Vascular Endothelial Growth Factor and its Receptors in COPD

Based on: Kranenburg AR, de Boer WI, Alagappan VKT, Sterk PJ and Sharma HS: Enhanced Bronchial Expression of Vascular Endothelial Growth Factor and Receptors (flk-1 and flt-1) in Patients with Chronic Obstructive Pulmonary Disease. Thorax 2005; 60:106-113
5.1 Summary

**Background:** Ongoing inflammatory processes resulting in airway and vascular remodeling characterize chronic obstructive pulmonary disease (COPD). Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) could play a role in tissue remodeling and angiogenesis in COPD.

**Methods:** We examined the cellular expression pattern of VEGF, Flt-1 and KDR/Flk-1 by immunohistochemistry in central and peripheral lung tissues obtained from (ex-) smokers with (FEV$_1$ <75% predicted; n=14) or without COPD (FEV$_1$ >85% predicted; n=14). The immunohistochemical staining of each molecule was quantified using a visual scoring method with grades ranging from 0 (no), 1 (weak), 2 (moderate) to 3 (intense).

**Results:** VEGF, Flt-1 and KDR/Flk-1 immunostaining was localized in vascular and airway smooth muscle (VSM and ASM) cells, bronchial, bronchiolar and alveolar epithelium and macrophages. Pulmonary endothelial cells abundantly expressed Flt-1 and KDR/Flk-1 but not VEGF. In COPD patients, bronchial VEGF expression was higher in microvascular VSM cells and ASM cells as compared to non-COPD patients (1.7 and 1.6 fold, p<0.01, respectively). VEGF expression in intimal and medial VSM (1.7 and 1.3 fold, p<0.05) of peripheral pulmonary arteries associated with the bronchiolar airways was more intense in COPD, as well as in small pulmonary vessels in the alveolar region (1.5 and 1.7 fold, p<0.02). In COPD patients, KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM (1.3, 1.9 and 1.5 fold, p<0.02), whereas endothelial Flt-1 expression was 1.7 times higher (p<0.03). Furthermore, VEGF expression was significantly increased in bronchiolar and alveolar epithelium as well as bronchiolar macrophages (1.5 fold, p<0.001). Additionally, expression of VEGF in bronchial VSM and mucosal microvessels as well as bronchiolar epithelium inversely correlated with FEV1 ($r < -0.45; p<0.01$).

**Conclusions:** Our results suggest that VEGF and its receptors Flt-1 and KDR/Flk-1 are involved in peripheral vascular and airway remodeling processes in an autocrine and/or paracrine manner. This system may also be associated with epithelial cell viability during airway wall remodeling in COPD.
3.2 Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible, usually progressive and associated with an abnormal inflammatory response of the lungs in response to noxious particles and gases (1). COPD is a major health problem with cigarette smoking as its main cause. One important pathological feature of COPD is chronic airway inflammation characterized by an influx of inflammatory cells predominantly neutrophils, macrophages and CD8+ T-lymphocytes in the lumen and wall of bronchial and bronchiolar airways and parenchyma (2-4). Furthermore, several studies reported a thickened bronchiolar wall and airway remodeling with peribronchiolar fibrosis, an increase in airway smooth muscle (ASM) mass and emphysema (3, 5, 6).

Vascular abnormalities have been associated with the development of COPD (7, 8). Wright et al. found an increase in wall area of small (< 500 μm) pulmonary vessels, by intimal thickening in mild to moderate COPD patients and medial thickening in severe cases as well, which was correlated with a decline in FEV1 (7, 9). Furthermore, recent observations indicated that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of CD8+ T-lymphocytes and have intimal thickening that was correlated to the amount of total collagen deposition (8, 10). Finally, emphysema may lead to loss of the pulmonary vascular bed and induce angiogenesis (11). Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

One of the potent proteins involved in vascular remodeling is vascular endothelial growth factor (VEGF). The VEGF family currently comprises six members (VEGF-A to F), of which the originally identified VEGF-A165 variant is the predominant form of five additional spliced variants (12). VEGFs are heparin-binding proteins and act via their high affinity, transmembrane receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). The receptors belong to the family of tyrosine kinases and are predominantly expressed by endothelial and epithelial cells (12). VEGF promotes an array of responses in the endothelium including hyperpermeability, endothelial cell proliferation and angiogenesis with new vessel tube formation in vivo (12, 13). The expression of VEGF can be induced under a variety of pathophysiological conditions, including pulmonary hypoxia and pulmonary hypertension with increased shear stress (13, 14). Both hypoxia and pulmonary hypertension are pathological features often seen in advanced COPD patients (2). We hypothesize, that increased VEGF expression perhaps under an influence of hypoxia-inducible transcription factors (HIFs) may
contribute to increased and abnormal proliferation of endothelial and VSM cells in pulmonary vessels leading to vascular remodeling.

