that was still further reduced in CS-treated patients (Figure 3).

**Low protease inhibitor, but high protease expression, in EoE; partial correction with CS treatment**

A balance between proteases and their inhibitors is essential for epithelial homeostasis and barrier function (23, 24). A reduced expression of filaggrin that is cleaved by serine proteases under the control of LEKTI (23), in EoE has previously been reported (13). Our results confirm the significantly lower expression of filaggrin in EoE as compared to controls (Figure 3). Interestingly, CS therapy did not have any effect on the filaggrin expression, thus suggesting that lowered filaggrin expression was owing to an effect of the disease rather than a consequence of therapy.

In agreement with previous observations (13), we found significantly decreased expression of LEKTI in CS-naïve EoE as compared to controls (Figure 3). In EoE patients with CS therapy, a slight increase of LEKTI was noted, suggesting that CS, most likely by confining the inflammation locally, augments LEKTI expression. In the opposite direction from LEKTI effects, we observed an increased expression of the proteases KLK5 and KLK7 in EoE patients, in particular in CS-naïve EoE patients, as compared to controls.
Decreased expression of intercellular junction proteins in EoE; no effect of CS

The complex system of intercellular junctions such as tight and adherence junctions, as well as desmosomes, is crucial for the epithelial barrier function (9). We studied the expression of intercellular junction proteins and observed a significantly decreased expression of E-cadherin ($p=0.0076$), claudin ($p<0.0001$), occludin ($p<0.0001$) and desmoglein-1 ($p<0.0001$) in EoE compared to control esophagus specimens (Figure 4). CS therapy did not have any effect on the expression of intercellular junction proteins, suggesting a disease-immanent low expression of these barrier components in EoE.

Furthermore, we observed sparse occludin adherens junctions in the upper epithelial layers of EoE specimens. In CS-naive EoE, but not CS-treated EoE, dendrites of LHC reached to this level and penetrated between cells. In normal esophagus only few LHC were found in the basal layers, separate from occludin which was abundantly distributed on epithelial cells in the upper layers (Figure 4B).

Discussion

Based on the observation that EoE patients were frequently sensitized to *Candida albicans*, we aimed to identify predisposing conditions and to investigate the composition of the esophageal epithelial barrier in patients with and without CS therapy. This study has revealed an increased expression of the antimicrobial peptide cathelicidin, but reduced HBD3, a decreased expression of LEKTI, while that of the proteases KLK5 and KLK7 was increased. Furthermore, we found a significantly lower expression of junctional proteins E-cadherin, claudin, occludin and desmoglein-1 in EoE patients as compared to controls. Strikingly, in CS-treated EoE patients, the number of LHC was below that found in controls, and cathelicidin expression was lower than in CS-naive EoE.

Most patients with EoE have elevated total IgE levels and associated atopic diseases (4). However, the preponderance of patients with elevated IgE levels (>0.35 kU/l) and the high mean levels of IgE specific for *Candida albicans* in EoE patients that had been treated with CS, were striking findings. In amendment with previously published data on *Candida albicans* sensitization in pediatric and adult
EoE patients (5, 6), the results of this study suggest CS therapy as an additional risk factor for *Candida albicans* sensitization.

In several trials, CS therapy has been shown to improve clinical and endoscopic signs as well as symptoms in most EoE patients (17, 25, 26). Accordingly, we observed less inflammation as reflected by low numbers of eosinophils and mast cells as well as TSLP expression in the esophageal tissue in patients under current or previous CS therapy. The number of LHC was significantly reduced in CS-treated as compared to CS-naïve patients with EoE. CS have been reported to affect the viability, maturation and immune function of epidermal LHC that are potent antigen-presenting cells and are required for an efficient T cell response to *Candida albicans* (27-29). In atopic dermatitis, activated LHC were shown to penetrate the tight junctions and were likely able to capture antigens from the outside (30). A similar observation was made in CS-naive EoE, but not CS-treated EoE. To summarize, CS therapy might directly affect LHC and thus promote *Candida albicans* infection and subsequent sensitization.

Cathelicidin LL-37 and HBD-3 exert antimicrobial activities against *Candida albicans* by inhibiting adhesion, disrupting its cell membrane, and initiating further immune responses (20, 21, 31). HBD-3 that was found to be highly expressed in normal esophagus (32) was decreased in all EoE patients, even in those treated with CS, in agreement with previous observations (33). Cathelicidin was highly expressed in specimens of CS-naive patients, but less so in tissues of CS-treated patients. These findings suggest an increased cathelicidin production in EoE that is reduced upon corticosteroid therapy. Thus, CS are assumed to inhibit antimicrobial peptide, in particular cathelicidin, production resulting in a reduced antimicrobial defense at the esophageal epithelial surface followed by a colonization and infection by *Candida albicans*.

