Title:
Bronchial vascular remodelling in COPD patients and its relationship with inhaled steroid treatment

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Abstract

**Background:** Up to now, only few studies have evaluated microvascular changes and proangiogenetic mediators in the bronchial mucosa of COPD patients and the results provided were discordant. Furthermore, in this regard, the role of ICS has been scarcely investigated in COPD.

**Objective:** This study was designed to evaluate vascular remodelling, its relationship with inflammatory cells and treatment effects in bronchial mucosa of COPD patients.

**Methods:** The study included 10 non-treated COPD patients (COPD), 10 COPD patients treated (COPD/ICS) with nebulized BDP 1600-2400 mcg daily (equivalent to 800-1200 mcg via MDI) and 8 control subjects (CS). Bronchial biopsies were evaluated for number and size of vessels and vascular area. Specimens were also examined for VEGF, bFGF and TGF-β expression. Moreover, inflammatory cell counts were performed.

**Results:** Vascular area and vessel size were significantly increased in COPD as compared to COPD/ICS and CS (p<0.05). VEGF+ cells, bFGF+ cells and TGF-β+ cells were significantly increased in COPD as compared to COPD/ICS and CS (p<0.05). In addition, bFGF+ cells were significantly increased in COPD/ICS as compared to CS. CD8+ and CD68+ cells were significantly increased in COPD as compared to COPD/ICS and CS (p<0.05).

In COPD, VEGF+ cells correlated with number of vessels (p<0.05), vascular area (p<0.01) and vessel size (p<0.05). Moreover, TGF-β+ cells significantly correlated with vascular area (p<0.05).

**Conclusion:** Bronchial vascular remodelling in COPD patients is mainly related to morphological changes of the mucosal microvessels rather than to new vessel formation, and may be reduced in patients under steroid treatment.
Key words: COPD; vascular remodelling; inhaled corticosteroids

Abbreviations used: VEGF, vascular endothelial growth factor, bFGF, basic fibroblast growth factor, TGF-β, transforming growth factor-beta.

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Introduction

Most of the literature regarding bronchial angiogenesis in chronic airway inflammation results from studies in asthmatic patients (1), since the vascular component of airway remodelling significantly contributes to the alteration of airway wall in asthma (2,3). Bronchial microvasculature may alter the bronchial wall by increasing vessel calibre, by oedema formation and by increasing the number of vessels (4,5).

It has been suggested that bronchial vascular changes may occur in COPD as well, although this phenomenon seems to be less evident than in asthma (6). There is no agreement on the presence of angiogenesis in the bronchial mucosa of patients with COPD, while an increased vascular area appears to be a common feature in these patients (7,8). Nevertheless, microvascular changes may contribute to increase airway wall thickness, and therefore they may be associated with the progression of COPD (9).

The pathologic mechanisms of bronchial vascular remodelling are not yet clarified, even if recent investigations emphasized the role of growth factors and cytokines. The expression of VEGF, bFGF and TGFβ is higher in COPD patients’ airways as compared to healthy subjects, and is related to the bronchial vascularity (10-12). In particular, VEGF, the most potent mediator of angiogenesis, can play a significant role in the pathophysiology of two major phenotypes of COPD, chronic bronchitis and emphysema (13). VEGF levels are significantly higher in patients with chronic bronchitis as compared to controls, while are significantly reduced in patients with emphysema (14). In addition, the bronchial epithelial expression of VEGF, FGF-1, FGF-2 and FGF receptor-1 (FGFR-1) is negatively correlated to lung function and positively correlated to the smoking history (10,11).

