Decreased CCAAT/Enhancer Binding Protein Transcription Factor Activity in Chronic Bronchitis and COPD*

Lukas Didon, MSc; Ingemar Qvarfordt, MD, PhD; Olof Andersson, MD, PhD; Magnus Nord, MD, PhD; and Gerdt C. Rüse, MD, PhD

Background: CCAAT/enhancer binding proteins (C/EBPs) are key regulators of cell differentiation and linked processes such as proliferation, apoptosis, and gene expression in several organs. C/EBPs are also central for inflammatory responses and infectious defenses, but so far little is known of their role in lung diseases. Chronic bronchitis (CB) and COPD are common smoking-associated lung diseases involving the airway epithelium.

Methods: Gelshifts were used to study C/EBP transcription factor activity in airway epithelial cells obtained by bronchial brush biopsy in four groups: healthy never-smokers (n = 115), asymptomatic smokers (n = 7), and smokers with CB and recurrent infectious exacerbations without COPD (n = 23) and with COPD (n = 13).

Results: C/EBP-binding activity was increased 4.6-fold in airway epithelial cells of healthy smokers compared with never-smokers. In contrast, C/EBP binding activity was not increased in the epithelium of smokers with CB or COPD. C/EBP-β was the dominant C/EBP in the airway epithelium in all groups.

Conclusions: We hypothesize that this lack of increase in C/EBP-β activity renders the epithelium incompetent of efficient regeneration and more sensitive to infection, suggesting a previously unknown role for C/EBPs in the pathogenesis of CB and COPD.

(CHEST 2005; 127:1341–1346)

Key words: airway epithelium; CCAAT/enhancer binding proteins; chronic bronchitis; COPD; transcription factors

Abbreviations: CB = chronic bronchitis; CC16 = Clara cell 16-kd protein; C/EBP = CCAAT/enhancer binding protein; DNA = complementary DNA

CAAT/enhancer binding proteins (C/EBPs) are intracellular proteins regulating cell differentiation and associated processes such as proliferation, apoptosis, and gene expression. They have been demonstrated to be critical for cellular differentiation in organs as diverse as liver, WBC, mammary gland, and fat tissue, and a similar role in lung is just beginning to be appreciated. In lung epithelium, the C/EBP-family members α, β, and δ are expressed; but so far, studies have mainly focused on their role in regulating lung gene expression and lung development, and little is known about their role in lung diseases.

Chronic bronchitis (CB) primarily affects smokers and is characterized by coughing and expectoration. COPD is characterized by airflow limitation that is not fully reversible. It is associated with a chronic inflammation leading to fixed narrowing of small airways and alveolar wall destruction (emphysema). The pathophysiology of CB includes mucus hypersecretion and ciliary damage as a result of the toxic and inflammatory effects of tobacco smoke. This results in impaired mucociliary clearance facilitating both bacterial infection and colonization. Epitheliotoxic products released by bacteria can further damage epithelial cells and cilia, resulting in additional decrease of mucociliary clearance in a vicious circle of respiratory decline first proposed by Cole and...
Wilson. Damaged epithelial cells are lost by sloughing, necessitating proliferation and subsequent differentiation to regenerate an intact airway epithelium. That C/EBPs are known to regulate cellular differentiation, proliferation, and apoptosis suggested to us that C/EBPs could have a role in the development of CB and COPD, especially as these transcription factors also are important for activation of early inflammatory genes and defense mechanisms against infection. To address the hypothesis that C/EBPs play a role in the pathogenesis of CB and COPD, we studied the activity of these intracellular proteins in the airway epithelium of healthy never-smokers, asymptomatic smokers, and smokers with CB and recurrent infectious exacerbations both without and with concomitant COPD.

**Materials and Methods**

**Design**

This report presents results derived from a cross-sectional bronchoscopic study of the airways of smokers with CB and COPD, the clinical protocol of which has been described in detail elsewhere. The clinical part of the study was performed at a tertiary teaching hospital in Gothenburg, Sweden, and was approved by the local Ethics Committee. All subjects were volunteers and were recruited from patient files and by advertisement in a daily newspaper. All subjects received both written and oral information before giving written consent.

