Coordinate Interaction between IL-13 and Epithelial Differentiation Cluster Genes in Eosinophilic Esophagitis


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Coordinate Interaction between IL-13 and Epithelial Differentiation Cluster Genes in Eosinophilic Esophagitis


We have previously proposed that the pathogenesis of eosinophilic esophagitis (EE) is mediated by an IL-13–driven epithelial cell response associated with marked gene dysregulation including eotaxin-3 overproduction. In this study, we compared epithelial responses between healthy patients and those with EE, aiming to uncover molecular explanations for EE pathogenesis. Esophageal epithelial cells could be maintained for up to five passages, with 67% and 62% of cell lines reaching confluence in healthy controls and EE cases, respectively. Both sets of epithelial cells avidly responded to IL-13 at similar levels as assessed by eotaxin-3 production. Acidic pH increased cellular release of eotaxin-3 (4.6 ± 1.98 ng/ml versus 12.46 ± 2.90 ng/ml at pH 7.4 and 4, respectively; p < 0.05). Numerous epidermal differentiation complex (EDC) genes, such as filaggrin and SPRR3, were downregulated both in IL-13–stimulated esophageal epithelial cells and in EE biopsies specimens compared with healthy controls. Whereas the filaggrin loss of function mutation 2282del4 was overrepresented in EE compared with control individuals (6.1%), eotaxin-3 was upregulated (p = 0.0172), the decreased filaggrin expression was uniformly seen in all EE cases in vivo. Indeed, expression of the EDC genes filaggrin and involucrin was strongly decreased directly by IL-13. These results establish that the epithelial response in EE involves a cooperative interaction between IL-13 and expression of EDC genes. The Journal of Immunology, 2010, 184: 000–000.

Eosinophilic esophagitis (EE) is a complex atopic disorder of the esophageal mucosa characterized by allergen-induced eosinophilic infiltration and epithelial cell hyperplasia (1). Yet the growth characteristics of normal (NL) and EE epithelial cells in vitro have not been studied, and there are limited data regarding the intrinsic properties of eosinophilic epithelial cells in patients with EE (1). Patients with EE typically exhibit symptoms that mimic gastroesophageal reflux disease (GERD), but GERD and EE are distinguished by the lack of histologic response to acid suppression therapy in EE (2). The

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The online version of this article contains supplemental material.

Abbreviations used in this paper: AD, atopic dermatitis; DN, dominant-negative; EDC, epidermal differentiation complex; EE, eosinophilic esophagitis; EV, empty vector; GERD, gastroesophageal reflux disease; hpf, high-power field; NL, normal; ns, nonsignificant; OR, odds ratio; SPRR, small proline-rich protein.

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EDC GENE REGULATION IN EOSINOPHILIC ESOPHAGITIS

mutations have been associated with AD (17–23); such mutations are only present in 0–5% of control individuals and 9–27% of AD individuals, depending upon the study (17, 24).

We have recently proposed that IL-13 is a key cytokine in EE disease pathogenesis. In particular, esophageal epithelial cells express all components of the IL-13 receptor including IL-4Rα, IL-13Rα1, and IL-13Rα2 (25). In addition, IL-13 induces prominent dysregulation of gene expression in the esophageal epithelium including marked overexpression of eotaxin-3, the most strongly induced IL-13 target gene, which is also highly overexpressed in vivo in the EE esophageal transcriptome (25). Furthermore, intratracheal IL-13 induces features of experimental EE in mice, and IL-13– and STAT6- deficient mice are protected from the development of experimental EE (26–28). A recent study has shown that in patients with AD, some EDC genes might be downregulated by IL-13 through a STAT6-dependent mechanism (29). In our initial analysis of the EE transcriptome, we have recently noted that filaggrin mRNA was dramatically decreased in the esophagus of EE compared with NL biopsy specimens using microarray analysis (30), suggesting abnormal epithelial differentiation in EE. In this study, we aimed to determine whether there is an intrinsic defect in epithelial cell responses in EE and to further determine which features of the disease can be mediated by IL-13. We report a profound dysregulation of EDC gene expression, identify the effects of IL-13 on EDC gene expression and the presence of gene variants in the EDC gene filaggrin in patients with EE.