Up to now, published data regarding the relationship between bronchial microvasculature and airway inflammation and remodelling in COPD are quite scanty, albeit inflammatory changes in the airways are central to the pathogenesis of COPD (15,16). In COPD patients, there is evidence that VEGF and its receptors Flt-1 and KDR/Flk-1 can be involved in peripheral vascular and airway remodelling processes and accordingly in the development of airway obstruction (10). Moreover, although there is evidence that inhaled corticosteroids (ICS) can be clinically effective in severe COPD patients (17), only few studies (18-20) have been conducted to assess the effect of ICS on airway inflammation and remodelling in COPD. In particular, no study has evaluated the effect of ICS on the vascular component of airway remodelling in COPD.

In this study, we hypothesised that the vascular component of the airway remodelling in COPD patients is associated to airway inflammation and we investigated whether it was related to the use of anti-inflammatory treatment. The aims of this cross sectional study were, therefore, to quantify bronchial microvascular changes in endobronchial biopsies of COPD patients in comparison with control subjects, to examine the relationship between vascularity, angiogenic factors and inflammatory cells, and to evaluate whether the bronchial vascularity in COPD patients is related to the use of ICS.
Methods

Subjects

In this study, we included COPD patients that were clinically stable and did not require long-term oxygen therapy. Twenty COPD patients were recruited from the Section of Respiratory Diseases of the Department of Clinical Sciences of the Parma University. The patients were all ex-smokers from at least ten years and had stable moderate to severe COPD, according to GOLD classification (21). They were divided into two groups: those not receiving treatment with ICS (COPD, n = 10) and those being treated with ICS (COPD/ICS, n = 10). COPD/ICS experienced more exacerbations in the last year than COPD. COPD/ICS patients were treated with nebulized beclomethasone dipropionate (BDP) at a daily dose of 1.6-2.4 mg, equivalent to 800-1200 µg via MDI (22), administered for at least two months. At the moment of the study all reliever medications, inhaled β₂ agonists, anticholinergics and theophylline, were continued as previously administered and required. Six out of COPD patients were under treatment with an inhaled long acting bronchodilator (formoterol, salmeterol or tiotropium) and inhaled salbutamol as needed, the remaining 4 patients were under treatment with oral sustained-released theophilline and inhaled salbutamol as needed. Eight out of COPD/ICS patients were under treatment with an inhaled long acting bronchodilator (formoterol, salmeterol or tiotropium) and inhaled salbutamol as needed, the remaining 2 patients were under treatment with oral sustained-released theophilline and inhaled salbutamol as needed. All patients were free from exacerbations in the previous 2 months and were non-atopic. Age-matched, non-atopic, life-long non smoking volunteers were enrolled as a control group. The control
subjects underwent fiberoptic bronchoscopy for diagnostic reasons (6 subjects for cryptogenic hemoptysis and 2 for solitary peripheral nodule). All study subjects denied any clinical history for allergic disease.

The study was approved by the University of Parma ethical committee, and subjects gave written informed consent to enter the study.

**Study design.**

This study was a cross-sectional study. On two different study days the subjects included in the three groups, (i.e. COPD patients COPD/ICS patients and control subjects) underwent clinical and functional evaluation as well as fiberoptic bronchoscopy. Subjects included in the 3 groups were recruited, sampled and analysed concurrently.

**Fiberoptic Bronchoscopy**

Fiberoptic bronchoscopy was performed according to a previously described protocol (23). Premedication consisted of atropine (0.5 mg) and diazepam (10 mg), both given by intramuscular injection. Local anaesthesia was obtained with a tetracaine tablet (20 mg) given 15 min before bronchoscopy. An additional aliquot of 2% lignocaine was aerosolized into the upper airways and applied topically to the pyriform sinuses and vocal cords to prevent coughing and as a local anaesthetic. Nebulized salbutamol (1.25 mg) and ipratropium bromide (0.25 mg) were administered 5 min prior to bronchoscopy (Model 1T10; Olympus, Tokyo, Japan). Three to five mucosal biopsy specimens were taken at the subcarinae in the middle and lower lobes of the right lung. Administration of nebulized salbutamol and ipratropium bromide was repeated after bronchoscopy when necessary. Patients were closely monitored after bronchoscopy in the outpatient clinic, and were discharged
when the effects of sedation disappeared and lung function returned to the baseline values.

