Chronic airways inflammation is one of the features of chronic obstructive pulmonary disease (COPD). We demonstrated previously that bronchiolar epithelium in COPD contains increased numbers of macrophages and mast cells. Transforming growth factor \(\beta_1\) (TGF-\(\beta_1\)) may be involved in this influx because it has chemotactic activity for macrophages and mast cells. In this study, we examined expression patterns of TGF-\(\beta_1\), TGF-\(\beta\) receptors type I and II (TGF-\(\betaRI\) and TGF-\(\betaRII\)) by immunohistochemistry and mRNA in situ hybridization in peripheral lung tissue of 14 current or ex-smokers with COPD (FEV\(_1\) < 75%) and 14 without COPD (FEV\(_1\) > 84%). In both groups, TGF-\(\beta_1\) and its receptors are present in airway and alveolar epithelial cells, airway and vascular smooth muscle cells, and tissue and alveolar CD68\(^+\) cells (considered herein to be macrophages). In subjects with COPD, a semiquantitative analysis revealed approximately twofold higher levels of TGF-\(\beta_1\) mRNA and protein in bronchiolar and alveolar epithelium (\(p < 0.02\)) as compared with subjects without COPD. With regard to bronchiolar epithelial cells, we found a significant correlation between TGF-\(\beta_1\) mRNA and protein expression (\(r = 0.62; p < 0.002\)), and between the FEV\(_1\) of all subjects together and TGF-\(\beta_1\) protein (\(r = -0.60; p < 0.0002\)) and mRNA (\(r = -0.67; p < 0.002\)) levels. The epithelial expression of TGF-\(\beta_1\) mRNA and TGF-\(\beta_1\) protein correlates with the number of intraepithelial macrophages (both: \(r = 0.44; p < 0.03\)) whereas intraepithelial mast cell numbers correlate with epithelial TGF-\(\beta_1\) mRNA expression. These data suggest a role for TGF-\(\beta_1\) in recruiting macrophages into the airway epithelium in COPD. de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, van Krieken JHJM. Transforming growth factor \(\beta_1\) and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease.

phages and mast cells in bronchiolar epithelium of current or ex-smokers with COPD compared with smokers or ex-smokers without COPD (6). Using sequential sections of the same tissue specimens, we found a significant increase in TGF-β1 mRNA and protein levels in airway epithelial cells in subjects with COPD as compared with those without COPD. These expression levels correlated with the increased numbers of intraepithelial macrophages (6).

**METHODS**

**Antibodies**

Polyclonal rabbit anti-human antibodies raised against synthetic peptides corresponding to either amino acids 158-179 of transforming growth factor β receptor type I (TGF-βRI) or amino acids 550-565 of TGF-β receptor type II (TGF-βRII) (both within the carboxyl terminal domain), and their neutralizing control peptides following the manufacturer’s instructions, and found to be negative.

**Subjects**

For this study, we used lung tissue specimens of subjects with or without COPD (6). Briefly, we selected tissue specimens from peripheral airways (airway diameter ranging from 1 to 3 mm) from current or ex-smokers who underwent lobectomy or pneumectomy for lung cancer. Fourteen subjects with COPD (FEV₁ < 75% of predicted value before bronchodilatation; reversibility in the FEV₁ of ≤ 13% of the predicted value after 400 μg inhaled salbutamol) were included, as well as 14 subjects without COPD (FEV₁ before bronchodilatation > 84% predicted). The total lung capacities (TLCs) were not below normal levels (TLC ≥ 80% predicted). Exclusion criteria included: (1) diffuse pulmonary infarction of fibrotic disorders; (2) absence of tumor-free or poststenotic pneumonia-free lung tissue specimens; and (3) obstruction of central bronchi due to the tumor. A II patients lack upper respiratory tract infection and did not receive antibiotics perioperatively. A II patients had not received glucocorticosteroids during 3 mo before resection, four patients received glucocorticosteroids only perioperatively. All patients had not received glucocorticosteroids during 3 mo before resection, four patients received glucocorticosteroids only perioperatively (Table 1). Data on lung function tests of these patients are presented in Table 1 and are described previously (6). Subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone.