Materials and Methods

Cell lines and primary cell culture

Primary esophageal epithelial cells were grown as previously described (25). Human esophageal epithelial cell line (TE) and squamous epithelial cells were provided by Dr. Hainaut (International Agency for Research on Cancer, Lyon, France) and were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Atlanta, J0138, Advantage) and 1% penicillin/streptomycin/ampicillin (Invitrogen). In preliminary studies, we optimized the culture conditions by modifying the source of the FBS, which had a potential effect on cell growth and responses (Supplemental Fig. 1). For primary cell culture, one or two distal esophageal biopsies were collected during routine endoscopy and with informed consent as approved by the institutional review board. The eosinophil level in each patient was quantified and corresponds to the maximum or peak eosinophil found in one high-power field (hpf). All sections of every biopsy were processed and counted. The samples were cut into sections and modified F-media (3:1 F-12/DMEM) supplemented with 5% FBS (Atlanta, J0138, Advantage), adenine (24.2 μg/ml), cholecalciferol (10 μM), insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), and human epithelial growth factor (10 ng/ml) in the presence of penicillin, streptomycin, and amphotericin (Invitrogen). Tissue was first digested twice with trypsin, followed by trypsin neutralization in F-media without epidermal growth factor. The cells were then plated onto 1.5 × 10^5 NH 3T3 J2 feeder cells irradiated with 6000 rad. The next day, the media was exchanged with F-media containing epidermal growth factor (10 ng/ml) and fresh media were added every other day. NL and EE patient-derived cells were cultured for 2 wk in this way, and feeder fibroblasts were then removed by differential trypsinization. Cells were stimulated with IL-13 (0, 1, 10, 100 ng/ml) obtained from Peprotech (Rocky Hill, NJ), as described (25). For acidification experiments, a pH 6 solution of HCl/NaCl (300 mosmol/kg H2O) or a pH 7.4 solution of NaCl (300 mosmol/kg H2O) was added for 4 h. After IL-13 stimulation, 250 μl saline or HCl/saline was added for 4 h. Supernatants were harvested and neutralized, and volumes were adjusted before eotaxin-3 quantification. Viable cells were counted using a hemocytometer and trypan blue staining.

RT-PCR

Total cellular RNA was extracted with Trizol (Invitrogen) according to the manufacturer’s instructions. The samples were DNase treated, and first strand cDNA was synthesized using Superscript II (Invitrogen). Total RNA from biopsy samples was extracted using the Mini RNA Extraction Kit (Qiagen, Valencia, CA), and reverse transcription was performed using Iscript (Bio-Rad, Hercules, CA). Real-time PCR was performed by rapid-cycling using the IQ5 (Bio-Rad) SYBR mix (Bio-Rad) as a ready-to-use reaction mix according to the manufacturer’s instructions. The following primers set were: GAPDH (350 bp), 5′-TGGAAAATCCCATCACCATC-3′ and 5′-GATGTGCTAGCCCTGATGTTG-3′; Involution (378 bp), 5′-GTTCCCTCCTCTGCACTAACCCTATC-3′ and 5′-CTTCACTCCAGTTGCTCCTCTC-3′; Eotaxin-3 (125 bp), 5′-GGAATTTTTCTTCTGTCCTG-3′ and 5′-ATCTGAGAGAAGAACCCCTCTC-3′; and 5′-AATCCGAA-CAATGTGCTAGCCTGAG-3′. PCR products were sequenced using the Cincinnati Children’s Hospital Medical Center (Cincinnati, OH) sequencing core facility.