Although the role of VEGF in the vascular biology is thoroughly studied, it has become clear that VEGF and its receptor system are involved in various other cellular events as well, including epithelial proliferation and survival, and the recruitment of mast cells, neutrophils and macrophages to sites of fibrosis (13, 15, 16). Recent studies indicate that VEGF is expressed in the lung by bronchiolar, submucosal glandular and alveolar type I and II epithelial cells, alveolar macrophages, airway and vascular smooth muscle (ASM and VSM) cells as well as myo-fibroblast in fibrotic lung lesions (14, 17, 18). In order to assess the role of EGF and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) in the pathophysiology of COPD, we first examined the expression of VEGF-A, Flt-1 and KDR/Flk-1 in central and peripheral lung tissue from (ex-)smokers with or without COPD. Furthermore, we investigated the relation of lung function with the expression data of VEGF and its receptors.

5.3 Materials and methods

Selection of patients

Central and peripheral lung tissues were obtained from current or ex-smokers who underwent lobectomy or pneumonectomy for lung cancer. Fourteen subjects with COPD (FEV₁ < 75% predicted) and fourteen subjects without COPD (FEV₁ > 84% predicted) were included as previously described (19-21). Total lung capacities (TLC) were not below normal levels (TLC >80% predicted). All patients lack upper respiratory tract infection and did not receive antibiotics perioperatively. Non of the patients had received glucocorticosteroids during 3 months period before resection, but four patients received glucocorticosteroids perioperatively. Based on these criteria, subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone. Clinical data are given in Table 1. Subjects were excluded if the obstruction of the central bronchi was due to the tumor, or if diffuse pulmonary inflammation or fibrosis was present, or if no tissue free from tumor could be obtained. Lung tissue specimens used in this study were obtained from the archival collection at the Department of Pathology (LUMC, Leiden, NL). Medical Ethics Committee of LUMC approved the study. The patients in these two groups participated in a larger research project, part of which has been published previously (19-21). Lung tissue specimens were routinely fixed in 10% neutral buffered formalin by inflation-immersion fixation and embedded in paraffin for histopathological examination and immunohistochemistry.
**Immunohistochemistry**

Paraffin sections (4 μm thick) of the lung tissues were cut and mounted on silane-coated glass slides. Immunohistochemistry was performed using a method as described earlier (20, 22, 23). In brief, after deparaffinization in xylene and rehydration through graded alcohol, slides were rinsed with phosphate buffered saline (PBS). Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. For VEGF, VEGFR-1, VEGFR-2 and Ki-67 staining, slides were pre-treated by boiling in citrate buffer (10 mM citrate buffer, pH = 6.0) for 10 minutes in a microwave oven. Subsequently, sections were preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (5% BSA/PBS, pH = 7.4), and afterwards incubated for 30 minutes at room temperature with affinity-purified rabbit polyclonal VEGF antibody in a dilution of 1:200 v/v. The VEGF antibody used was raised against a 20 amino acid synthetic peptide corresponding to residues 1-20 of the amino terminus of human VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). A different series of slides were incubated with a rabbit polyclonal antibody against a synthetic peptide corresponding to aa 1312-1328 of human Flt-1 (NeoMarkers, RB-1526, Fremont, CA, USA) in a dilution of 1:100 v/v. For VEGFR-2, a rabbit polyclonal antibody against aa 1326-1345 of mouse KDR/Flk-1 (NeoMarkers, RB-1527, Fremont, CA, USA) in a dilution of 1:200 v/v was used. To examine proliferation of cells in the airways, an antibody against Ki-67 (Dako Corporation, Glostrup, Denmark) of 1:400 v/v at 4°C overnight for was used as a marker. Consecutive tissue sections were also stained with a monoclonal mouse anti-human alpha-smooth muscle actin (α-SMA) antibody (clone 1A4: Biogenex, San Ramon, USA) in a dilution of 1:1000 v/v. The optimal dilution of the first antibody was identified by examining the intensity of staining obtained with a series of dilutions of the antibody from 1:50 to 1:1000. Negative controls were prepared by omission of the primary antibody. After washing with tris-base buffered saline (TBS, pH = 7.4), the test and control slides were incubated for 15 minutes with Powervision™ Post-antibody blocking solution (Immunovision Technologies, Daly City, CA, USA). Next, slides were washed and incubated with Powervision™ polymerized horseradish peroxidase conjugates (Immunovision Technologies, Daly City, CA, USA). Finally, the sections were stained with 3,3’-diaminobenzidine tetrahydrochloride (Sigma, Zwijndrecht, NL) as chromogen, counterstained with Mayer’s hematoxylin and visualized with light microscopy.