**Study Population**

The four different subject groups participating in the study consisted of healthy, lifelong never-smokers with normal ventilatory lung function; asymptomatic smokers with normal ventilatory lung function; smokers with CB; and smokers with COPD. CB was defined according to the American Thoracic Society criteria, ie, chronic or recurrent productive cough for a minimum of 3 months per year during the last 2 years (ie, corresponding to at least the Global Initiative for Chronic Obstructive Lung Disease stage 0: “at risk”). To identify subjects with COPD, a substantrification of subjects fulfilling the criteria for CB was done based on postbronchodilator FEV1; subjects with a postbronchodilator FEV1 < 80% predicted, ie, corresponding to Global Initiative for Chronic Obstructive Lung Disease COPD stage II, were included in the COPD group. All of the subjects within the three smoking groups were current smokers. CB exacerbations were defined according to Boman et al, in addition to mucopurulent or purulent sputum and cough, at least one of increased sputum thickness or production, increased difficulty of expectoration, symptoms of common cold, or pneumonia. Exclusion criteria have been described in detail previously. Briefly, subjects receiving airway, antiinflammatory, or immunomodulatory medications of any kind were not allowed to participate, nor were any with a history of asthma, atopy, or allergy, or any with symptoms of airway or systemic infection during 4 weeks prior to the investigation. Subjects with significant comorbidity of any other kind as judged by the investigators were also excluded. No subjects were receiving any regular inhalation therapy of any kind. No subject was receiving systemic corticosteroids or any other immunomodulatory or antiinflammatory treatment. Some patients with CB were receiving antihypertensive or antidepressive medications. Premedication preceding bronchoscopy was administered with morphine-scopolamine IM, and the local anesthetic was tetracaine.

**Bronchial Brush Biopsies**

Standardized bronchial brush biopsies from the right main bronchus were collected via bronchoscopy as described by Riise et al. Bronchoscopy was performed 0 to 12 weeks after spirometry, and all smoking subjects were asked to refrain from smoking for at least 8 h prior to bronchoscopy. Briefly, specimens were obtained with a sterile, single-sheathed, nylon cytology brush (Olympus BC 9C-26101; Olympus Optical; Tokyo, Japan). Three consecutive brushings from an approximately 5 cm2 intrabronchial mucosal area were taken. The brush was agitated in a vortex in 1 mL phosphate-buffered saline solution between each brushing, and the cell suspension was stored at −70°C awaiting further analysis.

**Analysis of C/EBP Transcription Factor Binding Activity**

Nuclear extracts were prepared from cells of the bronchial brush biopsies and C/EBP-binding activity measured by gelshift assays as described by Berg et al. Briefly, after preparation of nuclear extracts, identical amounts of nuclear protein were incubated in binding buffer using an end-labeled, double-stranded, synthetic oligonucleotide harboring a consensus C/EBP-binding site (5′-CGG GAT CCA TTG CGC AAT GGA TCC-3′) as probe for the gelshift. Where indicated, polyclonal antibodies (anti-C/EBP-α, anti-C/EBP-β, or anti-C/EBP-δ; Santa Cruz Biotechnology; Santa Cruz, CA) were included. The resulting protein-DNA complexes were resolved on nondenaturing 5% polyacrylamide gels. Gels were vacuum dried, and shifted complexes were visualized and quantified by phosphoimaging (FUJIX BAS 2000 phosphoimager; Fuji, Tokyo, Japan). For each lane, the radioactivity in the area indicated with a bracket in Figure 1, bottom was quantified. To allow for interassay comparisons, the radioactivity was normalized to an equal amount of overexpressed human C/EBP-β included in each assay. For use as positive controls and for normalization, human complementary DNAs (cDNAs) for C/EBP-α, C/EBP-β, and C/EBP-δ were cloned in the pCMV5 mammalian expression vector and overexpressed in COS-cells as previously described. The C/EBP-α cDNA was a kind gift from Per Antonsson, Department of Biosciences, Karolinska Institute NOVUM, Sweden. cDNAs for C/EBP-β and C/EBP-δ were from the I.M.A.G.E. Consortium (LLNL) [cDNA clones 4870789 and 3993934, respectively].

