Proliferation and inflammation in bronchial epithelium after allergen in atopic asthmatics


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Summary

Background The mechanisms that regulate epithelial integrity and repair in asthma are poorly understood. We hypothesized that allergen exposure could alter epithelial inflammation, damage and proliferation in atopic asthma.

Objective We studied epithelial cell infiltration, shedding, expression of the proliferation marker Ki-67 and the epithelial cell–cell adhesion molecules Ep-CAM and E-cadherin in bronchial biopsies of 10 atopic mild asthmatics 48 h after experimental diluent (D) and allergen (A) challenge in a crossover design.

Methods Epithelial shedding, expressed as percentage of not intact epithelium, Ki-67+, eosinophil/EG-2+, CD4+ and CD8+ cells were quantified by image analysis in bronchial epithelium, and adhesion molecules were assessed semi-quantitatively.

Results Epithelial shedding was not altered by A (D: 88.1 ± 3.1% vs. A: 89.2 ± 3.7%; P = 0.63). The numbers of Ki-67+ epithelial cells (D: 10.2 ± 0.2 vs. A: 19.9 ± 0.3 cells/mm; P = 0.03), EG-2+ (D: 4.3 ± 0.5 vs. A: 27 ± 0.3 cells/mm; P = 0.04) and CD4+ cells (D: 1.7 ± 1.2 vs. A: 12.3 ± 0.6 cells/mm; P = 0.04) were significantly increased after A, whilst CD8+ numbers were not significantly changed (P > 0.05). E-cadherin and Ep-CAM epithelial staining showed a similar intensity after D and A (P > 0.05). We found a positive correlation between EG-2+ and Ki-67+ cells in the epithelium (Rs: 0.63; P = 0.02).

Conclusion Our study indicates that allergen challenge increases epithelial proliferation in conjunction with inflammation at 2 days after exposure. This favours the hypothesis that long-lasting epithelial restitution is involved in the pathogenesis of asthma.

Keywords asthma, epithelium, proliferation and inflammation

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Introduction

The mechanisms underlying airway epithelial damage as observed in patients with asthma have attracted a great deal of interest [1, 2]. Several inflammatory products may induce epithelial damage in asthma, including eosinophil granule proteins [3], reactive oxygen species [4], mast cell proteolytic enzymes [5] and metalloproteases [6, 7]. The epithelial damage that results from inflammation is normally followed by a repair process, aimed at restoring epithelial integrity. Processes of epithelial damage and repair are a complex interplay of cell proliferation, migration and differentiation [8].

Kiel 67 (Ki-67) is a human cell-cycle-related antigen (a nuclear non-histone protein) expressed solely by cycling cells [9]. The cell-cycle marker Ki-67 has been widely used to assess cell proliferation in human tissue [9]. Increased epithelial proliferation in bronchial biopsies of mild stable asthmatics has recently been reported [10]. Previously, it has also been noted that epithelial metaplasia in inflammatory conditions of the airways is usually associated with proliferative and reparative processes [11]. Furthermore, it has been shown in guinea-pig trachea that epithelial proliferation was increased 24 h after allergen challenge in association with the formation of epithelial restitution cells, which is followed by differentiation towards a normal epithelium [12].

Adhesive mechanisms, such as epithelial adhesion molecules, are fundamental for the maintenance of epithelial integrity. Epithelial-cadherin (E-cadherin) [13] and epithelial cell adhesion molecule (Ep-CAM) [14] are two epithelial cell–cell adhesion molecules that display lateral immunostaining of cell membranes, consistent with the location of ‘intermediate/adherence junctions’, in human bronchial epithelium [1, 14]. E-cadherin, a calcium-dependent glycoprotein member of the cadherin superfamily, is essential for the induction and maintenance of polarized and differentiated epithelial phenotypes [14]. Ep-CAM, a calcium-independent glycoprotein, is expressed exclusively in epithelial cells and neoplasias.
derived from the epithelia [15, 16]. E-cadherin and Ep-CAM, markers of epithelial integrity, are also differently associated with active proliferation and dedifferentiation of epithelial cells [16, 17]. Goto et al. [18] showed decreased levels of E-cadherin during late asthmatic response (LAR) after allergen challenge in an animal model of asthma, suggesting that loss of E-cadherin would increase airway permeability. The expression of E-cadherin, that has been found in human bronchial epithelium localized to epithelial contacts close to the luminal surface [1], and Ep-CAM in bronchial biopsies of asthmatics has not been studied yet.