**Quantitative scoring analysis of immunohistochemistry**

Prior to screening, sections were coded so that the observers were unaware of the clinical details of the case under study. Expression of VEGF, Flt-1 and KDR/Flk-1 was analyzed semi-
quantitatively, using a visual scoring method with grades ranging from 0 to 3 (0 = no staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining) as previously described (8, 19, 20, 24). The entire section of a tissue block was investigated and scored at the same magnification. The staining intensity of VEGF, Flt-1 and KDR/Flk-1 was scored blindly by two independent observers, who were unaware of the clinical data of the case under study, in bronchial and bronchiolar airways as well as alveolar parenchyma in cells of epithelial, endothelial and smooth muscle origin as well as macrophages. We examined errors within and between observers by correlating the expression scores using Pearson’s analysis and found a very high correlation ranging from 0.8 to 0.9. In the bronchial airways staining was assessed in the bronchial epithelium, mucosal microvasculature, submucosal bronchial wall vessels, airway smooth muscle (ASM) cells and macrophages in the bronchial airway wall. In peripheral lung tissues the staining of VEGF and receptors was analyzed in bronchiolar an alveolar epithelium, bronchiolar ASM cells, and bronchiolar and alveolar macrophages. The vasculature in the peripheral lung was further subdivided into the larger pulmonary vessels associated with the bronchiolar airways and smaller vessels situated within the alveolar parenchyma. In each the VEGF and receptor staining of endothelial, intimal and medial VSM cells were assessed. Since TGF-β1 may also induce VEGF expression in epithelial cells (25, 26), we assessed the correlation between the epithelial VEGF expression from the current study and epithelial TGF-β1 expression from one of our previous studies (20). In both studies the same patient groups were used and the staining was performed on adjacent or near sections.

**Statistical Analysis**

Data were analyzed for statistical significance using the unpaired, two-tailed Students’ t-test as well as the non-parametric Mann-Whitney test, where appropriate. The expression data for VEGF and its receptors were expressed as mean ± SEM. Furthermore, VEGF and its receptors staining for different compartments were correlated with FEV₁ using Pearson’s correlation analysis. Differences with p ≤ 0.05 were considered to be statistically significant.

5.4 Results

**Clinical Parameters**

The clinical and lung function characteristics of all subjects included in the study are listed in Table 3.1. As defined, the COPD group demonstrated decreased FEV₁ and FEV₁/FVC values, (p<0.001) as has been described previously (19-21). The subjects in the two groups did not differ significantly in age and smoking status (pack-years) or steroid use (Table 3.1).
TABLE 5.1. A summary of the clinical characteristics of subjects with and without chronic obstructive pulmonary disease

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex (M/F)</th>
<th>Age</th>
<th>PY</th>
<th>FEV\textsubscript{1} (% Pred.)</th>
<th>FEV\textsubscript{1}/FVC (%)</th>
<th>Steroid treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-COPD</td>
<td>10/4</td>
<td>64 (3.7)</td>
<td>42 (7.7)</td>
<td>101 (3.3)</td>
<td>0.72 (0.02)</td>
<td>None</td>
</tr>
<tr>
<td>COPD</td>
<td>14/0</td>
<td>64 (2.3)</td>
<td>44 (0.8)</td>
<td>63 (2)</td>
<td>0.54 (0.02)</td>
<td>4</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.84</td>
<td>0.82</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** COPD = chronic obstructive pulmonary disease; Forced expiratory volume in 1 second (FEV\textsubscript{1}) and Forced vital capacity (FVC) are given as percentages of the predicted values (% Pred.) before bronchodilatation. M = Male; F = Female. PY = number of pack years. Data shown represent means with standard deviation in brackets. The patients in these two groups participated in a larger project, part of which has been published previously (19-21).