Chromosome location

The probe sets of the EE transcriptome and IL-13–induced genes were subjected to David ontology 2.0 DAVID (database for annotation, visualization, and integrated discovery) and expression analysis systematic explorer analyses, a Web-based (http://david.abcc.nim.nih.gov/) application (Laboratory of Immunopathogenesis and Bioinformatics, SAIC-Frederick, Frederick, MD) that allows access to a relational database of functional annotations (31, 32). Chromosome locations of the transcripts were obtained, and p values were calculated based on the total number of genes present on each chromosome. Microarray data are available online at www.ncbi.nlm.nih.gov/projects/geo; the accession number is GSE88553 (25).

Filaggrin mutation genotyping

Patient DNA was isolated from saliva, peripheral blood leukocytes, or oral buccal swab samples. The history of allergic diseases (asthma, AD, atopic rhinitis) was recorded in our EE population. EE was defined as ≥24 eosinophils per hpf in at least 1 hpf of the esophageal biopsy as previously described (33). For R501X, the following primers were used 5′-TCG GAG GAA GAC AAG GAT CG-3′ and 5′-TTG TCT GCT TGC ACT GTG G-3′ to amplify a 245-bp fragment as previously described (34). The PCR product was digested for 1 h with NlaIII at 37°C, and electrophoresis was performed on a 4% agarose gel with ethidium bromide. An allele with no mutation digested into two fragments of 213 bp and 32 bp. An allele with the R501X mutation digested into three fragments of 176 bp, 37 bp, and 32 bp. For 2282del4, the following primers were used 5′-AAT AGG CTC GAG TCA GGT-3′ and 5′-GATGTGCTAGCCCTGATGTTG-3′; Involucrin (378 bp), 5′-GTTCCCTCCTCTGCACTAACCCTATC-3′ and 5′-CTTCACTCCAGTTGCTCCTCTC-3′; and 5′-AATCCGAA-CAATGTGCTAGCCTGAG-3′. PCR products were sequenced using the R501X mutation digest into three fragments of 213 bp, 37 bp, and 32 bp. For 2282del4, the following primers were used 5′-TCG GAG GAA GAC AAG GAT CG-3′ and 5′-TTG TCT GCT TGC ACT GTG G-3′ to amplify a 245-bp fragment as previously described (34). The PCR product was digested for 1 h with NlaIII at 37°C, and electrophoresis was performed on a 4% agarose gel with ethidium bromide as previously described (17–23).

Statistical analysis

Statistical significance comparing different treatments or groups was determined by the Student t test (normal distribution equal variance), Welch t test (normal distribution, unequal variances), Mann-Whitney U test (nonparametric test, two groups), or ANOVA and Kruskal-Wallis test followed by a Dunn’s multiple comparison test (nonparametric test, three groups or more) using Prism 5 Software (GraphPad, San Diego, CA). For the casecontrol study, χ², odds ratio (OR), and 95% confidence interval were calculated using Prism 5 Software (GraphPad).

Results

Growth characteristics of primary epithelial cells from NL and EE patients

We aimed to determine whether primary esophageal epithelial cells from NL and EE patients had similar growth characteristics. The percentage of cells able to reach 80–100% confluency by 35 d was similar with 67 and 62% of NL and EE cell lines, respectively (Fig. 1A). No differences were found in the number of cells at confluence or after 35 d in culture with 1.8 ± 1.3 × 10^5 and 1.3 ± 1.0 × 10^5 in NL and EE cells, respectively (Fig. 1B). An average of 19 ± 8 d was sufficient to reach confluence (80–100%) in the NL group compared with 21 ± 8 d in the EE group (Fig. 1C).