As it has been reported that inflammatory cell infiltration increases 24 h after allergen exposure in bronchial biopsies of atopic asthmatics [19, 20], we postulated that allergen exposure could enhance epithelial inflammation, damage and proliferation in the bronchial biopsies of atopic asthmatics at 48 h. Therefore, we quantified the amount of epithelial shedding, as a marker of epithelial damage, the numbers of epithelial inflammatory (eosinophil/EG-2, CD4+ and CD8+) and proliferating (Ki-67+) cells, the presence of epithelial metaplasia and the expression of the adhesion molecules Ep-CAM and E-cadherin in bronchial mucosal biopsies of 10 atopic mild asthmatics 48 h after experimental diluent and allergen challenge.

Methods

Subjects

Ten non-smoking house dust mite (HDM) atopic individuals with mild intermittent asthma [21] participated in the study (Table 1), which was part of a larger project. The subject characteristics have been previously published [22]. The subjects had a documented early asthmatic response (EAR) and late asthmatic response (LAR) to inhaled HDM extract [22]. The study had a randomized, placebo-controlled and cross-over design. Bronchoscopy was performed in each patient 2 days after either allergen or diluent exposure. Each exposure was separated by a wash-out interval of at least 2 weeks.

Table 1. Characteristics of participants [22]

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Atopic status*</th>
<th>FEV1 (D) (%) predicted</th>
<th>FEV1 (A) (%) predicted</th>
<th>PC20 FEV1 histamine (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>20</td>
<td>5</td>
<td>92</td>
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<td>2</td>
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<td>20</td>
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<td>85</td>
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<td>3</td>
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<td>4</td>
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<td>85</td>
<td>82</td>
<td>1.77</td>
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<tr>
<td>5</td>
<td>M</td>
<td>26</td>
<td>4</td>
<td>98</td>
<td>95</td>
<td>1.0</td>
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<tr>
<td>6</td>
<td>F</td>
<td>21</td>
<td>4</td>
<td>101</td>
<td>100</td>
<td>1.33</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>20</td>
<td>5</td>
<td>105</td>
<td>102</td>
<td>0.36</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>19</td>
<td>4</td>
<td>98</td>
<td>98</td>
<td>4.23</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>26</td>
<td>4</td>
<td>101</td>
<td>100</td>
<td>1.94</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>24</td>
<td>3</td>
<td>100</td>
<td>104</td>
<td>3.34</td>
</tr>
</tbody>
</table>

FEV1 – forced expiratory volume in 1 s at baseline on diluent (D) and allergen (A) day.

*Atopic status as determined by the number of weal responses to 10 common allergen extracts (Vivodiagnost, ALK, Benelux).
†Baselines in percentage of predicted values in the screening period.
‡Provocative concentrations of histamine causing a 20% fall in FEV1 in the screening period.

Table 2. List of monoclonal antibodies used in the study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
<th>Antigen retrieval</th>
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</thead>
<tbody>
<tr>
<td>CD4</td>
<td>F6</td>
<td>1:50</td>
<td>Novocastra †</td>
<td>EDTA</td>
</tr>
<tr>
<td>CD8</td>
<td>B11</td>
<td>1:400</td>
<td>Novocastra †</td>
<td>EDTA</td>
</tr>
<tr>
<td>Eosinophil/ECP</td>
<td>EG-2</td>
<td>1:200</td>
<td>Pharmacia †</td>
<td>Trypsin</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Mb1</td>
<td>1:400</td>
<td>Immunotech †</td>
<td>Citrate</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>36</td>
<td>1:30000</td>
<td>BD-Transduction §</td>
<td>Citrate</td>
</tr>
<tr>
<td>Ep-CAM</td>
<td>323/A13</td>
<td>1:10000</td>
<td>Dr S. Litvinov †*</td>
<td>Trypsin</td>
</tr>
</tbody>
</table>

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§Lexington (KY, USA).
| Leiden University Medical Center (the Netherlands).
of 200 μm, and the results expressed as the number of immunostained cells per millimetre of BM. The presence of Ep-CAM and E-cadherin in the IE was scored by a semiquantitative method. A score of staining intensity ranging from 0 (absence) to 3 (maximum) on the basis of the extent of epithelial metaplasia for each section. The presence of ‘epithelial metaplasia’ [defined as multiple layers of round or polygonal cells, with large cytoplasma, in areas where the surface columnar cells were not present (Fig. 1)] was scored by a semiquantitative method ranging from 0 (absence) to 3 (maximum) on the basis of the extent of epithelial metaplasia for each section.

