Decreased expression of TGF-β type II receptor in bronchial glands of smokers with COPD

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Running title: TGF-β₁ signalling and mucus hypersecretion

Keywords: airflow limitation; cytokines; inflammation; mucus hypersecretion
Abstract

Rationale The role of transforming growth factor-β1 (TGF-β1) in chronic obstructive pulmonary disease is still controversial. Of interest, it has been proposed that TGF-β1 may protect from mucus hypersecretion, since it is able to down-regulate mucin production.

Objectives We performed this study to investigate the expression of transforming growth factor-β1 and its type II receptor in bronchial glands of smokers with COPD.

Methods With immunohistochemical methods we examined the expression of TGF-β1 and TGF-β type II receptor (TGF-β RII) in bronchial glands of 24 smokers undergoing lung resection for solitary peripheral nodules: 12 with airflow limitation (smokers with COPD) and 12 with normal lung function.

Measurements and main results The expression of TGF-β1 in bronchial glands was similar in the 2 groups of subjects examined, while that of type II receptor was decreased in smokers with COPD as compared to smokers with normal lung function (p=0.004). Moreover, the expression of TGF-β type II receptor was inversely correlated with the values of Reid’s index, a measure of gland size (p=0.02, r=-0.50).

Conclusions This study shows that, in bronchial glands of smokers with COPD, there is a decreased expression of TGF-β type II receptor which is associated to bronchial gland enlargement. These findings support the view that the lack of TGF-β signalling may induce structural changes in the bronchial glands which, in turn, may promote mucus hypersecretion.

Words: 224
Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a public health problem worldwide, being a major cause of chronic morbidity and mortality (1, 2). Although it is well established that cigarette smoking is the main risk factor for the development of COPD, the pathogenesis of the disease is still incompletely understood (3, 4). It has been recently suggested that transforming growth factor-β (TGF-β) may be involved in the development of COPD (1), even if its role remains controversial.

In the past there has been considerable interest in TGF-β1, with a few studies reporting an increased expression of this growth factor in the airways of smokers with COPD (5-7). It was therefore proposed that TGF-β, through fibrosis and thickening of the airway wall, may contribute to the development of airflow limitation in smokers. However, more recently, several studies pointed out that TGF-β1 may have a protective role as well. Indeed, in animal models, it has been clearly shown that alterations that abrogate TGF-β1 signalling result in an increased risk of pulmonary emphysema (8,9). In humans, genetic studies have shown that polymorphisms of the TGF-β1 gene, associated with a higher production of this growth factor, are less common among smokers with airflow limitation than among healthy smokers, suggesting that TGF-β1 may protect against the development of COPD (10,11).

One of the possible mechanisms through which TGF-β1 may exert a protective role is by switching off the inflammatory response, preventing its perpetuation. Indeed, it has been shown that TGF-β1 orchestrates the resolution of inflammatory responses, acting on both T and B-lymphocytes (12-14). Moreover, TGF-β1 attenuates the response to virus and bacteria, by inhibiting the upregulation of mucins induced by activation of the Toll like receptors (15). In this context, it has been reported that TGF-β1 is crucial for down regulating mucin production after infection with Haemophilus influenzae, which is a common pathogen in COPD (16).

While the role of TGF-β1 signalling on mucin production has been investigated in vitro, to the best of our knowledge, no study has addressed the relevance of TGF-β1 pathway on mucus hypersecretion in vivo. In a previous study we have shown that, in smokers with COPD, mucus hypersecretion was associated with an inflammatory process localized within the bronchial glands (17). In the present report we aimed to extend those findings by analyzing the expression of a cytokine involved in mucus production, such as TGF-β1, specifically in the gland compartment.