Epithelial cell responses to IL-13 and pH

To test whether epithelial cells from NL and EE individuals have the same propensity to respond to IL-13, cells were treated with IL-13 (0–100 ng/ml). The fold change of eotaxin-3 mRNA expression was 281 ± 513 and 64 ± 84-fold increase at 1 ng/ml, 7783 ± 14,689 and 2893 ± 2381-fold increase with IL-13 at 10 ng/ml and 34,174 ± 23,974 and
22,910 ± 24,389-fold increase with IL-13 at 100 ng/ml in NL and EE cells, respectively (Fig. 1D). Eotaxin-3 secretion was dependent on the culture conditions, especially the type of serum present (Supplementary Fig. 1). In addition, eotaxin-3 secretion into the supernatant was affected by proton concentrations. In particular, 4.6 ± 2.0 ng/ml and 12.4 ± 2.9 ng/ml were released into the supernatant of EE-derived cells stimulated with IL-13 (100 ng/ml) when cells were incubated 4 h at pH 7.4 and 4, respectively, although cell viability was not affected (data not shown). In addition, eotaxin-3 release was increased by heparin, as 12.9 ± 3.8 ng/ml eotaxin-3 was obtained after heparin treatment compared with the 4.6 ± 2.0 ng/ml in the absence of heparin. Similar results were obtained in primary epithelial cell cultures derived from NL individuals (Fig. 1E). These results suggest that the esophageal responses in EE may be a cell-extrinsic phenomenon in the presence of IL-13 and microenvironmental components, such as the levels of protons and heparin, affect eotaxin-3 levels.

**Downregulation of EDC genes in EE**

The EE transcriptome, defined as the 574 probes significantly different (p < 0.001) in esophageal biopsies of EE patients compared with NL patients (30), was analyzed for the chromosomal locations of the respective genes. Chromosome 1 was the most significantly (p = 0.0002) affected chromosome with 48 genes altered (corresponding to 61 probes, or 10.6% of the EE transcriptome; Fig. 2A). We noted the modulation of several EDC genes (such as filaggrin) clustered on chromosome 1q21 (12), a region linked with atopy (36). Using microarray results in NL and EE patient biopsies, we studied the expression pattern of the EDC genes at the location 1q21 (Fig. 2B). Most of the genes in this cluster showed significant decreased expression or trends toward decreased expression (Fig. 2B). Filaggrin mRNA was downregulated 16-fold in EE compared with normal individuals (Fig. 3A). We verified our microarray findings using real-time PCR by examining normal and EE patients to determine the relative expression of filaggrin in different disease states. Using a cohort of 144 individuals, filaggrin mRNA level was downregulated by 16-fold in patients with active EE compared with healthy patients. The esophageal specific SPRR gene esophagin (SPRR3) was highly expressed in the esophagus and significantly decreased 6-fold in patients with EE compared with healthy patients (Fig. 3B). In contrast, the EDC marker involucrin was not significantly downregulated in patients with EE compared with...
healthy patients (0.58 [0.017 - 0.086] and 0.13 [0.057 - 0.49], respectively; median [25–75 interquartile]; Fig. 3C). These data show that the esophagus of patients with EE has dysregulated expression of select EDC genes, including filaggrin and SPRR3.

Effect of therapy on EDC gene expression

We examined filaggrin expression in treated patients with EE to gain molecular insight into the reversibility of the EDC gene expression changes (Fig. 3A). In patients with EE who responded to
therapy (glucocorticoids and/or diet modification), the filaggrin expression level was not significantly different compared with the control level and was significantly \( p < 0.01 \) different compared with active EE. The same trend as filaggrin expression was observed with \( \text{SPRR3} \) gene expression; no significant decrease in involucrin gene expression in healthy patients versus patients with active or inactive EE was observed (Fig. 3B, 3C). These results show that abnormal EDC gene expression in EE is reversible following successful therapy.