**Immunolocalization of VEGF, Flt-1 and KDR/Flk-1**

**Bronchial airways**

Examples of VEGF expression in central airways of non-COPD and COPD subjects are given in Figure 3.1A and 3.1B, whereas 3.1C and 3.1D (both taken from COPD subjects) show the VEGF receptors Flt-1 and KDR/Flk-1, respectively. In all subjects, within the airways VEGF, Flt-1 and KDR/Flk-1 were localized in the bronchial epithelium and airway smooth muscle (ASM) cells, bronchial microvasculature of mucosa and submucosa and on inflammatory cells, predominantly macrophages, (Figure 3.1A-D). In the vessel wall, vascular smooth muscle (VSM) cells were positive for VEGF, Flt-1 and KDR/Flk-1, whereas endothelial cells did not stain for VEGF protein but were positive for the Flt-1 and KDR/Flk-1 (Figure 3.1). To assess the intensities of VEGF, Flt-1 and KDR/Flk-1 expression in various bronchial airway compartments, we opted for a visual scoring method as previously described (8, 19, 20, 24). VEGF expression was increased in bronchial airway smooth muscle cells of COPD patients as compared to non-COPD subjects (1.6 fold, p<0.01) but not in bronchial epithelial cells and macrophages (Figure 3.2A). In the central airways of patients with COPD as compared to non-COPD subjects, VEGF staining was more intense in VSM of microvasculature the bronchial mucosal (lamina propria) (1.7 fold, p<0.001) and bronchial VSM in the submucosa (1.4 fold, p<0.01, Figure 3.2A). No significant differences were observed when considering the expression levels of KDR/Flk-1 and
Flt-1 between COPD subjects and non-COPD patients (Figure 3.2B and 2C, respectively). In all subjects VEGFR-2 (KDR/Flik-1) expression was more intense than VEGFR-1 (Flt-1) expression, except for the expression in endothelial cells of bronchial microvessels and on bronchial macrophages, which were comparable (Figure 3.2B and 3.2C).

**Bronchiolar airways**

Figure 5.3 shows photographs of peripheral lung tissues from non-COPD and COPD subjects for VEGF (5.3A and 5.3B), KDR/Flik-1 (5.3C and 5.3D) and Flt-1 (5.3E and 5.3F), respectively. In bronchiolar epithelial cells VEGF (1.5 fold, p<0.001, Figure 5.4A) and Flt-1 expression (1.4 fold, p<0.04, Figure 5.4C) were increased in COPD patients as compared to non-COPD subjects, whereas the staining for KDR/Flik-1 was unchanged between both patient groups (Figure 5.4B).

---

**Figure 5.1** Immunohistochemical localization of VEGF (A-B), KDR/flik-1 (C) and flt-1 (D) in bronchial tissues from non-COPD (ex-) smoking subjects (A) and patients with COPD (B, C, D). Immunoreactive VEGF, KDR/flik-1 and flt-1 were localized in bronchial epithelial cells, airway smooth muscle (ASM) cells and in macrophages, endothelial and vascular smooth muscle (VSM) cells. Color is developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown color) and counterstained with Mayer's hematoxylin. Arrows indicate sites of positivity for VEGF, flt-1 or KDR/flik-1. Original magnification: x100; Scale bar = 50 μm.
Airway smooth muscle cells showed slightly increased VEGF expression in bronchiolar region (1.3 fold, p<0.05), whereas the expression of both the receptors remained unchanged in two patient groups. However, the expression of KDR/Flik-1 was more intense than Flt-1 in all patients (Figure 5.4B and 5.4C).

**Figure 5.2**  Graphic representations of VEGF (panel A), KDR/flik-1 (panel B) and flt-1 (panel C) protein expression in different cell types in bronchial airways using visual scoring.
The immunostaining score ranges from 0 (no staining) to 3 (very intense staining). Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean ± S.E.M. An asterisk indicates a significant difference (p<0.05, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchial epithelium (Epi), bronchial microvessels (MV) in the mucosa, bronchial vascular smooth muscle cells (VSM) in the submucosa, airway smooth muscle (ASM) and macrophages (Mφ).