To this aim, we examined the expression of TGF-β1 and TGF-β type II receptor (TGF-β RII) in bronchial glands of 24 smokers undergoing lung resection for solitary peripheral nodules. Twelve of them had COPD and 12 had normal lung function. Even if we had tissue available only from a few nonsmoking subjects (n=3), we decided to quantify the expression of TGF-β1 and its receptor also in these subjects for comparison purposes. Some of the results of this study were presented in abstract form (18,19).
METHODS

Subjects

The study population included 24 subjects who underwent lung resection for a solitary peripheral carcinoma. All had a history of cigarette smoking, 12 had airflow limitation (i.e. FEV1/FVC <70% after bronchodilator inhalation) (smokers with COPD) and 12 were asymptomatic with normal lung function (control smokers). Among COPD patients, 10 had symptoms of chronic bronchitis, defined as cough and sputum production occurring on most days of the month for at least 3 months a year during the 2 years prior to the study. Subjects with COPD had no exacerbations, defined as increased dyspnea associated with a change in the quality and quantity of sputum, that led the subject to seek medical attention, during the month preceding the study.

Moreover, for comparison purposes, we included in the analysis specimens obtained from 3 nonsmoking subjects who also underwent lung resection for a solitary peripheral carcinoma. They had no symptoms of chronic bronchitis nor airflow limitation (average FEV1/FVC: 81±8%).

All subjects included in the study had been free of acute upper-respiratory-tract infections and none had received glucocorticoids or antibiotics within the month preceding surgery, or bronchodilators within the previous 48 h. The subjects were nonatopic (i.e., they had negative skin tests for common allergen extracts), and had no past history of asthma or allergic rhinitis. The study conformed to the Declaration of Helsinki, and informed written consent was obtained for each subject undergoing surgery. Each subject underwent an interview, chest radiography, electrocardiography, routine blood tests, skin tests with common allergen extracts, and pulmonary function tests in the week before surgery.

Some of the subjects of this study were also included in a previous report (17).

Pulmonary Function Tests

Pulmonary function tests were performed as previously described (17). Briefly, they included measurements of FEV1 and FVC in all the subjects examined. The predicted normal values used were those from Communauté Europeene du Carbon e de l’Acier (CECA) (20). In order to assess the reversibility of airway obstruction in subjects with COPD, the pulmonary function measurement was repeated 15 min after the inhalation of 200 µg of salbutamol. Moreover, subjects with normal lung function underwent inhalation challenge with methacholine.

Histology

Bronchial rings were taken from the lobar or segmental bronchus of the lobe obtained at surgery, away from the tumor site. One bronchial ring was selected for each subject, fixed in 4% formaldehdyde and, after dehydration, embedded in paraffin wax. Bronchial rings were then oriented, and 5 µm-thick serial sections were cut for immunohistochemical analysis of TGF-β1 and TGF-β RII expression. Briefly, sections were subjected to antigen retrieval, to unmask antigens in formalin-fixed and paraffin
embedded tissue sections, by heating in a microwave oven on high power for 8 minutes in 0.01 mol/L citrate buffer (pH 6.0) and then incubated with a mouse monoclonal antibody anti TGF-\(\beta_1\) (dilution 1:20; Genzyme Diagnostics, Cambridge, MA) or with a polyclonal antibody anti TGF-\(\beta\) RII (dilution 1:200; Biotechnology Inc. Santa Cruz, CA). Before incubation with primary antibody, the sections were treated with a biotin blocking kit (Vector Laboratories, Peterborough, UK) to inhibit endogenous biotin. The detection system was performed using the Vectastain ABC kit (Vector Laboratories) with 3-amino-9-ethylcarbazole as the chromogenic substrate. Sections were counterstained with Mayer’s hematoxylin.

Morphometric measurements were performed by a single observer using a computerized image analyzer (Casti Imaging SC processing, Venice, Italy). Briefly, in each patient, the total gland area was identified at lower magnification (20X) and outlined with the computer cursor. Then, at higher magnification (40X) the number of acini positive for TGF-\(\beta_1\) or TGF-\(\beta\) RII staining was quantified by the observer and the results were expressed as percentage of the total number of acini. For an acinus to be positive, specific staining had to be present on at least a quarter of the total acinus. In addition, the area positive for TGF-\(\beta_1\) or TGF-\(\beta\) RII staining was outlined with the computer cursor in each acinus and the sum of these areas was computed by the image system. The results were expressed as percentage of positive area over the total gland area.