**Association of EE with filaggrin gene polymorphisms**

We propose that the observed decrease in filaggrin mRNA levels could reduce filaggrin function and cause associated disease phenotypes; this is consistent with the finding that filaggrin levels normalized after successful therapy. However, two loss-of-function genetic variants (R501X and 2282del4) in the filaggrin gene have recently been associated with AD; these may not affect the level of filaggrin mRNA, but instead the resulting inactive truncated proteins may compromise epidermal integrity and barrier function. Interestingly, no significant difference in filaggrin mRNA expression was observed between allergic and nonallergic patients with active EE (Fig. 4; Supplemental Table II).

![FIGURE 4. Filaggrin expression in patients with active EE with or without allergic symptoms or sensitization. Filaggrin expression was assessed by real-time PCR and normalized to GAPDH in nonallergic (\( n = 7 \)) and allergic patients with EE (\( n = 25 \)). Each data point corresponds to a patient and the lines show the median with the interquartile range.](image)

**Table I. 2282del4 and R501X mutations in the EE and control population**

<table>
<thead>
<tr>
<th>Deletion 2282del4</th>
<th>Patients with EE (( n = 329 ))</th>
<th>Controls (( n = 157 ))</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>309 (93.9%)</td>
<td>155 (98.7%)</td>
<td>( p = 0.0172 )</td>
</tr>
<tr>
<td>Het</td>
<td>20 (6.1%)</td>
<td>2 (1.3%)</td>
<td>( p &lt; 0.05 ) ; OR [CI], 5.0 [1.157-21.74]</td>
</tr>
<tr>
<td><strong>Allele frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No deletion</td>
<td>638 (97.0%)</td>
<td>312 (99.4%)</td>
<td>( p = 0.0185 )</td>
</tr>
<tr>
<td>Deletion</td>
<td>20 (3.0%)</td>
<td>2 (0.6%)</td>
<td>( p = 0.4591 )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SNP R501X</th>
<th>Patients with EE (( n = 339 ))</th>
<th>Controls (( n = 164 ))</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>324 (96.2%)</td>
<td>159 (97.0%)</td>
<td>( p = 0.4591 )</td>
</tr>
<tr>
<td>CT</td>
<td>15 (4.4%)</td>
<td>5 (3.0%)</td>
<td>( p = 0.4637 )</td>
</tr>
<tr>
<td>C</td>
<td>663 (97.8%)</td>
<td>323 (98.5%)</td>
<td>( p = 0.4637 )</td>
</tr>
<tr>
<td>T</td>
<td>15 (2.2%)</td>
<td>5 (1.5%)</td>
<td>( p = 0.4637 )</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism.

We genotyped 164 controls and 365 patients with EE for these mutations. Reliable genotype data for 2282del4 were obtained in 329 of the 365 patients with EE and 157 of all the 164 controls. We found 6.1% heterozygotes for 2282del4 in the EE group and 1.3% in the control group, corresponding to 3% and 0.6% allele frequency in the EE and control group, respectively (Table I). Of the patients with heterozygote EE with the deletion, 60% had a history of eczema and 70% of food allergy (Supplemental Table I). Significant associations (\( p = 0.0172 \); OR, 5.016; and \( p = 0.0185 \); OR, 4.89) were found between this single nucleotide polymorphism and EE. No overlap of mutations was found between heterozygote patients; a total of 11% of our EE population had one or the other mutation compared with 4.3% in the control population (\( p = 0.0363 \), Table II). The prevalence of atopy (Table III) was similar across the EE R105X positive, the EE 2282del4 positive, and EE WT groups (86.6%, 85.0%, and 83.3%, respectively). The prevalence of AD was 33%, 60%, and 44% (\( p = 0.11 \)) across the EE R105X positive, the EE 2282del4 positive, and EE WT groups, respectively. Interestingly, 2282del4 mutation was still associated with EE when EE patients without any history of AD were analyzed (\( p = 0.0315 \); Table III). As such, while filaggrin mutations (2282del4 and R501X) were increased in EE compared with control individuals; they were present in only 11% of patients with EE, suggesting that germline mutations in the filaggrin gene are not the primary etiology of filaggrin reduction.

