Sputum and BAL Clara cell secretory protein and surfactant protein D levels in asthma


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Keywords
asthma; clinical immunology; remodelling.

Abstract
Clara cell secretory protein (CC16) is associated with Th2 modulation. Surfactant protein D (SPD) plays an important role in surfactant homeostasis and eosinophil chemotaxis. We measured CC16 and SPD in sputum supernatants of 84 asthmatic patients and 12 healthy controls. In 22 asthmatics, we additionally measured CC16 and SPD levels in BAL and assessed smooth muscle area (SMA), reticular basement membrane (RBM) thickness, and epithelial detachment (ED) in bronchial biopsies. Induced sputum CC16 and SPD were significantly higher in patients with severe asthma (SRA) compared to mild–moderate and healthy controls. In 22 asthmatics, we additionally measured CC16 and SPD levels in BAL and assessed smooth muscle area (SMA), reticular basement membrane (RBM) thickness, and epithelial detachment (ED) in bronchial biopsies. Induced sputum CC16 and SPD were significantly higher in patients with severe asthma (SRA) compared to mild–moderate and healthy controls. BAL CC16 and SPD levels were also higher in SRA compared to mild–moderate asthma. CC16 BAL levels correlated with ED, while SPD BAL levels correlated with SMA and RBM. Severity represented a significant covariate for these associations. CC16 and SPD levels are upregulated in SRA and correlate with remodeling indices, suggesting a possible role of these biomarkers in the remodeling process.

Introduction
Patients with severe refractory asthma (SRA) often have remodeling airway changes (1). Airway smooth muscle mass, smooth muscle hypertrophy, and subepithelial fibroblasts have been shown to be the strongest predictors of airflow obstruction in SRA (2). Thickening of the reticular basement membrane due to extracellular matrix deposition is a particularly distinctive feature of SRA (3). Biomarkers in sputum supernatant have been previously associated with the remodeling process in SRA (4).

Clara cell secretory protein (CC16) modulates Th2 response, as it downregulates Th2 differentiation of human naive neonatal T cells through dendritic cell mediation (5). Induced sputum and serum CC16 levels have been shown not to differ significantly between patients with asthma or rhinitis and controls, but without further stratification of asthma according to severity (6, 7).

Surfactant proteins SPA and SPD downregulate airway allergic reactions by inducing a Th2 to a Th1 response shift (8), whereas SPD in particular inhibits eosinophil chemotaxis and eotaxin-triggered degranulation (9). SPD specifically interacts with the CD32 (Fc-gamma II) eosinophil receptor, inhibiting eosinophil cationic protein degranulation (10).

The aim of this study was to evaluate CC16 and SPD levels in sputum supernatants of asthma patients of different severity and to investigate any possible correlations with indices of remodeling derived from endobronchial biopsies.

Methods
We recruited 96 subjects, 40 SRA patients, 44 mild-to-moderate asthmatics, and 12 healthy, nonatopic, nonsmoking controls. SRA was defined according to American Thoracic Society criteria. Subjects with any other respiratory, malignant, heart, renal, liver, or collagen disease as well as subjects with a respiratory tract infection or asthma exacerbation in the past 8 weeks prior to admission were excluded.
Sputum was induced as previously described, using all modifications for safe measurements according to asthma severity (11).

Bronchoscopy was performed for the acquisition of bronchoalveolar lavage (BAL), and six bronchial biopsy specimens were obtained from various sites of the subsegmental carinae bilaterally.

Slides stained for hematoxylin–eosine, smooth muscle actin (SMA), and periodic acid–Schiff (PAS) stain were analyzed with the Image Pro Plus v5.1.2.59 system (Media cybernetics, Silver Spring, Maryland, USA) for assessing smooth muscle area (SMA) percentage of biopsy area, reticular basement membrane (RBM) thickness, and length of epithelial detachment (ED) as a percentage of the total epithelial length.