**Immunohistochemistry**

Serial paraffin-embedded tissue sections (3 μm thick) were alternately used for TGF-β1 and TGF-β1 in situ hybridization and immunohistochemistry on TGF-β1, TGF-βRI, and TGF-βRII. Detection of cell-specific markers on adjacent sections was performed in order to confirm the type of cell. Immunocytochemistry was performed on serial sections essentially as described earlier (6, 21). A titer deparaffinization, endogenous peroxidase was inactivated with 0.3% hydrogen peroxide. Sections to be stained with anti-CD68 were treated with proteinase K. Subsequently, sections were preincubated with 1% (wt/vol) bovine serum albumin (BSA). A nitogen expression was demonstrated with appropriate dilutions of the primary antibodies in conjugated immunoenzyme assays using a secondary biotin-conjugated antibody and a tertiary complex of streptavidin–avidin–biotin conjugated to horseradish peroxidase, and 3-amino-9-ethyl-carbazole (AEC) as chromogen. Finally, the sections were counterstained with Mayer’s hematoxylin. Incubation with phosphate-buffered saline (PBS) supplemented with 1% BSA instead of the primary antibody served as a negative control.

The possibility of false-positivity with anti-TGF-βRI and anti-TGF-βRII antibodies was verified by preabsorption of the first antibodies with their specific neutralizing control peptides following the manufacturer’s instructions, and found to be negative.

**TABLE 1**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Pack-Years</th>
<th>FEV₁</th>
<th>FEV₁/FVC</th>
<th>Steroid</th>
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<tr>
<td>Non-COPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>M</td>
<td>79</td>
<td>47</td>
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<tr>
<td>2</td>
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</tr>
<tr>
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</table>

**COPD**

<table>
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<tr>
<th>Mean ± SEM</th>
<th>64 ± 3.7</th>
<th>42 ± 7.7</th>
<th>101 ± 3.3</th>
<th>0.72 ± 0.02</th>
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* FEV₁ and FVC are given as percentage of predicted values.

The TGF-β1 and its receptor expression were assessed in a semi-quantitative analysis using a visual analogue scale. A subset was analyzed twice to assess the intra-observer variability (kappa = 0.4; p < 0.05; deviation in staining grade ranged from 0 to 0.5). The staining intensity in each of the following cell types was scored in a blinded manner: epithelial cells, subepithelial cells (CD3+ and tryptase+ cells, fibroblasts), smooth muscle cells, macrophages (CD68+), and endothelial cells of larger blood vessels. The staining intensity was graded and expressed as: 0 = absence of staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining.

**In Situ Hybridization**

The in situ hybridization was performed on paraffin-embedded sections adjacent to sections on which TGF-β1, TGF-βRI, TGF-βRII, and cell type-specific immunoreactivity were assessed. For in situ hybridization, we used a Smal-BamHI fragment of TGF-β1 complementary DNA (cDNA) cloned into pBluescript KS (Stratagene, La Jolla, CA) as described (20). The specific copy RNA (cRNA) probes were labeled with digoxigenin following the manufacturer’s protocol (Boehringer, Mannheim, Germany). The in situ hybridization was performed essentially as described (20). Briefly, after pretreatment sections were hybridized with 50 ng per slide during 16 h at 42°C. Subsequently, sections were washed in 2× standard saline citrate (SSC) with 50% formamide at 37°C, then in 0.1× SSC with 20 mM β-mercaptoethanol at 42°C, and finally treated with 2 U/ml ribonuclease (RNAse) T1 (Boehringer, Mannheim, Germany) in 2× SSC plus 1 mM ethylenediaminetetraacetic acid (EDTA) at 37°C. The immunodetection of digoxigenin-labeled hybrids was done using nitro blue tetrazolium (NBT) as chromogen and bichloroindolyl phosphate (BCIP) as coupling agent (Boehringer, Mannheim, Germany). The sense riboprobes were included as negative controls, and in general did not show staining. If staining of sense riboprobes was detected (which was
always far less than the staining of antisense probes), we subtracted this immunostaining score from the equivalent antisense immunostaining score. The staining intensity was expressed as described for the immunocytochemistry.