**Biopsy Processing**

Mucosal biopsies were immediately transferred into ice-cold acetone containing the protease inhibitor iodoacetamide (20 mM) and phenylmethylsulfonylfluoride (2 mM) for fixation; they were then stored at –20°C for 24 hours and processed into the water-soluble resin, glycolmethacrylate (Polysciences, Northampton, UK), for embedding (24). Biopsies were considered suitable for examination when there was at least 2.0 mm of basement membrane length and 0.2 mm² of sub-epithelial area.

Sections of 2 μm were cut and incubated with primary antibodies for 16-20 h overnight at room temperature. We used monoclonal antibodies directed against the following markers: endothelial basement membrane (collagen IV, 1:400, Novocastra Lab., Newcastle, UK), VEGF (121, 165 and 189 isoforms of VEGF, 1:50, NeoMarkers, Fremont, CA USA), bFGF (1:25, BD Biosciences Pharmingen, San Jose, CA USA), TGFβ (1:25, Novocastra Lab., Newcastle, UK), lymphocytes (CD3, CD4, CD8, 1:50, DAKO, Glostrup, Denmark), macrophages (CD68, 1:50, DAKO, Glostrup, Denmark), neutrophils (anti-elastase, 1:50, DAKO, Glostrup, Denmark), mast cells (tryptase, 1:200, DAKO, Glostrup, Denmark) and eosinophils (1:1000, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). After washing in Tris-buffered saline (TBS), the sections were incubated for 2 h with biotin-conjugated secondary antibody (Vector Lab, Burlingame, CA USA). Light microscopy was performed with a Leica microscope (Leica DMLB, Werzlar, Germany) at 1000x magnification.
As previously described (25), the vascularity and number of cells, including both inflammatory cells and cells positive for VEGF, bFGF and TGFβ, were quantified in all nonoverlapping high power fields in the sub-epithelial lamina propria. The latter is defined as a zone 100 µm below the epithelial basement membrane, and expressed as number of vessels and cells per square millimeter of lamina propria. In each section, all available nonoverlapping high power fields with intact tissue covered by intact basement membrane were examined. Vascular area was expressed as percentage of the area of the assessed lamina propria. The mean size of vessels was estimated by dividing the total vascular area by the total number of vessels. Final values represent the mean of at least two sections from two different biopsies in each subject. A single observer (AZ) who had no knowledge of patient characteristics evaluated tissue sections using an image analysis system (Image-Pro Plus; MediaCybernetics, Inc. Silver Spring, MD 20910 USA). An average of eight power fields were examined for each subject included in the study. The mean coefficient of variation for repeated measurements ranged from 6% to 11% for the inflammatory cells and the growth factor-positive cells, and from 4% to 7% for vascular data.

**Statistical Analysis**

Values are presented as mean±standard deviation (SD) or median (IQR). Differences among the groups was performed by a non-parametric Kruskal-Wallis ANOVA test, followed, where significant, by the Mann-Whitney U test for comparisons between groups. Relationships were estimated by the Spearman’s rank correlation coefficient ($r_s$). A $p$ value ≤0.05 was taken as significant. Intra-observer variation was determined by counting the mean coefficient of variation for repeated measurements.
Results

Characteristics of COPD patients and control subjects are reported in Table 1. In COPD patients, lung function values were recorded after bronchodilator administration.