**Involvement of IL-13 in EDC gene expression**

We next aimed to investigate the effect of IL-13 on epithelial cell growth and differentiation. Chronic exposure of esophageal epithelial cells to IL-13 (100 ng/ml) for 9 d increased cell growth modestly (mean \( \pm \) SD, 44.5 \( \pm \) 3.1 to 65.2 \( \pm \) 11.4 \( \times \) 104 cells; \( p < 0.05 \); Fig. 5A). In contrast, a similar stimulation with IL-13 for 48 h did not increase the cell number (data not shown). We aimed to determine whether the expression of the EDC genes such as involucrin, \( \text{SPRR3} \), and filaggrin were downregulated by IL-13 (Fig. 5B–D). Microarray analysis revealed that filaggrin, involucrin, and \( \text{SPRR4} \), 1A, and 3 were significantly downregulated in IL-13 treated cells. Using real time PCR, IL-13 (100 ng/ml) decreased filaggrin and involucrin mRNA expression by more than 9-fold and 5-fold in primary esophageal epithelial cells, respectively (Fig. 5C, 5D). Interestingly, pretreatment with glucocorticoids was not able to inhibit IL-13 downregulation of filaggrin mRNA (data not shown). Using a dominant negative form of STAT6, we examined the role of STAT6 in the decreased expression observed after IL-13 stimulation. Expression of a dominant negative form of STAT6 partially rescued the IL-13 mediated repression of involucrin (Fig. 5E), suggesting that STAT6 functionally contributes to IL-13–regulated involucrin expression in esophageal epithelial cells.
regulated by IL-13, the increased number of involucrin-expressing cells might be responsible for the involucrin mRNA expression level not significantly changing in EE biopsy samples. We have previously shown a strong overlap between the IL-13-stimulated esophageal epithelial cell transcriptome and the EE gene signature, suggesting a major involvement of IL-13 in EE (25, 30). Chronic exposure of esophageal epithelial cells to IL-13 induces a modest increase in cell number in vitro. IL-13 has been shown in vivo to induce esophageal epithelial hyperplasia in mice (38). Our results also demonstrate that NL and EE epithelial cells both avidly respond to IL-13 by overexpressing eotaxin-3. Although these results are representative of only one gene, they suggest that the IL-13/IL-4 receptor/STAT6 pathway is not dramatically altered in NL versus EE. Our data are thus in line with the increase in IL-13 levels in EE biopsies previously reported (25) and argue against the pathogenic involvement of keratinocyte-intrinsic defects. In addition, filaggrin is restored to normal levels when patients respond to glucocorticoids. This glucocorticoid effect is most likely caused by the decrease of IL-13 in vivo, as in vitro experiments have shown that glucocorticoids do not modify IL-13–induced filaggrin expression in esophageal epithelial cells (data not shown). Murine models have shown the potentially important role of IL-13 in EE, as IL-13–deficient mice have a decrease in eosinophil infiltration. Similar observations are seen in IL-4/IL-13 double-deficient and STAT6 deficient mice (27). EE is thus a multifactorial disease that certainly involves IL-13, (at least in part), but also numerous other components (genetic, immunologic, or environmental) that may partially overlap in their role and function in EE pathogenesis.

Acid reflux and reflux diseases are confounders in EE diagnosis, and acid neutralization resolves esophageal eosinophilia in some patients (2). The release of eotaxin-3 by protons may explain why proton pump inhibitors often have partial clinical effects in EE (1, 2). Protons are known to modify barrier function of the squamous cell barrier.