Moreover, Reid's index, which measures bronchial gland size, was calculated by computing the ratio between the maximum thickness of each bronchial gland and the thickness of the bronchial wall (17). Finally, to quantify mucin producing acini, Alcian Blue-Periodic Acid Schiff (PAS) staining was performed, which identifies acidic and neutral mucopolysaccharides, respectively. The results were expressed as ratio between Alcian Blue-PAS\(^-\) and Alcian Blue-PAS\(^+\) acini, which thereafter will be referred to as mucin-negative and mucin-positive acini.

Statistical Analysis

Group data were expressed as means ± SEM, or as medians and ranges when appropriate. Differences between groups were analyzed with the unpaired Student’s t test for clinical data and the Mann-Whitney U test for morphologic data. Correlation coefficients were calculated using Spearman’s rank method. Values of \(p < 0.05\) were accepted as significant. At least three replicate measurements of morphometric parameters were performed by the same observer, and the intraobserver variability was assessed with the coefficient of variation (CV) for repeated measurements. For each subject CV was calculated as the ratio between the standard deviation (SD) and the mean of the three measurements performed: (SD/mean) \(\times\) 100.

RESULTS

Clinical Findings

The characteristics of smokers with COPD and control smokers are reported in Table 1. The two groups of subjects were similar with regard to age, sex and smoking history (pack-years). Ex-smokers were seven among smokers with COPD and eight
among control smokers. As expected from the selection criteria, smokers with COPD had a significantly lower value of FEV\textsubscript{1} (% predicted) and FEV\textsubscript{1}/FVC ratio (%) than did control smokers, while the two groups of subjects were similar with regard to PaO\textsubscript{2} and PaCO\textsubscript{2} values (Table 1). According to the GOLD criteria, three smokers with COPD were classified in stage I and nine in stage II (1). All control smokers had normal lung function and reactivity to methacholine within the normal range (PD\textsubscript{20} FEV\textsubscript{1} > 1.44 mg methacholine, corresponding to 7 \(\mu\)moles).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69 ± 2</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>12/0</td>
<td>12/0</td>
</tr>
<tr>
<td>Pk-yrs</td>
<td>55 ± 6</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>Smoking (Ex/Current)</td>
<td>7/5</td>
<td>8/4</td>
</tr>
<tr>
<td>FEV\textsubscript{1}/FVC (%)</td>
<td>67 ± 2 *</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>FEV\textsubscript{1} (% pred)</td>
<td>75 ± 5 *</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>PaO\textsubscript{2} (mmHg)</td>
<td>85 ± 3</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>PaCO\textsubscript{2} (mmHg)</td>
<td>39 ± 2</td>
<td>38 ± 2</td>
</tr>
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* p< 0.01 as compared with controls subjects.

**Histological Findings**

Quantification of TGF-\(\beta\)\textsubscript{1} and TGF-\(\beta\) RII was satisfactory in all the subjects included in the study, except in one control smoker in whom quantification of TGF-\(\beta\) RII could not be performed because only a small amount of tissue was available. The total number of acini examined was not different in smokers with COPD and in control smokers [median (range): 172 (52-386) vs 122 (55-384)]. Similarly, the total gland area was not different between the two groups examined [1 (0.4-3) vs 0.6 (0.3-2) mm\textsuperscript{2}].

The percentage of TGF-\(\beta\) RII\textsuperscript{+} acini over total acini was decreased in smokers with COPD when compared to control smokers [48 (7-90) vs 69 (54-100) %; p = 0.004]. When the results were expressed as percentage of positive area over total gland area, smokers with COPD had a decreased percentage of TGF-\(\beta\) RII\textsuperscript{+} area as compared to control smokers [7 (2 – 12) vs 11 (5 –22) %; p= 0.012] (Figure 1 and figure 2). By contrast, the expression of TGF-\(\beta\)\textsubscript{1} was not significantly different between smokers with COPD and control smokers, both when the results were expressed as percentage of positive acini or as percentage of positive area (Figure 3).