Statistical analysis

All data are reported as mean ± SEM unless otherwise noted. Two-tailed paired t-tests were applied to explore the differences in FEV$_1$ values. Non-parametric statistical analysis (Wilcoxon rank test) was applied to examine the effect of allergen on epithelial damage and metaplasia, EG2, CD4$,^+$, CD8$^+$ and Ki67$^+$ cells, E-cadherin and Ep-CAM immunostaining. Cellular counts were log transformed before analyses. Correlation analyses were carried out by means of Spearman rank correlation testing (Rs). Statistical significance was accepted for a $P$-value less than 0.05.

Results

Pulmonary function

Baseline FEV$_1$ was not different between the diluent and allergen days (Table 1) [22]. The maximum percentage fall in FEV$_1$ from baseline during the EAR (mean ± SEM) was 43 ± 3%, whilst during the LAR (mean ± SEM) it was 32 ± 4%. Diluent challenge did not affect baseline FEV$_1$ values.

Epithelial shedding, cellular inflammation, proliferation, metaplasia and adhesion molecules

The sections of bronchial biopsies had a mean BM length of 3.84 ± 0.53 and 5.60 ± 0.46 mm after diluent and allergen challenge, respectively. The length of intact epithelium analysed was on average 0.68 ± 0.54 mm after diluent and 0.99 ± 0.84 mm after allergen exposure. The degree of bronchial epithelial shedding was similar after diluent (88.1 ± 3.14% BM) and after allergen challenge (89.2 ± 3.69% BM, $P = 0.63$).

The numbers of EG-2$^+$ cells (paired data: $n = 5$) in the epithelium were significantly higher after allergen challenge (27 ± 0.3 cells/mm) as compared to diluent (4.3 ± 0.5 cells/mm) ($P = 0.04$) (Table 3, Figs 2a, 2a') and 3). The numbers of CD4$^+$ cells (paired data: $n = 5$) increased after allergen challenge (12.3 ± 0.6 cells/mm) in comparison with diluent (1.7 ± 1.2 cells/mm) ($P = 0.04$) (Table 3 and Fig. 3). The numbers of CD8$^+$ (paired data: $n = 6$) were not significantly different after diluent and allergen challenge ($P = 0.46$) (Table 3).

Nuclear staining of Ki-67 (paired data: $n = 10$) was present in epithelial cells, mainly close to the sites of epithelial loss. The number of Ki-67$^+$ epithelial cells was significantly increased after allergen challenge (19.9 ± 0.3 cells/mm BM) compared to that after diluent (10.2 ± 0.2 cells/mm BM; $P = 0.03$) (Table 3, Figs 2b, 2b' and 3). The epithelial metaplasia score in IE was not different after diluent (0.28 ± 0.28) or allergen (1.14 ± 0.55) ($P = 0.11$).

The staining of Ep-CAM (paired data: $n = 6$) and E-cadherin (paired data: $n = 7$) in bronchial biopsies of both the groups was characterized by a membrane-bound lateral labelling mainly in columnar cells, without cytoplasmic staining. Basal cells were positively stained for both adhesion molecules, even though Ep-CAM was expressed only in occasional basal cells. We did not observe any significant difference between diluent and allergen with respect to intensity of immunostaining of Ep-CAM (Table 3, Figs 2c and 2c') and E-cadherin (Table 3, Figs 2d and 2d').

Relationship between EG-2$^+$ cells, Ki-67$^+$ cells and epithelial metaplasia

The numbers of EG-2$^+$ cells were positively correlated with the numbers of Ki-67$^+$ epithelial cells in the bronchial epithelium of atopic asthmatics (Rs $= 0.63$; $P = 0.02$) (Fig. 4). Furthermore, ‘epithelial metaplasia’ score was positively related to the numbers of Ki-67$^+$ epithelial cells (Rs $= 0.53$; $P = 0.029$), and tended to be significantly associated with EG-2$^+$ cells in the epithelium (Rs $= 0.53$; $P = 0.06$).