Figure 5.3 Immunohistochemical localization of VEGF (A-B), KDR/flk-1 (C-D) and flt-1 (E-F) in peripheral tissues from non-COPD (ex-) smoking subjects (A, C, E) and patients with COPD (B, D, F). Immunoreactive VEGF, flt-1 and KDR/flk-1 were localized in bronchiolar and alveolar epithelial cells, airway smooth muscle (ASM) cells, macrophages and in endothelial and intimal/medial vascular smooth muscle (VSM) cells. Color is developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown color) and counterstained with Mayer's hematoxylin. Arrows indicate sites of positivity for VEGF, flt-1 or KDR/flk-1. Original magnification: x100; Scale bar = 50 μm.
Figure 3.4 Graphic representations of VEGF (panel A), KDR/flk-1 (panel B) and flt-1 (panel C) protein expression in different cell types in bronchiolar airways and associated pulmonary arteries using visual scoring. Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean ± S.E.M. An asterisk indicates a significant difference (p<0.05, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchiolar epithelium (Epi), endothelial cells (EC), intimal and medial vascular smooth muscle cells (VSM int. and med.), airway smooth muscle (ASM) and bronchiolar macrophages (Mφ).
When considering the expression of VEGF in the larger pulmonary arteries associated with the bronchiolar airways, the fold in intimal and medial VSM staining was 1.7 and 1.3 (p<0.05, Figure 3.4A) between COPD and control subjects respectively, whereas endothelial cells did not express VEGF. KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM (1.3, 1.9 and 1.5 fold, p<0.02, Figure 3.4B), whereas the corresponding value in endothelial cells for Flt-1 expression is 1.7 (p<0.03, Figure 3.4C). In both patient groups, the intimal VSM stained 2-3 times less intense than medial VSM for VEGF, Flt-1 and KDR/Flk-1. Moreover, the vascular Flt-1 expression was lower than KDR/Flk-1 and VEGF in each of the investigated vessel wall areas (p<0.002, Figure 3.4). Staining of VEGF in bronchiolar macrophages (1.5 fold, p<0.001, Figure 3.4A) was increased in COPD as compared to non-COPD subjects, whereas the staining on macrophages of Flt-1 or KDR/Flk-1 expression in bronchiolar airways as well as VEGF, Flt-1 or KDR/Flk-1 in the alveolar region remained unchanged (Figure 3.5).

**Alveolar parenchyma**

Staining of alveolar epithelial cells (type I and II) for COPD was more intense than for non-COPD controls (1.5 fold, p<0.0001, Figure 3.5A). KDR/Flk-1 and Flt-1 expression, were not changed in alveolar epithelial cells (Figure 3.5B and 3.5C). VEGF expression was increased in intimal and medial VSM (1.5 and 1.7 fold, p<0.01, Figure 3.5A) of small pulmonary vessels in the alveolar region whereas the corresponding values for KDR/Flk-1 were 2.0 and 1.8 (p<0.02), respectively (Figure 3.5B). Furthermore, the expression of both KDR/Flk-1 and Flt-1 were increased in endothelial cells of small pulmonary vessels in lung parenchyma (1.7 and 2.1 fold, p<0.001, Figure 3.5B and 3.5C).

**Correlation between staining and clinical data**

We examined the relation between FEV₁ values of patients in both groups and the staining scores of VEGF, Flt-1 and KDR/Flk-1 in the investigated areas. Within the bronchial airways, FEV₁ values were inversely correlated with VEGF staining scores in bronchial mucosal microvasculature (r = -0.65; p<0.001, Figure 3.6A), bronchial ASM cells (r = -0.45; p<0.01, Figure 3.6B) if all subjects were analyzed together.
Figure 5.5  Graphic representations of VEGF (panel A), KDR/flk-1 (panel B) and flt-1 (panel C) protein expression in different cell types in alveolar parenchyma and pulmonary vasculature using visual scoring. Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean ± S.E.M. An asterisk indicates a significant difference (p<0.05, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchiolar epithelium (Epi), endothelial cells (EC), intimal and medial vascular smooth muscle cells (VSM int. and med.), and alveolar macrophages (Mø).
Figure 5.6  Correlation with FEV$_1$ (% predicted) of VEGF protein expression in microvessels (MV) in the bronchial mucosa (A), bronchial airway smooth muscle (ASM) cells (B), bronchiolar epithelial (Epi) cells (C) and medial vascular smooth muscle (VSM) cells of pulmonary arteries associated with the bronchiolar airways (D). Correlation was assessed for the combined patient groups (non-COPD and COPD). Correlation coefficient ($r$) was obtained using linear regression (Pearson's) analysis.