### Discussion

In this study, we compared epithelial responses between healthy patients and those with EE to uncover molecular explanations for EE pathogenesis. Esophageal epithelial cells from both sets grew with comparable characteristics and avidly responded to IL-13 at similar levels. Numerous EDC genes, such as filaggrin, and SPRR3 were downregulated in both IL-13–stimulated esophageal epithelial cells and EE compared with NL biopsies specimens. Whereas a filaggrin loss of function mutation (2282del4) was overrepresented in a subset of patients with EE compared with control individuals, the acquired decrease in filaggrin expression was uniformly seen in all patients with EE in vivo, suggesting a generalized importance. The EDC genes filaggrin and involucrin expression were differentially increased during esophageal epithelial cell differentiation (Supplemental Fig. 2) and strongly decreased by IL-13.

In our cell culture condition, NL- and EE-derived biopsy specimens had similar growth characteristics. These results suggest that the esophageal hyperplasia observed in EE may result from an extrinsic phenomenon. It has been shown that esophageal epithelial cells differentially express a multitude of genes in EE compared with healthy controls (30). Whereas the upregulated genes include proliferation markers, such as Ki67, the downregulated genes include members of the EDC (33, 37). In patients with EE, there is severe epithelial cell hyperplasia, and the number of epithelial cells with early esophageal differentiation markers (e.g., involucrin) is increased. In addition, there is a decrease in occurrence of well-differentiating cells. This finding may explain partially why the involucrin gene, expressed in early differentiating cells, is not significantly decreased in EE. In contrast, filaggrin, expressed in well differentiating cells, is highly downregulated in patients with EE. Although both are downregulated in patients with early esophageal differentiation markers (e.g., involucrin), the increased number of involucrin-expressing cells might be responsible for the involucrin mRNA expression level not significantly changing in EE biopsy samples.

### Table II. Combined analysis of R501X and 2282del4 mutation in EE and controls

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EE Population (n = 365)</th>
<th>Controls (n = 164)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>R501X</td>
<td>15 (4.7%)</td>
<td>5 (3.0%)</td>
<td>NS</td>
</tr>
<tr>
<td>2282del4</td>
<td>20 (6.3%)</td>
<td>2 (1.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>R501X and 2282del4</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>R501X or 2282del4</td>
<td>35 (11.0%)</td>
<td>7 (4.3%)</td>
<td>p = 0.0363 OR [CI], 2.379 [1.033-5.475]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EE Population (n = 365)</th>
<th>Controls (n = 164)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknownb R501X</td>
<td>26 (7.1%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Unknown 2282del4</td>
<td>36 (9.9%)</td>
<td>7 (4.3%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

bGenotype not determined.

### Table III. Frequency of AD and genotypes in the EE patients

<table>
<thead>
<tr>
<th></th>
<th>R501X Positive</th>
<th>2282del4 Positive</th>
<th>WT</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE without AD</td>
<td>10</td>
<td>8</td>
<td>131&lt;sup&gt;c&lt;/sup&gt;</td>
<td>p = 0.0315 (for 2282Del4) OR [CI], 4.794 [1.00-23.98]</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>2</td>
<td>157</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>R501X Positive</th>
<th>2282del4 Positive</th>
<th>WT</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 15)</td>
<td>(n = 20)</td>
<td>(n = 283)</td>
<td></td>
</tr>
<tr>
<td>AD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 (33.3%)</td>
<td>12 (60%)</td>
<td>102 (44.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Skin prick test positive&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 (46.7%)</td>
<td>10 (50.0%)</td>
<td>72 (69.9%)</td>
<td>NS</td>
</tr>
<tr>
<td>History of atopic disease&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13 (86.6%)</td>
<td>17 (85.0%)</td>
<td>210 (83.3%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup>Information unavailable for 50 of the genotyped patients.
<sup>b</sup>Past or present history of allergic dermatitis.
<sup>c</sup>History of at least one positive skin prick testing to food or aeroallergen.
epithelium by disrupting intercellular desmosomes. Interestingly, epithelial permeability to protons differs between healthy subjects and patients with active GERD (39). It is interesting to note that eotaxin-3 is associated with the outer surface of the cell membrane, possibly via CCR3, which has been reported to be expressed by skin keratinocytes (40, 41). Notably, heparin releases bound eotaxin-3 from the cell surface. Chemokines are known to bind to glycosaminoglycan side chains of proteoglycans, such as heparin, and heparin potentiates eotaxin-induced eosinophil recruitment in vivo (42). The role of heparin in eotaxin-3 release has been previously demonstrated in bronchoalveolar epithelial cells (43) and in blood where the level of eotaxin-3 in heparin-plasma is higher compared with EDTA plasma (33, 44). These findings emphasize the potential role of mast cell-derived heparin in eosinophil recruitment in EE. Consistent with the ability of external factors to regulate eotaxin-3 release, eotaxin-3 recovery in the cell media was dependent on the type of sera used. These results demonstrate that the external components in the microenvironment likely regulate eotaxin-3 levels and the propensity of eosinophils to infiltrate the tissue.