Table 3. Quantitative (numbers of cells/mm of basement membrane) and semiquantitative analysis (intensity score: 0–3) of immunostained cells in bronchial epithelium for the following markers

<table>
<thead>
<tr>
<th>Cellular markers</th>
<th>Number of paired data</th>
<th>Diluent</th>
<th>Allergen</th>
<th>$P$-value</th>
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</thead>
<tbody>
<tr>
<td>Ki67$^+$ cells/mm</td>
<td>10</td>
<td>10.2 ± 0.2</td>
<td>19.9 ± 0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>EG-2$^+$ cells/mm</td>
<td>5</td>
<td>4.3 ± 0.5</td>
<td>27 ± 0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>CD4$^+$ cells/mm</td>
<td>5</td>
<td>1.7 ± 1.2</td>
<td>12.3 ± 0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>CD8$^+$ cells/mm</td>
<td>6</td>
<td>18.2 ± 0.1</td>
<td>19.9 ± 0.3</td>
<td>0.46</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>7</td>
<td>1.75 ± 0.44</td>
<td>1.3 ± 0.29</td>
<td>0.24</td>
</tr>
<tr>
<td>Ep-CAM</td>
<td>6</td>
<td>2.28 ± 0.31</td>
<td>2.01 ± 0.26</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Values of quantitative analysis are expressed as geometric means.
Discussion

This study shows that the exposure to inhaled allergen increases epithelial proliferation and cellular inflammation in bronchial biopsies of atopic asthmatics 48 h after exposure to the sensitizing agent. Furthermore, the present data demonstrated that intra-epithelial eosinophil inflammation is related to the proliferation marker Ki-67 in the bronchial epithelium of asthmatic patients. For Ep-CAM, only sporadic basal cells were positively stained. No differences were observed for immunoreactivity of Ep-CAM (c') and E-cadherin (d') after allergen exposure. Arrows indicate immunoreactive cells. Arrowheads indicate basal epithelial cells negatively stained for Ep-CAM. Original magnification: × 400.

Fig. 2. Immunoreactivity for EG-2+, Ki-67+, Ep-CAM and E-cadherin in the epithelium of bronchial biopsies of atopic asthmatics 48 h after diluent (a, b, c and d, respectively) and allergen (a', b', c' and d' respectively) challenge showing increased numbers of EG-2+ cells (a') and Ki-67+ cells (b') after allergen exposure. Ep-CAM and E-cadherin antibodies immunostained lateral borders of columnar cells and some basal cells in the bronchial epithelium of asthmatic patients. For Ep-CAM, only sporadic basal cells were positively stained. No differences were observed for immunoreactivity of Ep-CAM (c') and E-cadherin (d') after allergen exposure. Arrows indicate immunoreactive cells. Arrowheads indicate basal epithelial cells negatively stained for Ep-CAM. Original magnification: × 400.

Fig. 3. Individual changes in the numbers of Ki-67+, EG-2+ and CD4+ cells in the epithelium of atopic asthmatics at 48 h after diluent (D) and allergen (A) challenge. Each bar indicates the geometric mean of each value in the respective group. *P<0.05 vs. diluent.
correlation test. This study explored inflammatory cell infiltration in bronchial biopsies of atopic asthmatics, showing a significant association between increased eosinophilic infiltration and epithelial proliferation. The present study also explored inflammatory cell infiltration in the epithelium of bronchial biopsies at 48 h after allergen exposure. It has been previously demonstrated that epithelial shedding persists after the resolution of the late-phase reaction. These data suggest that persistent activation of epithelial cells and abnormal repair process following proliferative repair may occur at 48 h after allergen exposure. This favours the hypothesis that persistent activation of epithelial cells and abnormal repair process following proliferative response are key features in the pathogenesis of atopic asthma.

In asthmatic airways, mucosal inflammation has been extensively studied [29], but few reports analysed inflammatory cell infiltration in the epithelium of bronchial biopsies at baseline and at 24 h after allergen challenge [20, 30]. The present study also explored inflammatory cell infiltration in the epithelium of bronchial biopsies in atopic asthma at 48 h after allergen exposure. It has been previously demonstrated that the levels of the eosinophil and CD4⁺ cell chemotactants eotaxin and IL-16 increased 24 h after allergen challenge in the airways of asthmatics [31, 32], possibly contributing to the influx of eosinophils and CD4⁺ cells in the epithelium. Previous studies on sputum [33] and BAL [34] reported an increase of eosinophils and CD4⁺ cells at 48 h after allergen challenge. Our present results extend these observations to the bronchial wall, showing that eosinophils and CD4⁺ cells are significantly increased in bronchial epithelium at 48 h after allergen. These data suggest that allergen-induced inflammatory cell infiltration in the epithelium persists after the resolution of the late-phase reaction.