**Collection of BAL Fluid and Analysis of Clara Cell 16-kDa Protein**

BAL fluid was obtained from a segmental bronchus of the middle lobe by instillation and aspiration of 20 mL plus 80 mL plus 60 mL of body-tempered, sterile, pyrogen-free, phosphate-buffered saline solution. The first aliquot of 20 mL was aspirated separately to minimize oropharyngeal contamination as described previously and not further analyzed. The aspirates from the remaining two aliquots (total, 140 mL) were pooled in a siliconized glass container on ice. The fluid was subsequently filtered through a nylon web (100 μm pore size) for retention of mucus. Evaluation of BAL was done as previously described by light microscopy of cytospin preparations and only samples containing <1% squamous epithelial cells were accepted as.
Figure 1. Top: C/EBP activity in nuclear extracts prepared from airway epithelial cells obtained by bronchial brush biopsy of healthy never-smokers, healthy smokers, smokers with CB, and smokers with COPD. ***p = 0.0003 (Kruskal-Wallis test). Bottom: Representative gelshift assays from each group: healthy never-smokers, healthy smokers, smokers with CB, and smokers with COPD. In indicated lanes, antibodies against the C/EBP family members present in the lung epithelium are included. Bracket indicates retarded complex; arrowhead indicates super-shift resulting from the inclusion of antibody; F indicates migration of free unbound probe. The first six lanes contain human C/EBP-α, -β, and -δ overexpressed in COS cells as positive controls. Super-shifts induced by the inclusion of the respective antibodies in these lanes confirm antibody reactivity.
Cell-free BAL fluid was obtained by separation of the supernatant after centrifugation (250 g, 10 min, + 4°C) and frozen in aliquots (−70°C) awaiting analysis. Clara cell 16-kDa protein (CC16) levels were measured by quantitative Western blot analysis as previously described. Briefly, CC16 was detected with a polyclonal CC16 antibody (Dako; Copenhagen, Denmark) and antibody-antigen complexes were visualized and quantified with 125I-protein A (NEN; Boston, MA) and phosphoimager plates (Fujix BAS-2000; Fuji).

**Statistical Methods**

Data are presented as median (range) or as standard box plots. The nonparametric Kruskal-Wallis test was used for comparisons of C/EBP activity and CC16 protein levels between multiple groups. Mann Whitney U tests were used for pair-wise comparisons of C/EBP activity and CC16 protein levels. Analyses of correlations were performed using Spearman rank correlation analysis; p < 0.05 defined statistical significance.

**RESULTS**

**C/EBP Transcription Factor Activity in Bronchial Epithelial Cells**

Demographic and clinical data of the study population are given in Table 1. C/EBP-binding activity was analyzed in bronchial epithelial cells obtained by brush biopsies. As seen in Figure 1, top, healthy smokers displayed 4.6-times higher levels of C/EBP activity than healthy never-smokers. When antibodies against different C/EBP family members were included in the gelshift assay, use of C/EBP-β antibody resulted in clearly diminished shifts and the appearance of super shifts. Weaker effects were seen when antibody against C/EBP-δ was included, and no effects on inclusion of C/EBP-α antibody (Fig 1, bottom). The same pattern was observed in all groups. Together this indicates that C/EBP-β is the dominant C/EBP in the airway epithelium in all groups, and that C/EBP-δ is also active, but to a lesser extent. In comparison with healthy smokers, C/EBP-binding activity in smokers with CB and COPD was markedly lower (Fig 1, top). In the smokers, C/EBP activity displayed a correlation with FEV1 (p = 0.044, p = 0.31, Spearman rank correlation), and there was also a negative correlation between C/EBP activity and smoking history in pack-years (p = 0.019, p = −0.36, Spearman rank correlation).

**CC16 Levels in BAL Fluid**

To address whether the differences in C/EBP activity between the smoking groups result from differences in the extent of epithelial injury, BAL fluid levels of the epithelial injury marker CC16 were analyzed. No difference between healthy smokers and smokers with CB or COPD was found (Fig 2), indicating similar insult to the airway epithelium in all three groups of smokers. As expected, never-smokers exhibited higher CC16 levels than the smoking groups (Fig 2).