When we subdivided the subjects according to the presence or absence of symptoms of chronic bronchitis (instead of airflow limitation) we observed a decreased expression of TGF-\(\beta\) RII in smokers with chronic bronchitis (n=10)
compared to smokers without chronic bronchitis (n=14). Indeed, patients with chronic bronchitis had a decreased percentage of TGF-β RII⁺ acini as compared to smokers without chronic bronchitis [48 (32-90) vs 66 (7-100); p=0.01], as well as a decreased percentage of TGF-β RII⁺ area [7 (3-10) vs 11 (2-22); p=0.01].

When we subdivided the subjects according to the current smoking status, we didn’t observe any significant difference between current smokers (n=9) and ex smokers (n=15) in the percentage of TGF-β RII⁺ area [6 (2-22) vs 9 (3-18)] nor in that of TGF-β₁⁺ area [5 (0-11) vs 6 (0-16)].

In the three nosmoking subjects that were analysed, the values of TGF-β RII and TGF-β₁ expression were in the same range of control smokers. In particular, the median value for the percentage of TGF-β RII⁺ area was: 12 (10-12%) and that for the percentage of TGF-β₁⁺ area was: 13 (6-16%).

Bronchial gland size, as expressed by Reid’s index, was not significantly different between smokers with COPD and control smokers [45 (27-64) vs 38 (17-76%)]. Similarly, the ratio between mucin-negative and mucin-positive acini was not significantly different in the two groups of subjects examined [9 (0-55) vs 6 (0-97%)]. However, when all the smoking subjects were considered together, both the percentage of TGF-β RII⁺ acini and the percentage of TGF-β RII⁺ area were negatively correlated with the values of Reid’s index (p=0.02, r=−0.50 and p=0.01, r=−0.54 respectively) (figure 4). Similarly, a trend for a correlation to be significant was observed between the percentage of TGF-β RII⁺ acini and the ratio between mucin-negative and mucin-positive acini (p=0.06, r=0.40). Mucin-positive acini were identified in sections stained with Alcian blue-PAS staining, where they can be stained either in blue (acidic mucopolysaccharides) or in purple (neutral mucopolysaccharides) (figure 5). In serial sections, expression of TGF-β₁ and TGF-β RII was not restricted to a particular type of acinus but was observed in either mucin-positive or in mucin-negative acini.

The number of neutrophils infiltrating the bronchial glands was increased in smokers with COPD compared to control smokers [42 (12-88) vs 11 (5-57) cells/mm²; p=0.01], however this neutrophilic infiltration was not significantly correlated to either TGF-β₁ or TGF-β RII expression.

When we examined the expression of TGF-β₁ and its receptor in the bronchial epithelium, we observed that, either in smokers with COPD or in control smokers and even in nonsmokers, these proteins were present at extremely high levels in both ciliated and goblet cells. For this reason a detailed quantification in the epithelium could not be performed.

The mean coefficients of variation (CV) for repeated measurements were: 2.9% for TGF-β RII⁺ acini; 1.3% for TGF-β RII⁺ area; 2.7% TGF-β₁⁺ acini; 11% for TGF-β₁⁺ area.
DISCUSSION

This study shows that, in smokers with COPD, there is a decreased expression of TGF-β type II receptor in the bronchial glands, a reduction which is associated to bronchial gland enlargement. These findings suggest that an impaired TGF-β signalling might induce structural changes in the bronchial glands which, in turn, may promote mucus hypersecretion.