EDC genes and filaggrin are strongly downregulated in patients with EE (30). It has also been shown that a strong association exists between filaggrin mutations and AD (45). Because a large part of the EE population (∼43%) has eczema or a history of eczema, we hypothesized that these mutations would be associated with EE. Although the filaggrin mutations would be expected to be enriched in an atopic population, the prevalence of atopic diseases was the same in EE R105X positive, EE 2282del4 positive, and EE WT groups. In addition, EE patients without AD had a significantly increased frequency of the 2282del14 mutation compared with

FIGURE 5. Physical map and gene expression of the EDC gene cluster on the chromosome 1 in IL-13–stimulated primary esophageal epithelial cells. A. The effect of chronic exposure of IL-13 on esophageal epithelial cell number is shown. Cells (10% confluence) were stimulated every 3 d with IL-13 (0, 10, 100 ng/ml) for 9 d (80–90% confluence). Cell numbers are presented as mean ± SD. The results are representative of at least three independent experiments. B. Schematics of the physical map of the 1q21 gene cluster and the microarray expression profile primary esophageal epithelial cells stimulated with IL-13. B and C. Esophageal primary epithelial cells from patients with EE were stimulated with IL-13 (100 ng/ml) for 48 h. Total mRNA was extracted and subjected to real-time RT-PCR for involucrin (D) and filaggrin (C). D. The esophageal epithelial cell line TE-7 (<10% confluence) was transfected with a dominant-negative (DN) form of STAT6 or the empty vector (EV). Total mRNA was extracted and submitted to real-time RT-PCR for involucrin mRNA quantification. Results are normalized to a housekeeping gene (GAPDH). Bars are mean ± SD. Experiments were performed in triplicate. *p < 0.05. DN, dominant-negative; EV, empty vector.
controls (Table III), suggesting that the association of the 2282del4 mutation with EE was not dependent on the high rate of AD in EE. The significant association found between EE and 2282del4 suggests enhanced percutaneous sensitization in EE (21). It is interesting to note that the frequency of the RS01X was below that expected in such a highly atopic population, and this needs further investigation. Our results demonstrate that mutations in filaggrin may separately contribute to EE, but are present in only a small subset of the EE population despite the profound downregulation of filaggrin gene expression seen in nearly all patients.

In conclusion, we report that esophageal epithelial cells from patients with EE are functionally normal compared with cells isolated from control individuals, as assessed by growth properties ex vivo. IL-13 responsiveness based on eosin-3 production. Although a filaggrin loss of function mutation (2282del4) was overrepresented in EE compared with control individuals, the acquired decrease in filaggrin expression was uniformly seen in all EE cases in vivo. Indeed, EDC gene (filaggrin and involucrin) expressions were strongly decreased directly by IL-13. As such, these results establish that the primary defect in epithelial responses in EE is not intrinsic to the epithelium, rather it is likely secondary to the effects of IL-13, which regulates expression of EDC genes. Our results support a model of coordinated interaction between IL-13 and epithelial differentiation cluster genes in EE.

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Disclosures
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References