The bronchiolar epithelium ($r = -0.67$; $p < 0.001$, Figure 3.6C) and medial VSM of larger pulmonary arteries associated with bronchiolar airways ($r = -0.50$; $p < 0.01$, Figure 3.6D) also showed an inverse correlation with FEV$_1$ values from the total group. Additionally, VEGF expression in medial VSM was correlated with KDR/Flk-1 expression in endothelium of pulmonary arteries ($r = 0.41$; $p < 0.01$) as well as smaller alveolar vessels ($r = 0.48$; $p < 0.01$). Furthermore, we found correlation for the expression pattern of KDR/Flk-1 and Flt-1 in the endothelium of pulmonary arteries ($r = 0.67$; $p < 0.001$) as well as in alveolar vessels ($r = 0.80$; $p < 0.0005$).
Additionally, we examined correlation between the epithelial VEGF expression from the current study and epithelial TGF-β1 expression from one of our previous studies (20). In both studies the same patient groups were used and the staining was performed on adjacent or near sections. With regard to the bronchiolar epithelium, Pearson’s analysis revealed a significant positive correlation between the VEGF protein and TGF-β1 protein levels \( (r = 0.55; p<0.004) \) and VEGF protein and TGF-β1 mRNA expression \( (r = 0.45; p<0.02) \). With regard to the alveolar epithelium, the VEGF protein levels correlated significantly with the TGF-β1 mRNA expression only \( (r = 0.58; p<0.002) \), but not with the TGF-β1 protein levels \( (r = 0.31; p<0.12) \).

5.5 Discussion

In this study we show that COPD is associated with an increased expression of VEGF in the bronchial, bronchiolar and alveolar epithelium and in bronchiolar macrophages as well as ASM and VSM cells in both bronchiolar and alveolar region. KDR/Flk-1 and Flt-1 were increased in COPD as compared to non-COPD in endothelial, intimal and medial VSM cells of larger pulmonary arteries and of smaller caliber alveolar vessels. Interestingly, we observed a significant inverse correlation of VEGF with FEV\(_1\) in bronchial mucosal microvessels and ASM cells, bronchiolar epithelium and medial VSM of larger pulmonary arteries associated with bronchiolar airways. TGF-β1 staining in the bronchiolar epithelium also correlated with VEGF in the same patients as described in our previous study (20).

Our results indicate that VEGF and its receptors Flt-1 and KDR/Flk-1 are localized within the airways and vasculature in endothelial and epithelial cells as well as smooth muscle cell origin and furthermore on various inflammatory cells, predominantly macrophages. The localization of VEGF and its receptors in the lungs of our patient groups is in agreement with earlier reports, which described a similar staining pattern in human developing and normal adult as well as in emphysematous lungs (17, 27, 28). In contrast to Kasahara et al. (28), where authors showed in emphysematous lungs that VEGF and its receptor VEGF-R2 were decreased in total lung extracts, as measured with ELISA or western blot analysis, we found that the epithelial and endothelial cells in the alveolar spaces and in the most distal airways were intensely positive for VEGF and KDR/Flk-1 in COPD patients. Furthermore, our patient groups could be considered as mild to moderate COPD whereas, in the study of Kasahara the selected patients were solely emphysematous in origin. Our findings of increased VEGF expression in viable cell populations represent in part a successful attempt to repair sustained damage and perhaps contribution to vascular remodeling and their participation in the establishment and maintenance of the functional blood-gas interface, maturation, survival and proliferation of
capillary endothelial cells (29). In adult lungs, VEGF and its receptor system could contribute in the maintenance of endothelial and epithelial cell viability in response to injury (31).

