10

Summary and General Discussion
10.1 Summary & General Discussion

COPD is a chronic disorder of the lungs, with an abnormal inflammatory response of airways, parenchyma and vasculature in response to noxious particles and gases (1). The disease is becoming a major health problem with increasing trend of morbidity and mortality (1). COPD is a complex disease, which is influenced by genetic as well as environmental factors. Historically, the relation of α₁-antitrypsin deficiency and the development of COPD emphasized the role of genetics (2). Among factors like childhood respiratory infections, air pollution and occupational exposures, tobacco smoking is clearly the most important environmental trigger for COPD (2). Furthermore, strong relations of decline in lung function (as measured by FEV₁ % predicted) with the number of pack years as well as the beneficial effects of smoking cessation have been established (3). Yet of all smokers, only 10-20 percent actually develops COPD and in all subjects with diagnosed COPD only roughly 1% is associated with α₁-antitrypsin deficiency (3). The mechanisms determining these discrepancies as well as their underlying molecular mechanisms during the development and progression of the disease are not yet fully understood.

Tobacco induced injury is responsible for the process of chronic airway inflammation by an influx of inflammatory cells in the lumen and wall of bronchial and bronchiolar airways and as well as in the lung parenchyma. Structural abnormalities, in turn, will result in progressive airflow limitation and decreased gas exchange, in patients leading to breathlessness and eventually death. Yet, how smoke-induced injury can lead to the development of deregulated tissue repair with scar tissue formation is not completely understood.

We hypothesized that altered molecular events caused by differential expressed genes underlie the observed structural changes in COPD. Summarizing, therefore, the aims of studies presented in this thesis were:

- What characterize the structural alterations in the development of COPD in the peripheral as well as central vasculature and airways.
- What is the role of growth factors like FGF-1, FGF-2 and their receptor FGFR-1 as well as VEGF and its two receptors flt-1 and KDR/flk-1 in the development of vascular structural abnormalities in patients with COPD.
- What is the expression pattern of extracellular matrix (ECM) proteins such as collagens, laminins and fibronectin in the central and peripheral that could also contribute to airflow limitations in COPD.
What is the effects of fibroblast growth factors and transforming growth factor-β₁ (TGF-β₁) on proliferation and production of ECM components by cultured ASM cells, as contributing cells to airway wall thickening in COPD.

**Thesis** provides an overview of the clinical characteristics, pathogenesis and pathological changes in COPD. The inflammatory and structural abnormalities in COPD are described and the role of cytokines and growth factors in airway as well as vascular remodeling, pulmonary angiogenesis and the development of fibrosis in the peripheral and central airways are introduced. The aims of this thesis are outlined at the end of the chapter. We focused on the vascular alterations in the peripheral lungs of COPD patients. We found structural abnormalities and increased protein expression of the fibroblast growth factor/receptor (FGF/FGFR) system in the peripheral pulmonary vasculature of COPD patients. COPD patients showed increased thickness of the pulmonary vessel walls in vessels with several sizes from 100 to 400 μm and above but not with vessels of smaller lumen diameter. Surprisingly, no significant differences were observed in the percent of smooth muscle content of the wall, as indicated by α-smooth muscle actin staining divided by vascular wall area. Interestingly, we observed that in COPD patients FGF-1 was significantly increased in medial VSM cells of pulmonary vessels > 200 μm, whereas FGF-2 was more intense in endothelial and medial VSM cells of small caliber vessels (< 200 μm). Moreover, the expression of their receptor FGFR-1 was more pronounced on endothelial and medial VSM cells of each size categories in COPD patients. Furthermore, we observed an inverse correlation of FEV₁ with the medial VSM expression of both ligands and with vascular wall area. Therefore, the FGF/FGFR system could play an important role in the regulation of vascular remodeling, in COPD.