Figure 1  (A) CC16 values (ng/ml) in sputum supernatants in patients with asthma (all, severe refractory [SR], mild to moderate) and healthy subjects, *P < 0.001 values indicate difference between asthma and healthy subjects, **P < 0.001 values indicate difference between SRA, mild to moderate, and healthy subjects, ***P < 0.001 values indicate difference between SRA and mild to moderate, ^P < 0.001 values indicate difference between SRA and healthy subjects, and $P < 0.001 values indicate difference between healthy subjects and mild to moderate. Values are presented as median interquartile ranges. (B) SPD values (ng/ml) in sputum supernatants in patients with asthma (all, severe refractory [SR], mild to moderate) and healthy subjects, *P < 0.001 values indicate difference between asthma and healthy subjects, **P < 0.001 values indicate difference between SRA, mild to moderate, and healthy subjects, ***P < 0.001 values indicate difference between SRA and healthy subjects, and $P < 0.001 values indicate difference between healthy subjects and mild to moderate. Values are presented as median interquartile ranges. CC16, Clara cell protein; SPD, surfactant protein D.
**Table 1** Inflammatory variables of study participants

<table>
<thead>
<tr>
<th>Variables</th>
<th>SRA</th>
<th>Mild-to-moderate asthma</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 40 for sputum</td>
<td>n = 44 for sputum</td>
<td>n = 12 only</td>
</tr>
<tr>
<td>Induced sputum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells $\times 10^6$/ml IS</td>
<td>2.6 (1.4–5)*,#</td>
<td>2 (0.9–3.6)*</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>Eosinophils (%) IS</td>
<td>7 (3–14)*,#</td>
<td>2 (0–7)</td>
<td>0.5 (0–1)</td>
</tr>
<tr>
<td>Neutrophils (%) IS</td>
<td>34 (21–47)*,#</td>
<td>25 (19–39)</td>
<td>25 (18–29)</td>
</tr>
<tr>
<td>Macrophages (%) IS</td>
<td>54 (36–66)*,#</td>
<td>60 (33–73)</td>
<td>68 (58–75)</td>
</tr>
<tr>
<td>Lymphocytes (%) IS</td>
<td>2 (1–2)</td>
<td>0.5 (0.5–1)</td>
<td>0.25 (0–1)</td>
</tr>
<tr>
<td>CC16 (ng/ml) IS</td>
<td>288 (65–999)*,#</td>
<td>99 (50–324)*</td>
<td>33 (21–53)</td>
</tr>
<tr>
<td>SPD (ng/ml) IS</td>
<td>30.5 (17–63)*,#</td>
<td>8 (4–29)*</td>
<td>5 (3.4–7)</td>
</tr>
<tr>
<td>BAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells $\times 10^6$/ml IS</td>
<td>18 (14–21)</td>
<td>19 (15–21)</td>
<td>0.621</td>
</tr>
<tr>
<td>Eosinophils (%) IS</td>
<td>10 (6.5–17)</td>
<td>3 (1–4.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils (%) IS</td>
<td>4 (3–9)</td>
<td>2 (2–3.5)</td>
<td>0.036</td>
</tr>
<tr>
<td>Macrophages (%) IS</td>
<td>70 ± 16</td>
<td>82 ± 7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lymphocytes (%) IS</td>
<td>9.5 ± 5</td>
<td>11 ± 3</td>
<td>0.187</td>
</tr>
<tr>
<td>CC16 (ng/ml) IS</td>
<td>277 (176–925)</td>
<td>164 (90–209)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SPD (ng/ml) IS</td>
<td>2135 (1705–2782)</td>
<td>1050 (680–1351)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endobronchial biopsies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMA %</td>
<td>13.5 ± 5.0</td>
<td>4.6 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RBM μm</td>
<td>10.5 ± 4.5</td>
<td>4.7 ± 1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Epithelial detachment %</td>
<td>24 ± 4</td>
<td>20 ± 2</td>
<td>0.031</td>
</tr>
</tbody>
</table>

CC16, Clara cell protein; SPD, surfactant protein D; BAL, bronchoalveolar lavage; SMA, smooth muscle area; RBM, reticular basement membrane; ED, epithelial detachment; SRA, severe refractory asthma.

Normally distributed data are presented as mean ± standard deviation (SD), whereas skewed data are presented as median (interquartile ranges). *Statistically significant difference compared to healthy subjects; #Statistically significant difference compared to mild-moderate asthma; bold numbers indicate significant differences. $P$-values indicate differences across the three groups for IS and across the two groups for BAL and biopsies.