Interestingly, immunoreactivity for VEGF in intimal and medial VSM cells and for Flt-1 as well as KDR/Flk-1 in endothelial cells of pulmonary arteries and alveolar vessels was elevated in patients with COPD. The highest levels of VEGF expression in the pulmonary vasculature were observed in the medial VSM cells and of KDR/Flk-1 in endothelial cells of arteries with a diameter of approximately 200 μm which are known to play an important role in pulmonary blood pressure regulation and vascular resistance (14, 30). Pulmonary hypoxia and hypertension with increased shear stress are pathophysiological conditions that have been shown to increase the expression of VEGF in VSM cells (13, 14). Blockade of KDR/Flk-1 is associated with oblitative endothelial cell proliferation in pre-capillary arterioles with abnormal vessel development and at the same time with induction of capillary endothelial and cell death by apoptosis, together leading to death in rat embryos, similar to that seen in human primary pulmonary hypertension subjects (13, 18, 31, 32). In a follow-up study they found that after VEGFR-2 blockade apoptosis predominated in areas of oxidative stress and that apoptosis blockade by a broad spectrum caspase inhibitor markedly reduced the expression of markers of oxidative stress (33). Hypoxia, oxidative stress and pulmonary hypertension are pathological features often seen in advanced COPD patients and increased VEGF expression may lead to increased or even abnormal proliferation of endothelial and VSM cells in pulmonary vessels. This suggests a potential role of this endothelial mitogen in peripheral angiogenesis and vascular remodeling, possibly in orchestration with other smooth muscle specific growth factors like FGF-2, PDGF and TGF-β1 (12, 34-36).

We observed increased expression for VEGF and unchanged expression levels for Flt-1 and KDR/Flk-1 in bronchiolar and alveolar epithelial cells as well as in airway smooth muscle cells in COPD. It has been previously documented that the expression of VEGF and receptor KDR/Flk-1 can also be induced by stimuli like hypoxia and oxidative stress in other than endothelial cells, such as epithelial and smooth muscle cells (33, 37, 38). In a recent report, Kanazawa and colleagues (39) have demonstrated that VEGF levels in induced sputum were higher in patients with bronchitis and lower in emphysema as compared to normal controls. Moreover, VEGF levels in bronchitis patients were inversely correlated with FEV₁ values. Our data on inverse correlation of VEGF levels in various airway and vascular cells is in agreement with this report. In our study subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone. Furthermore, the nature of the human material examined (sputum) in the study of Kanazawa and colleagues is different than
the lung tissue where we immunohistochemically localize and quantify the VEGF and its receptor levels.

Recent studies indicated that the expression of VEGF was increased in bronchial and alveolar epithelial cells and also was induced in α-SMA positive (myo-)fibroblasts in bleomycin induced fibrosis in the rat and in human patients with pulmonary fibrosis and that these fibrotic regions were densely populated by mast cells and macrophages with elevated KDR/Flk-1 expression (15, 17). We have shown earlier that mast cells and macrophages were increased in bronchiolar airway epithelium and reported an increased expression of TGF-β1 in bronchiolar and alveolar epithelial cells in patients with COPD (20, 21). We found a significant correlation between VEGF expression in epithelial cells with the expression of TGF-β1 published on same patient groups earlier (20) suggesting that the VEGF/Flk-1 system, possibly together with TGF-β1, represents a molecular link between inflammatory cell accumulation and proliferation of myo-fibroblasts. Summarizing, the elevated VEGF and TGF-β1 expression on bronchiolar epithelial cells and macrophages and the presence of KDR/Flk-1 and Flt-1 suggests a mechanism of initiating and perpetuating fibrosis at sites of tobacco induced injury contributing to airway remodeling in COPD. As inhaled corticosteroids could decrease the VEGF expression levels (40), but this was not the case in our study as none of the patient received inhaled corticosteroid therapy except 4 patients received corticosteroids perioperatively. However, caution must be exercised in extrapolating the expression data based on fourteen patients in each group as the increased trend of VEGF expression in bronchial airways and KDR/Flk-1 in bronchial and bronchiolar airway smooth muscle could reach significance if more patients would have been examined.

Taken together, these findings strongly suggest a role for VEGF and its receptors in airway and vascular remodeling, and thereby in the development of airway obstruction in COPD. At present, our knowledge of airway and vascular remodeling during the development of COPD is far from complete. Probably, many growth factors, among them VEGF, play an essential role in the pulmonary and vascular viability and repair in response to tissue injury. The increased pulmonary VEGF expression in airways, parenchymal lining and small-diameter pulmonary vessels in COPD may reflect an, in part unsuccessful, attempt to stimulate tissue repair mechanisms caused by tobacco-induced injury.

Acknowledgements
The authors thank Drs. J. Stolk and J.H.J.M. van Krieken for their help in the analysis of the clinical data and pathology, respectively. Technical assistance of Mrs. A. Willems-Widyastuti
is acknowledged. This study was supported in part by The Netherlands Asthma Foundation (grants #97.73 & 95.49).

5.6 References