We described the pulmonary expression of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), which could also play a role in tissue remodeling and angiogenesis in COPD. We examined the immunohistochemical staining of VEGF, flt-1 and KDR/flk-1 in central as well as peripheral lung tissues obtained from (ex-) smokers with or without COPD. 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. Additionally, endothelial cells throughout the lungs abundantly expressed flt-1 and KDR/flk-1. Within the bronchial airways VEGF expression was enhanced in VSM cells of microvessels in the bronchial mucosa and submucosa as well as in ASM cells as compared to patients without COPD. VEGF expression was more intense in COPD in intimal and medial VSM of the peripheral pulmonary arteries associated with the bronchiolar airways and in small pulmonary vessels in the alveolar region as well. Moreover, KDR/flk-1 expression was enhanced in endothelial cells, intimal and medial VSM of the peripheral pulmonary arteries,
whereas flt-1 expression in endothelial cells only. Furthermore, VEGF staining was significantly increased in bronchiolar, alveolar epithelium and bronchiolar macrophages as well as the flt-1 receptor in the bronchiolar epithelium. VEGF expression in bronchial microvessels in the mucosa, bronchial ASM cells and bronchiolar epithelium inversely correlated with FEV₁ values. Taken, together, these results implicate also VEGF and its receptors, flt-1 and KDR/flk-1 in peripheral vascular and airway remodeling processes in COPD.

In order to extrapolate these findings we investigated the role of FGF-1 and FGF-2 and their receptor FGFR-1 in the central bronchial airways. FGF-1, FGF-2 and FGFR-1 were quantified with digital image analysis and were localized in bronchial epithelium, airway and vascular smooth muscle (ASM and VSM). In COPD as compared to non-COPD patients, elevated levels of FGF-1 and FGFR-1 were observed in bronchial epithelium and of FGFR-1 in only ASM. Interestingly, our results revealed increased expression of FGF-2 in COPD patients in the cytoplasm of the bronchial epithelium and nuclear localization in ASM cells. This latter observation could pinpoint towards an alternative functional mechanism for FGF-2. Moreover, we found a positive correlation of FGF-1 expression in the bronchial epithelium with packyears as well as inverse correlation of FEV₁/FVC with FGF-2 and FGFR-1 expression in ASM cells. Furthermore, in cultured human ASM cells, FGF-1 and/or FGF-2 induced cellular proliferation. Steady state mRNA levels of FGFR-1 were elevated in human ASM cells treated with either FGF-1 or FGF-2. Increased bronchial expression of fibroblast growth factors and their receptor in COPD cases, and the mitogenic response of human ASM cells to FGFs in vitro, suggest a potential role for FGF/FGFR-1 system in the remodeling of bronchial airways in COPD.

Furthermore, we showed that COPD is associated with increased deposition of extracellular matrix (ECM) molecules including collagens subtypes I, III, IV, fibronectin and laminin in the central airways, contributing to airway wall thickening. Staining for ECM components was observed surface epithelial basement membrane (SEBM) at sites of intact or damaged epithelium, interstitial space and vessels of lamina propria and adventitia of the bronchial airways. Total collagen was increased in the SEBM at sites of intact bronchial epithelium, but was not changed in the interstitial space and microvasculature of the lamina propria and adventitia of the airway in COPD as compared to non-COPD. Deposition of Collagen I and III, however, was enhanced in the SEBM both at damaged and intact epithelium, lamina propria and bronchial adventitia in COPD. Deposition of collagen IV was not different between the two groups, whereas expression of fibronectin was only increased in vessels of the lamina propria in COPD. Increased expression of laminin was observed in ASM and microvasculature in COPD as compared to non-COPD. FEV₁ values inversely correlated with collagen I and III in SEBM and lamina propria, respectively. When considering co-localization of total collagen with subtypes for collagen I, III
and IV, we found a significant correlation between total collagen and collagen III in the SEBM at both damaged and intact epithelium but not between total collagen and collagen I or IV localization. We conclude that smokers with COPD exhibit increased bronchial deposition of collagens, fibronectin and laminin and this could be involved in airway remodeling leading to airflow limitation.

We further investigated whether the altered extracellular matrix (ECM) deposition in the central airways of COPD patients could be partly ASM cell derived. In this study, therefore, we examined the mRNA expression of ECM proteins such as collagen I, III and fibronectin in cultured human ASM cells stimulated with FGF-1, FGF-2 or TGF-β1. Densitometric analysis of Northern blots showed increased mRNA expression of collagen I and III in ASM cells stimulated for 24h with TGF-β1 or FGF-1, whereas the levels for these mRNAs did not change in FGF-2 stimulated cells. ASM cells constitutive expressed fibronectin mRNA, which remained unaltered after each of the stimuli. TGF-β1 did not induce cell proliferation as determined by ³H-thymidine incorporation assay and cell count, this in contrast to FGF-1 and FGF-2. Total protein over DNA ratio in ASM cells, as a measure for cellular hypertrophy remained unaffected by each stimulus. Interestingly, increased levels of TGF-β1 were observed in the conditioned medium of FGF-2 but not FGF-1 stimulated ASM cells with a maximum after 2-4 hours of incubation. We conclude that TGF-β1 and FGF-1 stimulate mRNA expression of collagen I and III in ASM cells. Taken together, induced cell proliferation by FGF-1 and FGF-2 and increased ECM synthesis by FGF-1 and TGF-β1 in ASM cells in vitro implicate these growth factors in ASM cell accumulation by hypertrophy and/or hyperplasia during COPD.