Statistics

The immunohistochemistry and in situ hybridization data were expressed as mean ± SEM. Significance levels were obtained using the unpaired, two-tailed Student’s t test. Correlation analysis and statistics between expression levels and intraepithelial numbers of mast cells and macrophages was done using Stata Statistical Software 5.0 (StataCorp., College Station, TX). A t p < 0.05 differences were considered to be statistically significant.

RESULTS

In subjects without COPD, TGF-β₁ mRNA and protein are localized predominantly in bronchial, bronchiolar, and alveolar epithelial cells, vascular and airway smooth muscle cells, and in CD68⁺ cells (Figures 1 and 2; Table 2). A faint staining was seen in subepithelial cells including inflammatory cells. Although high levels of TGF-β₁ transcripts were found in endothelial cells (Figure 2; Table 2), we did not detect TGF-β₁ immunoreactivity within endothelial cells.

In subjects with COPD, we observed significantly higher (p < 0.02) TGF-β₁ protein (up to 2 times) and mRNA (1.5 times) levels in bronchial, bronchiolar, and alveolar epithelial

Figure 1. Micrographs of lung tissue sections from subjects without COPD (A–C) and with COPD (D, E). TGF-β₁ mRNA localization (blue/purple) in bronchioli using the antisense probe are shown in (A, D). The adjacent section of (A) is shown in (B, C) after incubation with the sense probe either before CD68- (B) or after CD68- immunostaining (C). An example of TGF-β₁ protein staining in the airways is shown (E). Small arrows indicate pneumocytes, large arrows CD68-positive cells; O = bronchiolar lumen. Only sections in (E) are counterstained with hematoxylin. Original magnification: ×50 (A-D) or ×100 (E).
cells as compared with subjects without COPD (Figure 2; Table 2). In vascular smooth muscle cells within alveoli of subjects with COPD, we also noted 1.5 times higher (p < 0.002) TGF-β1 protein but not mRNA levels (Table 2). Endothelial cells in airway walls but not in alveolar walls exhibit 1.5 times higher (p < 0.01) TGF-β1 mRNA levels as compared with patients without COPD (Figure 2; Table 2). No differences in expression levels of TGF-β1 were noticed in CD68+.

Because a difference in TGF-β1 levels may also contribute to TGF-β1-mediated effects that underlie the pathogenesis of COPD, we examined both TGF-βRI and TGF-βRII protein expression levels. In subjects without COPD, both receptors are present on the same cells that produce TGF-β1. The highest expression levels of both receptors are found in bronchial, bronchiolar, and alveolar epithelium as well as in alveolar macrophages (Table 3). Subepithelial cells (fibroblasts, inflammatory cells) exhibited less expression of TGF-βRI and TGF-βRII as compared with epithelial, smooth muscle, or endothelial cells (Table 3). In contrast, endothelial cells display a moderate expression of TGF-βRI and low levels of TGF-βRII (Table 3).

Definition of abbreviation: SMC = smooth muscle cells.
* Semiquantitative analysis of the TGF-β1 mRNA and protein levels per cell type in bronchial and bronchiolar airways (airways) or in the alveoli (alveoli) in subjects without COPD (Non-COPD) or with COPD. The mean immunostaining score ± SEM ranges from 0 (no staining) to 3 (very intense staining).
† Significant difference (p < 0.02, Student’s t test) with comparable data from subjects without COPD.
In subjects with COPD, we did not detect expression levels and patterns of TGF-βRI different from those in subjects without COPD. However, a twofold increase (p < 0.001) in TGF-βRII expression in alveolar macrophages was observed as compared with subjects without COPD (Table 3).

We found a positive and significant correlation between TGF-β1 mRNA and protein levels in bronchiolar epithelial cells (r = 0.62; p < 0.002). In addition, the bronchiolar endothelial TGF-β1 mRNA levels correlated well with the bronchiolar TGF-β2 protein levels in vascular smooth muscle cells (r = 0.58; p < 0.006). In the bronchiolar epithelium, the numbers of intraepithelial CD68+ cells were correlated with both TGF-β3 mRNA (r = 0.44; p < 0.03) and protein (r = 0.44; p < 0.03) levels (Figure 3). The number of intraepithelial tryptase+ cells (considered to be mast cells) also correlated with the TGF-β3 mRNA levels in the bronchiolar epithelium (r = 0.58; p < 0.002), but did not correlate significantly with TGF-β3 protein levels (r = 0.13; p = 0.48) (Figure 3). Finally, if considering all subjects with and without COPD together, then the FEV1 values correlate with both TGF-β1 mRNA (r = −0.67; p < 0.0002) and protein (r = −0.60; p < 0.002) expression in the bronchiolar epithelium.