**DISCUSSION**

C/EBPs are key regulators of cell differentiation, proliferation, and apoptosis, as well as inflammatory and immune responses, but so far little is known of their role in lung diseases. In the present study, we investigated C/EBP transcription factor binding activity in common smoking-associated disorders. Increased C/EBP-β activity was demonstrated in airway epithelial cells of healthy smokers compared with healthy never-smokers. In contrast, C/EBP-β activity was not increased in the epithelium of smokers with CB or COPD. This raises the possibility that injury to the epithelium caused by tobacco smoke induces C/EBP-β activity in healthy smokers. We hypothesize that this serves as a protective mechanism by stimulating regeneration of the lung epithelium through promotion of cell proliferation. It would also inhibit apoptotic signals induced by the tobacco smoke, and activate early inflammatory genes and defense mechanisms against infection. Following this, activation of C/EBP-β would be of

**Table 1—Demographic and Clinical Data of the Study Population**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy Never-Smokers</th>
<th>Asymptomatic Smokers</th>
<th>Smokers With CB</th>
<th>Smokers With COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, No.</td>
<td>10</td>
<td>7</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Age, yr</td>
<td>55 (37–64)</td>
<td>41 (34–52)</td>
<td>48 (38–64)</td>
<td>57 (37–67)</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>102.5 (91–120)</td>
<td>112 (95–129)</td>
<td>88 (80–111)</td>
<td>74 (61–79)</td>
</tr>
<tr>
<td>Smoking history, pack-years</td>
<td>20.3 (11.3–25)</td>
<td>36.3 (12.5–87.5)</td>
<td>20 (10–40)</td>
<td>20 (10–30)</td>
</tr>
<tr>
<td>Years of smoking</td>
<td>28 (18–35)</td>
<td>33 (23–50)</td>
<td>38 (23–46)</td>
<td></td>
</tr>
<tr>
<td>Male/female gender, No.</td>
<td>5/5</td>
<td>2/5</td>
<td>6/17</td>
<td>3/10</td>
</tr>
<tr>
<td>Duration of CB, yr</td>
<td>7 (4–25)</td>
<td>10 (6–30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infectious CB exacerbations over 2 yr</td>
<td>7 (4–20)</td>
<td>6 (4–10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data are presented as median (range) unless otherwise indicated.
importance to maintain a functional epithelium and adequate defenses against bacterial colonization. It also implies that the low C/EBP-activity we observe in smokers with CB and COPD could reflect an inability to activate C/EBP. This would then have pathogenic implications, as it renders the epithelium incompetent of efficient regeneration and more sensitive to infection, accentuating the vicious circle in the development of CB.

What causes this inability to activate C/EBP can only be speculated on, but it could involve direct effects of long-term smoking on airway epithelial cells, as C/EBP activity in the smokers displays a negative correlation with smoking history in pack-years. This is also reflected in the difference in smoking history between the smoking groups (Table 1). An alternative hypothesis for the role of C/EBP in CB and COPD could be that the development of disease is independent of C/EBP-activity. Instead, C/EBP-activity would be diminished as a result of increasing epithelial cell dysfunction as the disease progresses, further promoting progression of disease. Thus, irrespective of the mechanism, the decreased C/EBP activity observed in the current study could have a pathogenic role in CB and perhaps COPD. Also, the correlation between C/EBP activity and FEV₁ observed in the smokers lends support for a role in COPD pathogenesis. In order to address whether the differences in C/EBP activity between the smoking groups result from differences in the extent of epithelial injury, we analyzed levels of the secretory protein CC16, a useful molecular marker for smoking-induced injury to the airway epithelium.16,17 No difference between healthy smokers and smokers with CB or COPD was found, indicating similar insult to the airway epithelium in all three groups of smokers.

For this study, we used gel shift assays to analyze C/EBP transcription factor activity in airway epithelial cells obtained by bronchial brush biopsies. A brush biopsy yields > 90% epithelial cells and is thus a useful means to sample the airway epithelium.18 Since this method yields free intact epithelial cells, it is well suited for functional analysis of intracellular proteins such as transcription factors as demonstrated in the current study. The inflammation in CB, and especially in COPD, typically exhibits partial resistance to the antiinflammatory action of glucocorticoids.7,8 Interestingly, C/EBPs have been suggested to be involved in mediating the effects of glucocorticoids in lung epithelial cells5,14 and also in bronchial smooth-muscle cells.19 One can thus speculate that the decrease in C/EBP activity in CB and COPD can have a causative role for the resistance to glucocorticoids seen in these diseases.

CONCLUSION

In conclusion, this study shows increased C/EBP-activity in the airway epithelium of healthy smokers, but not in smokers with CB and COPD. Together with the proliferative and antiapoptotic role of C/EBP- and its role in activating defense mechanisms,1,5,9 this suggests C/EBPs as previously unknown players in the pathogenesis of CB and COPD. Clearly, further studies are needed; however, our results open new vistas to investigate the pathogenesis of these common and severe lung diseases.

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