Subjects also underwent atopy and lung function assessment. Sputum induction for cell count identification and CC16-SPD measurement in sputum supernatants were performed within 1 week from the initial assessment. Patients in the bronchoscopy group underwent bronchoscopy within 2 weeks from their initial assessment and after performing a sputum induction.

For additional methods, see the Methods section of the online Supporting Information.

**Results**

Patients with asthma had significantly higher levels for both CC16 and SPD in sputum supernatants compared to healthy subjects (Fig. 1A and B). Patients with SRA had significantly higher sputum levels of CC16 and SPD compared to those with mild-to-moderate asthma and healthy controls ($P < 0.001$ for both comparisons; Table 1, Fig. 1A and B). Patients with SRA had also significantly higher levels for both CC16 and SPD in BAL compared to patients with mild-to-moderate asthma (Table 1, Fig. 1C and D). Mild-to-moderate asthmatics had significantly higher levels compared to healthy subjects for either CC16 or SPD measured in sputum supernatants (Table 1, Fig. 1A and B).

Patients with SRA presented significantly increased SMA, RBM thickness, and length of epithelial detachment in endobronchial biopsies compared to those with mild-to-moderate asthma (Table 1).

**Associations of CC16 with SPD**

CC16 in sputum supernatant presented a significant positive association with sputum SPD. CC16 in BAL showed a significant positive association with ED. SPD in BAL was positively associated with SMA and RBM. The presence of SRA represented a significant covariate for both CC16 and SPD associations either in sputum and/or in BAL.

**Discussion**

We have shown that both CC16 and SPD levels in sputum supernatants and BAL are significantly higher in patients with SRA compared to mild-to-moderate asthmatics and healthy subjects. Recent evidence suggests that Clara cells regulate the immunomodulatory response and airways’ remodeling through the production of specific factors such as CC16. CC16 is secreted by the nonciliated Clara cells which reside predominantly in the respiratory bronchioles and by nonciliated columnar cells of the large and small airways (12). After airway injury, Clara cells in stem cell niches proliferate and migrate to replenish the injured terminally differentiated epithelial cells (8). In fact, after alveolar injury,
Clara cells can be seen in the alveolus, a process characterized as alveolar bronchiolization (13). Accordingly, CC16 has been proposed as a biomarker for epithelial cell dysfunction (14). The positive correlation of increased CC16 levels with the percentage of epithelial detachment in our study supports a possible role of CC16 as a marker of epithelial injury and regeneration. This process is more intense in severe compared to mild asthma and may explain the higher CC16 levels found in induced sputum and BAL of SRA.

Surfactant protein D is mainly produced by type II pneumocytes, although Clara cells can also produce small amounts of SPD, partly explaining the modest correlation between CC16 and SPD sputum levels in our study. In a bleomycin-induced fibrosis mouse model, alveolar SPD regulates numbers of macrophages and fibrocytes, profibrotic cytokine expression, and fibrotic lung remodeling, indicating a close relationship between SPD and airway remodeling (15). We found increased sputum and BAL SPD levels in SRA patients that correlated positively with SMA and RBM, further supporting a possible role of SPD in the remodeling process.

The increased SPD amounts in the sputum of patients with SRA could reflect an overproduction of SPD by type II pneumocytes, an altered turnover of SPD, or both. Despite evidence linking SPD with eosinophil chemotaxis and alveolar macrophages, no correlation was found between SPD and sputum cellular populations. Nevertheless, eosinophils were increased in the SRA group compared to mild-to-moderate asthma.

In conclusion, induced sputum and BAL SPD and CC16 levels are increased in SRA. Additionally, BAL SPD and CC16 levels correlate with bronchial remodeling indices. Our findings warrant further investigation for the evaluation of SPD and CC16 as candidate remodeling markers in this particular phenotype of asthma.

Conflicts of interest
None of the authors has any funding support or any financial relationship with a biotechnology and/or pharmaceutical manufacturer to declare.

 Supporting Information
Additional Supporting Information may be found in the online version of this article: Data S1. Materials and methods.

References
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