The minor expression of filaggrin in the esophagus compared to skin (13) was further reduced in EoE tissue irrespective of CS therapy. The expression of both filaggrin and zonula occludens-3 were shown to negatively correlate with spongiosis in EoE (15). Recently, a deficit of the protease inhibitor LEKTI was shown to increase protease activity that might result in PAR-2 activation, subsequent TSLP
production, the influx of eosinophils and mast cells and atopic dermatitis-like lesions (34). Our results lead us to assume a similar scenario in EoE with low LEKTI, but increased KLK-5 and KLK-7 expression, elevated TSLP as well as eosinophil and mast cell infiltration of the esophagus. A dysregulated KLK expression has been demonstrated in esophageal epithelial cells exposed to IL-13 and in EoE (35).

The significantly reduced expression of the tight and adherens junction proteins E-cadherin, claudin and occludin as well as desmoglein-1 in EoE as compared to non-inflamed control esophagus, suggested a defective expression owing to the disease rather than CS therapy. Even so, the expression was similar in CS-naive and CS-treated EoE assuming that reducing inflammation by CS had no effect on these barrier proteins. The discrepancy to recently reported normal desmoglein-1 expression in inactive EoE might be due to the selection of patients and the protocol for staining analysis (36). The impact of a decreased desmoglein-1 expression on the impaired epithelial barrier structure and function in EoE has been demonstrated (11). These authors did not observe a decreased expression of tight and adhesion junction proteins in active EoE (11), in contrast to our findings and those of others (12, 15). Recently, an increased expression of genes encoding for desmoglein-1 and filaggrin upon therapy with fluticasone or elemental diet in association with an improved functional esophageal mucosal integrity has been reported (37, 38). Notably, Candida albicans itself may contribute to tissue damage by secreting aspartic proteinases that detaches intercellular junctions such as desmosomes (39) and by enhancing the degradation of E-cadherin (40, 41).

Taken together, our study provided further evidence for an disease-inherent impaired epithelial barrier in EoE that could be only partially restored in patients treated with CS. Thus, we hypothesize the following scenario: A decreased expression of LEKTI leads to an increased activity of KLK5 and KLK7 that, by activating the PAR2 receptor on epithelial cells, induces TSLP production. In addition to kallikreins, a decreased expression of intercellular adhesion proteins results in a defective epithelial structure. The impaired epithelial barrier promotes the colonization by Candida albicans that further detaches junction proteins and thus assists microbial invasion followed by innate and adaptive immune responses. TSLP is able to stimulate eosinophils to generate extracellular DNA traps that directly kill...
microbes, but may also initiate a T helper type 2 inflammation involving dendritic cells, T cells and B cells and subsequent sensitization to *Candida albicans*. CS seem to have selective effects on improving the epithelial barrier. Instead, CS reduce the expression of cathelicidin as well as the numbers of LHC and eosinophils, thereby possibly even further promoting *Candida albicans* colonization and invasion.

**Acknowledgment**

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**References**


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Figure legends

Figure 1  Levels of total IgE and Candida albicans-specific IgE. Bars represent mean values (+SEM) of serum IgE of EoE patients without (black, n=15) or with corticosteroid therapy (grey, n=33) compared to controls (white, n=19). *, p<0.05; **, p<0.01.

Figure 2  Eosinophilic inflammation is striking in corticosteroid (CS)-naive, but reduced in CS-treated EoE patients. The graphs show mean numbers (+SEM) of eosinophils, mast cells, and Langerhans (CD1a+) cells as well as expression of TSLP in esophageal biopsy specimens of CS-naive (n=9), CS-treated (n=6), previously CS-treated (n=6) EoE patients and controls (n=9). *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. Representative images of immunofluorescence staining are shown. Magnification x630 (cells), x400 (TSLP), bars 20 μm.

Figure 3  Abnormal expression of antimicrobial peptides, filaggrin, proteases and protease inhibitor in EoE. The graphs show mean (+SEM) expression of cathelicidin, HBD3, filaggrin, LEKTI, KLK5 and KLK7 in esophageal biopsy specimens of CS-naïve (n=9), CS-treated (n=6), previously CS-treated (n=6) EoE patients and controls (n=9). Representative images of immunofluorescence staining are given. Magnification x400, bars 20 μm.

Figure 4  Abnormal expression of junctional proteins in EoE. Graphs in panel A show mean (+SEM) expression of E-cadherin, claudin, occludin and desmoglein-1 in esophageal biopsy specimens of CS-naïve (n=9), CS-treated (n=6), previously CS-treated (n=6) EoE patients and controls (n=9). Representative images of immunofluorescence staining are shown. Magnification x400, bars 20 μm. Panel B shows Langerhans cells (LHC, CD1a+, green) in basal and abundant occludin in upper epithelial layer in controls, but few LHC and scarce occludin expression in CS-treated EoE. In CS-naïve EoE, LHC are spread through the entire epithelium and their dendrites penetrate the upper cell layers between tight junctions (occludin, red) (insert). Magnification x1000.
Figure 1
Figure 2
Figure 3

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Figure 4