Table 1  Characteristics of COPD patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>COPD/ICS</th>
<th>Control Subjects</th>
<th>p^a</th>
<th>p^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>N°</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>77±8</td>
<td>75±11</td>
<td>66±17</td>
<td>.236</td>
<td>.360</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>0/10</td>
<td>3/7</td>
<td>5/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1 (% pred)</td>
<td>51±16</td>
<td>50±14</td>
<td>104±22</td>
<td>.001</td>
<td>.001</td>
</tr>
<tr>
<td>FEV1/VC (%)</td>
<td>49±10</td>
<td>48±14</td>
<td>76±3</td>
<td>.001</td>
<td>.001</td>
</tr>
<tr>
<td>TLC (% pred)</td>
<td>106±14</td>
<td>123±27</td>
<td>119±17</td>
<td>.203</td>
<td>.408</td>
</tr>
</tbody>
</table>

p^a when compared COPD with control subjects

p^b when compared COPD/ICS with control subjects

Fiberoptic bronchoscopy with endobronchial biopsy specimens was performed successfully and was well tolerated in all subjects.

Data on vascularity, growth factor expression and inflammatory cells are summarized in Table 2.
Table 2. Median (IQR) of vascular area, vessel size, number of vessels, VEGF+ cells, FGF+ cells, TGFβ+ cells and inflammatory cells in COPD patients and control subjects.

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>COPD/ICS</th>
<th>Control Subjects</th>
<th>p^a</th>
<th>p^b</th>
<th>p^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular area (%)</td>
<td>6.2 (5-6)</td>
<td>4.0 (3-6)</td>
<td>4.0 (4-4.1)</td>
<td>.043</td>
<td>.001</td>
<td>.896</td>
</tr>
<tr>
<td>Vessel size (µ²)</td>
<td>424 (389-490)</td>
<td>323 (294-398)</td>
<td>305 (284-327)</td>
<td>.023</td>
<td>.003</td>
<td>.408</td>
</tr>
<tr>
<td>Vessel number (n/mm²)</td>
<td>140 (90-165)</td>
<td>134 (109-146)</td>
<td>120 (113-128)</td>
<td>.853</td>
<td>.633</td>
<td>.315</td>
</tr>
<tr>
<td>VEGF+ cells (n/mm²)</td>
<td>142</td>
<td>102</td>
<td>80</td>
<td>.043</td>
<td>.003</td>
<td>.274</td>
</tr>
</tbody>
</table>
Vascular area and vessel size were significantly increased in COPD, as compared to COPD/ICS (p=0.043) and CS (p=0.001), whereas no difference was found in vessel number among COPD, COPD/ICS and CS (Figure 1). VEGF+ cells, were significantly increased in COPD, as compared to COPD/ICS and CS (p=0.043 and p=0.003, respectively). bFGF+ cells and TGF-β+ cells were significantly increased in COPD, as compared to COPD/ICS (p=0.035 and p=0.001, respectively) and CS (p=0.001). In addition, bFGF+ cells were significantly increased in COPD/ICS, as compared to CS (p=0.001).

When cell profiles among groups were examined, CD8+ cells and CD68+ cells were significantly increased in COPD, as compared to COPD/ICS (p=0.023 and p=0.001, respectively) and CS (p=0.007 and p=0.001, respectively), whereas no difference was found in the number of CD3+ cells, CD4+ cells, neutrophils and eosinophils. In COPD the number of mast cells tended to be higher than in healthy subjects (p=0.055).

In COPD, the number of VEGF+ cells was related to the vascular area (r_s=0.82, p<0.01) (Figures 2 and 3), the number of vessels (r_s=0.75, p<0.05), and the vessel size (r_s=0.68, p<0.05). Moreover, in the same group of patients the number of TGF-β+ cells was significantly related to the vascular area (r_s=0.66, p<0.05) (Figures 2 and 3). In no study group significant correlations were observed between vascularity data and inflammatory cells or lung function data.
Discussion

In this study, we assessed the bronchial vascular remodelling in the central airways of COPD patients and examined its relationship both with angiogenic growth factor expression and inflammatory cells. In addition, we evaluated the association between bronchial microvascularity and a long-term treatment with inhaled BDP. We showed that in the airways of untreated COPD patients, the bronchial vascular area and vessel size were increased, as compared to those of COPD patients treated with BDP and to those of control subjects. Moreover, we found a higher bronchial expression of VEGF, bFGF and TGF-β in untreated COPD patients, as compared to those of COPD patients treated with BDP and of control subjects. Finally, we provided the first evidence that in untreated COPD patients, the vascular component of airway remodelling was positively related with the bronchial expression of VEGF and TGF-β.