10.2 Vascular alterations and the role of growth factors

Vascular abnormalities including pathological angiogenesis and vascular remodeling resulting from tobacco induced injury have been associated with the development of COPD (4-6). Early reports from Wright et al. described an increased wall area of small (< 500 μm) pulmonary vessel within the intima in mild to moderate COPD patients and additionally in the media in severe cases (4, 7). The wall thickening has been attributed to a chronic inflammatory process with ongoing fibrosis and an increased adventitial infiltration of inflammatory cells, predominantly CD8⁺ T-lymphocytes (6, 8). The emergence of smooth muscle cells within the intima of small pulmonary arterial branches and the extension of medial vascular smooth muscle (VSM) distally into pulmonary arteries, arterioles and veins that are normally devoid of smooth muscle have also been described (9). We have looked at the vascular alterations in COPD and have shown vascular wall thickening in the peripheral pulmonary vessels of mild COPD patients compared to non-smoking controls (chapter 2).
Angiogenesis in COPD

Tobacco smoking imposes severe oxidative stress on the lungs directly via reactive oxygen species in the smoke as well as indirectly through activation of inflammatory cells leading to a repetitive cycle of oxidant stress and protease activation. Occluded capillaries and loss of the pulmonary vascular bed by emphysema has been suggested to lead to the formation of new vessels (angiogenesis) and an increased number of broncho-pulmonary arterial anastomoses (9). Hypoxia is an important trigger for angiogenesis in order to (re-)supply tissues with oxygen and detecting as well as responding to hypoxia are therefore of pathophysiological and clinical relevance (10). Sustained alveolar hypoxia can cause pulmonary vasoconstriction with pulmonary hypertension and pulmonary angiogenesis with the formation of collateral vessel sprouting and remodeling of existing vessels (9).

In COPD patients we observed increased expression of FGF-2 and receptor FGFR-1 in endothelial and VSM cells in many small calibre (50-200 mm) alveolar vessels (Chapter 2). Additionally, VEGF and its receptors, flt-1 and KDR/flk-1 were increased on these kind of pulmonary vessels (Chapter 3). Angiogenic sprouting is a mechanism, in which VEGF and FGF-2, play an important role (11). It is assumed that tobacco-induced tissue injury to the endothelium with consecutive alveolar hypoxia leads to a series of events initiating angiogenesis in COPD (12, 13). In brief, myo-fibroblasts or vascular smooth muscle cells get activated by hypoxia and expression of hypoxia inducible transcription factors is induced, resulting in VEGF secretion (10, 14, 15). Endothelial and VSM cells activation leads to destabilization of the vessels by the actions of angiopoietin 2 and tie-2 receptor (16-18). In addition, VEGF increases vascular permeability, thereby allowing extravasation of plasma proteins which lays down a provisional matrix for proliferation and migration of endothelial cells (18). The increase in vascular permeability and as well as additionally secretion of proteinases by endothelial and VSM cells lead to liberation and activation of growth factors such as VEGF and FGF-2 from the surrounding matrix with prolongation of endothelial cell initiated tube formation (16-18). FGF-2 and platelet-derived growth factor also affect angiogenesis by recruiting mesenchymal progenitor cells (pericytes) or (myo-)fibroblast and smooth muscle cells, whereas angiopoetin-1 and transforming growth factor-β1 further stabilize the newly formed vessel (18). In COPD little is known about the exact role of angiogenesis, but the relevance of the blood vessels in COPD is emerging by recent observations, indicating that severe emphysema is associated with pulmonary endothelial cell apoptosis and increased levels of oxidative stress makers as well as decreased VEGF and type 2 receptor (KDR/flk-1) expression (19). Moreover, treatment with a blocker of VEGF type 2 receptor caused emphysema in experimental animals placed in hypoxic conditions (20). In contrast, we observed increased VEGF expression in pulmonary vessels in a patient group with mild to moderate
disease. It is possible that the kind of patients is responsible for the observed differences, mild COPD subjects in our case versus solely emphysema patients in case of the study above. These discrepancies could also pinpoint towards different stages of development or severity of the disease. In mild to moderate COPD patients increased expression of VEGF and receptors may indicate an active and partly successful response to tobacco induced injury, whereas the decreased expression observed by Voelkel and coworkers may represent a failing response at the end stage of the disease.