The epithelium is an essential target of inflammation in asthma [35], resulting in epithelial damage. It has been shown that epithelial shedding in vivo is instantaneously associated with intense restitution processes [12, 36]. Our results indicate that increased epithelial proliferation occurs in the absence of a detectable increase in epithelial shedding 48 h after allergen challenge. Even in processes where shedding is not directly involved, an increased proliferation of epithelial cells cocultured with autologous bronchoalveolar (BAL) cells from allergic asthmatics after segmental allergen challenge has already been demonstrated [37]. What are the mechanisms that mediate the increased epithelial proliferation following allergen exposure as observed in the present study? In addition to growth factors produced by epithelial and mesenchymal cells, other factors and inflammatory cells may also regulate epithelial proliferation. This is illustrated by the growth-promoting activities of neutrophil defensins and other neutrophil products [38], eosinophil-derived TGF-α [39], and the Th2 cytokines IL-4, IL-5 and IL-13 [40, 41]. In addition, the combination of allergen and Th2 cytokines appeared to enhance the release of TGF-α by cultured epithelial cells derived from patients with asthma [42].

Although we cannot totally exclude the fact that further epithelial shedding took place after allergen challenge, our findings support the hypothesis that allergen-recruited inflammatory cells may modulate airway epithelial proliferation [37]. This is confirmed by our results of a positive correlation between the numbers of Ki-67⁺ cells and EG-2⁺ eosinophils. Whether this relation is causal, and explained by the fact that eosinophils enhance epithelial cell proliferation directly or indirectly following injury, cannot be concluded from the present study.

The repair process in the airway epithelium is structurally characterized by the formation of multiple layers of polygonal and flat poorly differentiated basal cells ('epithelial reparative metaplasia') followed by the development of normal differentiated epithelium [36]. Keenan et al. [43] showed that reparative epidermoid metaplasia occurs within 48 h after mechanical tracheal injury. In this sense, epithelial metaplasia in the airways of atopic non-smoking asthmatics may be interpreted as an ongoing repair process [44]. Moreover, a previous study showed a correlation between increased proliferation activity of bronchial epithelium and the degree of squamous metaplasia in smoking chronic bronchitics [25].

The present study, in line with the latter, is the first demonstration of the relationship between epithelial proliferation and metaplasia in atopic asthma.

Epithelial shedding, a histological marker of epithelial damage, is considered to be a major feature in asthma [45], in particular atopic asthma [46]. The occurrence of shedding is supported by the findings of elevated numbers of epithelial cells in sputum [45], BAL fluids [47] and by histological observations in bronchial biopsies and tissues obtained at autopsy [48–50]. Conversely, other studies found a similar level of epithelial desquamation in normals and asthmatics [2, 51], even after allergen challenge [20]. We cannot exclude the possibility that mechanical injury induced during bronchoscopy may have interfered with our analyses of epithelial shedding by masking a further variation induced by allergen challenge. In addition, the high degree of epithelial desquamation precluded the analysis of intact epithelium in a substantial number of biopsies and was therefore a limiting factor for the inflammatory cell counts and cell adhesion molecule analyses in our study.
Ep-CAM and E-cadherin function in mediating epithelial cell–cell adhesion [15, 52]. A previous report revealed that E-cadherin protein localization diminished in adherence junctions between tracheal epithelial cells 6 h (LAR) after allergen challenge in sensitized guinea-pigs and that E-cadherin mRNA expression rapidly increased after immunochallenge [18]. Thus, epithelial inflammation disrupts epithelial adhesion molecules during LAR, but also allows E-cadherin regeneration [18]. In this study, performed on biopsies obtained 48 h after allergen challenge, we did not find any change in the intensity and pattern of staining of Ep-CAM and E-cadherin immunoreactivity between diluent- and allergen-stimulated biopsies. It is conceivable that at the time of bronchoscopy (48 h after allergen), the levels of E-cadherin returned to basal values showing no difference in the protein expression, in contrast with that previously reported 6 h after allergen [18]. We also point out that the semi-quantitative method may have a limitation in evaluating the epithelial immunostaining of Ep-CAM and E-cadherin as it is based on a score, instead of quantitative measurement, of protein expression.

In summary, our study shows that cellular inflammation and cell proliferation are increased at 48 h after allergen exposure in the airway epithelium of atopic asthmatics. Our findings strongly suggest that allergen-recruited inflammatory cells may modulate epithelial cell proliferation. Given the fact that allergen exposure is a well-known inducer of episodic worsening of airways inflammation and clinical symptoms in asthma [53], we postulate that the dynamic sites of epithelial repair can be relevant for the development of exacerbations and/or the maintenance of the disease.

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References