DISCUSSION

In the present study, we compared protein and mRNA distribution patterns of TGF-β1, and protein expression of TGF-βRI and TGF-βRII in the pulmonary bronchi, bronchioli, and alveoli of subjects with and without COPD. In subjects without COPD, TGF-β1 proteins and transcripts were seen predominantly in epithelial cells, smooth muscle cells, and both interstitial and intraluminal CD68+ cells (considered herein to be macrophages). The distribution patterns were similar for all airways examined. In subjects with COPD, a higher expression of TGF-β1 mRNA and protein was seen in airway and alveolar epithelial cells as compared with subjects without COPD. A high expression of both TGF-β receptor types was seen especially in macrophages in subjects with COPD. The higher expression of TGF-β1 in bronchiolar epithelial cells correlates with both the increased number of macrophages and mast cells in the bronchiolar epithelium in COPD (6), and with FEV1 values if all current or ex-smokers were taken together. These data indicate that TGF-β1 is implicated in the recruitment of macrophages and mast cells into the airway epithelium in COPD.

In vitro studies demonstrated that TGF-β mediates chemotaxis of different types of inflammatory cells including monocytes, mast cells, and T lymphocytes (9–12). TGF-β1 can also stimulate the expression of the cell–cell adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (22). ICAM-1 is necessary for diapedesis of mononuclear phagocytes through the endothelial and epithelial cell layers (23). The enhanced ICAM-1 expression seen on airway epithelial cells in smokers with chronic bronchitis (24) may be

![Figure 3](image-url)

Figure 3. Correlations between inflammatory cell numbers and TGF-β1 protein and mRNA expression both in bronchiolar epithelium. (A) Macrophage numbers and TGF-β1 mRNA levels. (B) Macrophage numbers and TGF-β1 protein expression. (C) Mast cell numbers and TGF-β1 mRNA levels. (D) Mast cell numbers and TGF-β1 protein expression. Cell numbers are given per millimeter of basement membrane (BM). Open circles represent data from subjects without COPD, closed circles from subjects with COPD. Data are obtained by linear regression analysis. Correlation (r) and significance level (p value) are given.
mediated by TGF-β1 and supposedly stimulates the diapedesis of pulmonary monocytes and macrophages. TGF-β1 can also induce the expression of cytokines like interleukin-1β by monocytes and thereby contributes to inflammatory processes (9, 25). In vivo studies on transgenic mice support the in vitro data as it was shown that targeted overexpression of TGF-β1 in the pancreas or in the central nervous system is accompanied by an influx of inflammatory cells including macrophages into these organs (26, 27). Hence, there is ample evidence that TGF-β is involved in inflammation.

Our present expression data on TGF-β1 in airways of subjects without COPD agree with previous studies on subjects without COPD (13, 14, 19). We also found TGF-β1 expression in pneumocytes both at the protein and at the mRNA level. In contrast, Aubert and coworkers (18), Magiann and coworkers (13), and Corrin and coworkers (15) did not observe any TGF-β1 protein immunoreactivity in morphologically normal-appearing pneumocytes. This may be due to the recognition of different epitopes by the anti-TGF-β1 antibodies. Furthermore, the TGF-β1 expression patterns in patients with COPD as observed in the present study agree with the two TGF-β1 expression studies done so far on three patients with emphysema (17) and 19 smokers with chronic bronchitis (19). In contrast, Aubert and coworkers (18) did not find any difference in TGF-β1 mRNA or protein expression between subjects with or without COPD. A possible explanation could be that Aubert and coworkers (18) did not analyze the TGF-β1 expression per cell type but rather used tissue homogenates. In addition, as pointed out earlier, the TGF-β1 protein localization was examined with different antibodies.