Interestingly, it has been recently shown that alterations in the TGF-β1 pathway may be involved in the pathogenesis of both chronic bronchitis and emphysema, which are the main clinical hallmarks of COPD (8,9,16). In this context, it is known that TGF-β1 acts as a negative regulator of mucin production induced by bacterial infections, as shown by Jono and coworkers (16). These authors clearly demonstrated that infection with *Haemophilus influenzae* induces a defensive response characterized by upregulation of mucins, and that TGF-β1 is crucial for attenuating this response after removal of the infectious agent. Indeed, these authors reported that suppression of the TGF-β RII pathway results in mucin hyperproduction in vitro. In vivo, it has been shown that a mouse knockout for Runx3, which is a transcription factor that mediates TGF-β signalling, develops mucus hypersecretion (21). Our finding of a decreased expression of TGF-β RII in smokers with COPD, the majority of whom had symptoms of chronic bronchitis, supports the view that an altered TGF-β pathway may result in mucus hypersecretion in humans as well.

Of interest, when we examined the relationship between the expression of TGF-β RII and Reid’s index we observed a negative correlation, indicating that the lower the expression of this receptor, the larger the bronchial gland size. Moreover, when we analysed the mucus content in bronchial glands, we observed a trend for a correlation to be significant showing that the lower the expression of TGF-β RII, the higher the number of mucin-positive acini. Taken together these observations further support the protective role of TGF-β pathway on mucus hypersecretion in smokers.

These findings are in line with the recent hypothesis that TGF-β1 could play a role in maintaining the lung homeostasis in physiologic conditions, an effect which may be lost in smokers who develop COPD (10, 22). Of interest, a similar role of TGF-β1 in maintaining tissue homeostasis has been proposed in other chronic diseases, since a decreased activity of TGF-β RII has been observed in the early phases of tumorigenesis and atherogenesis (23, 24).

The concept that TGF-β1 may have a protective role in smokers is also supported by two recent genetic studies showing that polymorphisms of the TGF-β1 gene, associated with higher production of this growth factor, were more frequent among healthy smokers than among smokers with COPD (10,11). Furthermore, studies on animal models strongly suggest that abnormalities in the activation and signalling of TGF-β1 are important in the pathogenesis of emphysema. Indeed, it has been shown that mice lacking activation of latent TGF-β1 develop severe pulmonary emphysema through alterations of macrophage metalloelastase MMP12 (8,9). Of interest, a recent study demonstrated that similar mechanisms occur in humans as well. Indeed, it has been shown that alveolar macrophages from patients with COPD exhibit a reduced production of TGF-β1, which is paralleled by a reduced production of TIMP-1 (25). These findings suggest that impairment of TGF-β1 is associated with
a reduced anti-proteolitic activity which in turn may result in emphysematous lesions. On the same line, we recently demonstrated that TGF-β RII expression in alveolar walls of patients with severe emphysema was negatively correlated with T lymphocyte infiltrate, which is thought to promote alveolar wall destruction (22). The intriguing concept which can be inferred from all these studies is that, by downregulating a single signalling pathway in the lung (TGF-β₁), it is possible to promote the development of the two main clinical components of COPD, i.e. emphysema and chronic bronchitis.

Nevertheless it should be emphasized that the role of TGF-β₁ in COPD is still controversial, with some studies reporting an increased TGF-β₁ signalling (5-7) and other studies being unable to replicate these findings (26,27). However, making a comparison between those and our report can be difficult because, at variance with previous ones, our study was focused specifically on bronchial glands. It is indeed conceivable that the expression of TGF-β₁ and its receptors could be under the control of different factors in the different lung compartments.

Among its different activities, TGF-β₁ is known to play a pivotal role in the resolution of immune responses (12-14) and it can be hypothesized that a decreased expression of TGF-β RII may result in a persistent inflammatory response. Indeed, in the present study we confirmed the prominent neutrophilia within the bronchial glands that we previously reported in smokers with COPD. However, when we examined the relationship between TGFβ RII expression and number of neutrophils, we found no significant correlation. These findings indicate that the lack of TGF-β signalling has a direct effect on gland hyperplasia and mucus hypersecretion, but not on neutrophilic infiltration within the glands.