Previous studies reported different findings on the bronchial vascularity in COPD (7,8,26). Kuwano et al (26) did not show any increase in bronchial mucosal vascularity in COPD patients, as compared to controls. By contrast, more recently Hashimoto et al (7) found that in inhaled steroid free COPD patients, the vessel number showed a tendency to increase and the vascular area in the inner zone of the small airways was significantly greater than that in control subjects. In addition, Calabrese et al (8) showed an increase in bronchial vascularity in the central airways of patients with COPD, expressed in terms of both number of vessels and vascular area, when compared to controls. Differences in patients selection criteria, in vascularity
assessment, in sampling site or in biopsy processing and immunostaining techniques can explain these different results. In particular, Kuwano et al. obtained tissue specimens from lung surgical resections and stained vessels with Factor VIII (26), while Calabrese et al (8) assessed the bronchial vascular component using fiberoptic bronchoscopy only in COPD patients who were currently smokers. In spite of the differences in methods and patient selection criteria, all studies (7,8,26) consistently found an increased vascular area in the airways of COPD patients.

Among angiogenic growth factors, VEGF is quite relevant to the angiogenic processes closely related to airway inflammation and remodelling in COPD (13). In the present study, we confirmed that inhaled steroid free COPD patients could have an higher bronchial airway expression of VEGF, as compared to healthy non smoking subjects. It has been previously demonstrated that the cellular VEGF expression was increased in the bronchial airways of smoking patients with COPD, in comparison with that of healthy non-smokers (8). Moreover, Kranenburg et al (10) demonstrated a higher expression of VEGF in central and peripheral airways of ex-smokers with COPD as compared to those without COPD. Interestingly, in this study we also provided the evidence that VEGF expression was directly correlated to the airway mucosal microvascularity, both in terms of vessel number and size and of vascular area. Taken together, these findings support the view that VEGF may be one of the main determinants of the vascular changes in the bronchial mucosa of COPD patients.

In this study, we found that also bFGF levels were higher in the airways of COPD patients than in control subjects. It is
of note that bFGF is a growth factor with the potential to contribute to the changes of the bronchial microvascularity and of pulmonary vessels of COPD patients. Kranenburg et al. (11) previously observed that COPD patients, when compared to non-COPD subjects, could have an increased expression of FGF-1 in the bronchial epithelium and of FGFR-1 in the bronchial epithelium cells, in the airway smooth muscle cells and in the vascular smooth muscle of the bronchial small vessels. Additionally, it has been found that the expression of FGF-2 is also increased in central airways of COPD patients, where it is correlated with the number of vessels (28).