Vignola and coworkers (19) reported a higher epithelial TGF-β1 expression in lung tissue from smokers with chronic bronchitis as compared with nonsmokers. They suggested that cigarette smoking accounts for the higher TGF-β1 expression. However, they did not include a subject group of smokers without chronic bronchitis. Now, we demonstrate that the epithelial TGF-β1 expression is increased in smokers with COPD as compared with smokers without COPD. Because the mean number of pack-years does not differ between our two subject groups (Table 1), our data indicate that smoking alone cannot fully account for the higher epithelial TGF-β1 expression in COPD. In addition, the higher epithelial TGF-β1 mRNA and protein expression in COPD correlates with a low FEV1. From our data, we cannot conclude that the TGF-β1 expression correlates with the severity of the disease. This is probably due to the lack of sufficient numbers of patients with severe COPD.

According to the linear regression analysis, the number of intraepithelial mast cells correlates significantly with the epithelial TGF-β1 mRNA but not the protein expression. At this moment, we do not have an explanation for this difference. The presence of TGF-β1 mRNA but absence of TGF-β1 protein in endothelial cells (Figure 2), and the good correlation between endothelial TGF-β1 mRNA levels and vascular smooth muscle cells TGF-β1 protein levels suggest that endothelial cells synthesize no or little TGF-β1 protein and/or that TGF-β1 proteins produced by endothelial cells are rapidly secreted and taken up by the surrounding smooth muscle cells. The relatively high levels of TGF-β1 and TGF-β1 mRNA on macrophages in COPD indicate that TGF-β1 may potentially stimulate the migration of these macrophages. Moreover, the elevated level of TGF-β1 in lung epithelial cells in COPD provides a concentration gradient of TGF-β1 which may mediate an even more prominent effect on macrophage migration as compared with subjects without COPD. Because TGF-β1 is chemotactic toward monocytes/macrophages, this strengthens the hypothesis that lung epithelial-derived TGF-β1 is involved in the chemotaxis of macrophages into the airway epithelium in COPD.

TGF-β1 is probably not the only factor in the recruitment of macrophages and mast cells into the bronchiolar epithelium in COPD. A nother potent chemotactic and activating protein for macrophages and murine mast cells is monocyte chemotactic protein-1 (MCP-1) which can be expressed by airway epithelial cells in situ (28–30). TGF-β1 has been shown to induce the expression of MCP-1 (31, 32). In addition, a recent study showed that intratracheal instillation of MCP-1 in mice resulted in a recruitment of macrophages into the lung interstitium and alveoli and enhanced cigarette smoke–induced emphysema (33). Whether the influx of macrophages and mast cells into the airway epithelium in subjects with COPD is mediated directly by TGF-β1 or indirectly via MCP-1, remains to be determined.

Finally, TGF-β1 may be involved in structural remodeling of the extracellular matrix. We found a higher TGF-β expression in airway epithelium and pneumocytes in smokers with COPD as compared with smokers without COPD (this study). Other studies have shown an increased mass of structurally disordered collagen bundles in the alveolar septae of subjects with emphysema (7, 8). In bronchial airways, the increased expression of TGF-β1 in chronic bronchitis is significantly correlated with the number of fibroblasts and the thickness of the basement membrane (19). Finally, TGF-β1 can induce extracellular matrix synthesis including collagens and fibronectin in various cell types including lung fibroblasts, macrophages, and epithelial cells in vitro (34, 35). These data support the hypothesis that TGF-β1 contributes to the airway and airspace remodeling in COPD.

In conclusion, in smokers without COPD TGF-β1 and its receptors are expressed differentially by airway epithelial, endothelial, and smooth muscle cells, as well as macrophages. In smokers or ex-smokers with COPD, the higher TGF-β1 expression in bronchiolar epithelial cells correlated with the increased numbers of intraepithelial macrophages in COPD. The present data strengthen the hypothesis that TGF-β1 is involved in macrophage influx in COPD. Although the correlation data suggest that TGF-β1 is not the only factor for this recruitment, our data may point to the importance of TGF-β1 antagonists in future strategies for therapy of COPD.

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References