We should acknowledge that, even if we favour the hypothesis that the decreased TGFβ RII expression reflects a decreased TGFβ signalling, an alternative explanation is that it would result from internalization of the receptor due to an excessive TGFβ binding. However, we think this possibility is unlikely because it has been reported that internalization of TGFβ RII occurs at extremely slow rates (28) and independently of the presence of the ligand (29).

A possible limitation of our study is that we could not include a group of healthy non-smoking subjects for comparison, because the majority of lung cancer cases are strictly related to the smoking habitus. Nevertheless, when we quantified the expression of TGF-β₁ and its receptor in the three nonsmoking subjects whose tissue was available, this expression was similar to that of smokers with normal lung function. Another possible limitation of our study is that potential confounding factors may interfere with the expression of TGF-β. For example, TGF-β is known to bind to different proteins in the extracellular matrix (30) and this interaction may mask the molecule to the specific antibody. This interaction may raise concerns when considering the expression of the ligand, since TGF-β₁ can be secreted outside the cell and interact with extracellular matrix proteins. By contrast, we feel rather confident that our findings of a decreased expression of TGF-β RII is valid because the receptor, at variance with the ligand, is mostly present in the cell and it is less likely to bind to extracellular matrix components. Finally, in the present study we examined only the expression of TGF-β₁ and its type II receptor, while the TGF-β family includes many different ligands and receptors. Therefore, we can not exclude that
other members of the TGFβ family, and in particular TGFβ₂ which is known to be
dysregulated in asthma, could also play a role in COPD (31).

In conclusion this study shows that, in bronchial glands of smokers with
COPD, there is a decreased expression of TGF-β type II receptor which correlates
with bronchial gland enlargement. These results suggest that TGF-β₁ signalling may
have a protective role in the development of mucus hypersecretion in COPD, a
finding which is somehow surprising. Intriguingly, a similar protective role has been
recently demonstrated in other chronic disorders, such as atherosclerosis and cancer,
where TGF-β₁ was traditionally thought to have detrimental effects.

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FIGURE LEGENDS

Figure 1
Individual counts for TGF-β RII⁺ acini (% total acini) (A) and TGF-β RII⁺ area (%
total gland area) (B) in the bronchial glands of smokers with COPD and control
smokers. Horizontal bars represent median values.

Figure 2
Microphotograph showing TGF-β RII⁺ acini in bronchial glands of a smoker with
COPD (A) and a control smoker (B). Arrows indicate TGF-β RII⁺ acini, which are
stained in brown. Original magnification: 630X

Figure 3
Individual counts for TGF-β₁⁺ acini (% total acini) (A) and TGF-β₁⁺ area (% total
gland area) (B) in the bronchial glands of COPD and control smokers. Horizontal bars
represent median values.

Figure 4
Relationship between TGF-β RII⁺ area (% total gland area) and values of Reid’s index
(%) (Sperman’s rank correlation, p = 0.01, r = -0.54). Smokers with COPD are
indicated by triangles and control smokers by circles.

Figure 5
Microphotograph showing Alcian blue-PAS staining in a bronchial gland of a smoker
with COPD. Mucin-positive acini can be stained either in blue (acidic
mucopolysaccharides) or in purple (neutral mucopolysaccharides) as indicated by the
arrows, while mucin-negative acini are indicated with the arrow head. Original
magnification 400X.
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Figure 3A, 3B

1. TGFβ+ acini/total acini (%)
   - COPD: [Graph showing data points and mean]
   - Smokers: [Graph showing data points and mean]

2. TGFβ+ area/total gland area (%)
   - COPD: [Graph showing data points and mean]
   - Smokers: [Graph showing data points and mean]
Figure 4

TGFβ RII⁺ area
(normal gland area (%) - 100)

Reid's Index (%)
Decreased expression of TGFβ type II receptor in bronchial glands of smokers with COPD

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