The TGF-β expression in the airways of COPD patients has been until now assessed by different methods. De Boer et al (12) used a semi-quantitative analysis by a visual analogue scale, by in situ hybridization and immunostaining techniques, while Takizawa et al (27) used an immunocytochemical analysis from cell culture. Both studies (12,29) were able to find an increased expression of TGF-β in COPD patients, as compared to controls. We analyzed the bronchial mucosal expression of TGF-β by immunohistochemistry and, in spite of the difference in methods, our results are in line with the previous ones (12,29). Furthermore, we showed a positive relationship between TGF-β and vascular area in COPD patients without steroid treatment, by suggesting a possible role of TGF-β in the vascular component of the airway remodelling in COPD. In vitro studies showed that TGF-β can act as a regulator in the maintenance of vascular homeostasis (30). Moreover, TGF-β can increase endothelial nitric oxide synthase (eNOS) and determine NO-dependent vasodilation, by binding with endoglin, a component of the TGF-β receptor complex (31).
Though the potential clinical benefits of inhaled steroids in COPD, i.e. reduced rate of exacerbations, improvement in quality of life, are well known (32), however the evidence regarding their effects on airway inflammation and remodelling in COPD is scanty (18,19,33). Hattotuwa et al. (18) observed a reduction in the CD8/CD4 ratio in the epithelium and of the numbers of subepithelial mast cells in COPD patients treated with high dose of fluticasone propionate (FP). Afterwards, by an ultrastructural biopsy study the same authors showed a mast cell reduction in COPD patients treated with inhaled steroids (19). In line with these previous studies, we found that in COPD patients under treatment with high doses of BDP had lower numbers of CD8+ T-lymphocytes and to a tendency for mast cells to be lower in the lamina propria, as compared to inhaled steroid free COPD patients. In addition, in agreement with Verhoeven et al. (33), who observed substantial reductions in CD68 cell number in the lamina propria of COPD patients treated with FP, we observed that COPD patients under BDP treatment had also less CD68+ cells compared to untreated COPD patients.

In the present study, we provide the first evidence that steroid naïve COPD patients have increased vascular area and vessel size as well as upregulation of VEGF, FGF and TGF-β, as compared to COPD patients treated with steroids. Our study suggests that the anti-inflammatory effect of ICS in COPD may be also due, at least in part, to a reduction in vascularity and expression of angiogenic factors in the airways. It must be acknowledged, however, that in a cross sectional study, Paredi et al (34) have previously found that in COPD patients with mild airway obstruction, the bronchial blood flow was not significantly
affected by a long-term treatment with a low daily dose of budesonide (34).

There are potential criticisms of our study. We acknowledge that this is a cross sectional biopsy study, which measured large airway vascularity in COPD patients, who were not randomised to treatment. Therefore, we do not infer from our study any causal nexus between steroid treatment and micro vascularity in COPD patients. However, we performed this study in order to generate an hypothesis, which has to be tested by a properly designed longitudinal study. Another limitation of our report is the low power of the study because of the small number of subjects in each group. However, a low number of subjects and a low number of biopsies per subject are common limitations in biopsy studies. Moreover, we think that the significant differences we observed are valid even if we cannot exclude the possibility of type 2 statistical errors (i.e. inability to detect small differences). Despite all of these limitations, we believe that studies on bronchial biopsies provide a unique opportunity to investigate airway inflammation and remodelling in COPD patients.

Up to now, the vascular component of the airway remodelling in COPD has been remarkably little studied for such an important condition. Our study shows that some aspects of vascular remodelling, such as bronchial vascular area and vessel size, are increased in the airways of inhaled steroid free COPD patients and an upregulation of VEGF, bFGF and TGF-β could play a key role in these changes. By contrast, angiogenesis does not seem to be a feature of COPD patients, who are not current smokers. Though COPD patients under treatment with high doses of nebulized BDP have reduced microvasularity, as compared to untreated COPD patients, further longitudinal intervention studies are required to assess the effect of inhaled steroids on the vascular component of airway remodelling in COPD.
Acknowledgments

The authors would like to thank Mrs. Iris Spanevello for the valuable help in the biopsy processing.
References


Legend for figures

**Figure 1.** Microphotographs from a COPD (*upper panel*) and from a COPD/ICS (*lower panel*) showing bronchial mucosa stained with antibody directed against Collagen IV to outline vessels. Original magnification x400.

**Figure 2.** Relationship between the vascular area and the number of VEGF+ cells (*upper panel, rs=0.82, p<0.01*) and the number of TGF-β+ cells (*lower panel, rs=0.66, p<0.05*) in ten COPD patients.

**Figure 3.** Microphotographs from a COPD patient showing bronchial immunostaining for VEGF (*upper panel*) and TGF-β (*lower panel*). Original magnification x400.