Thus, the presence of VEGF and its receptors, especially KDR/flk-1, in the lungs are associated with both maintenance, survival and the protection against apoptosis of endothelial cells and the initiation of repair by angiogenesis in response to tissue injury. Although further studies are necessary to elucidate the contribution of the formation of new vessels (angiogenesis) in COPD, our results suggest that VEGF and its receptors, flt-1 and KDR/flk-1 as well as FGF-2 and receptor FGFR-1 are important players in the peripheral lungs during the development of COPD (chapter 2 and 3).

*Vascular remodeling in COPD*

Many of the factors of normal vessel formation are also active during pathological vascular wall remodeling with deregulated repair as a consequence of either direct tobacco-induced injury, inflammation or increased shear stress in COPD (18). A pathological link has been establish between pulmonary hypertension and the development of vascular wall thickening and remodeling (13, 15, 21). Microvessels of the normal adult lungs contain a mixed population of partially muscular and muscular vessels, the latter consists of separated muscular segments where preexisting smooth muscle cells are defined by an internal and external elastic lamina (22).

The sources of the newly formed cells during vascular remodeling have been a key issue of investigation. Recent studies indicate that the existing VSM contribute only relatively little to the increase microvascular smooth muscle population as indicated by a low proliferation index (23, 24). Rather, vessel wall thickness increases by migration of interstitial fibroblast to the vessel wall and by cells derived from de-differentiated VSM or even endothelial cells. VSM and endothelial cell-derived VEGF, FGF-1 and FGF-2 stimulate fibroblast chemotaxis and proliferation (25-30). We have demonstrated increased expression of these ligands in our COPD patient group (chapters 2 and 3). Release of the mediators such as platelet-derived growth factor, and endothelin-1 may also contribute to chemotaxis and alignment of these cells, whereas transforming growth factor-β1 induced the expression of α-SMA in endothelial and fibroblast, the early marker of smooth muscle phenotype differentiation (18). Furthermore, we have shown that the ratio in the amount of α-SMA positive staining versus vascular wall area remained constant in growing vascular walls,
indicating an overall increase in all the individual cell types and extracellular matrix, rather than a shift towards a particular cell type. TGF-β1 is a potent inducer of ECM proteins synthesis in fibroblast and vascular smooth muscle cells such as collagens which may be involved in vascular wall thickening in COPD as indicated by a correlation with the amount of total collagen deposition in the vascular wall (5, 31). In addition, recent observations link alveolar hypoxia and the expression of hypoxia-inducible transcription factors with the actions of VEGF and FGF-2 on endothelial, VSM cells and fibroblasts in the ongoing process of vascular remodeling. Hypoxia and endothelial injury induce the expression of VEGF in VSM cells as well as VEGF and KDR/flk-1 in endothelial cells, whereas the expression and release of FGF-2 can be upregulated in endothelial cells by increased shear stress (10, 14, 17, 32, 33). The release of these growth factors leads to increased proliferation of endothelial and VSM cells. Furthermore, Rose and colleagues showed that hypoxic fibroblast showed increased HIF-1α expression and VEGF release, inducing both fibroblast recruitment and proliferation, which in turn activated and increased the proliferation of VSM cells (15). In addition, growth factors such as VEGF, FGF-2 and PDGF and TGF-β released from macrophages and mast cells upon hypoxia near sites of vascular lesions may contribute the vascular remodeling (27, 34, 35). Moreover, a shift in HSPG-side chain, which is acting as the potent co-receptor for the FGFR-1, leads to a remarkably enhanced responsiveness of FGF-2 on endothelial cells under influence of HIF-1α during hypoxia (36). The cellular interactions within the vascular wall and some of most the important mediators during vascular remodeling in COPD are summarized in the figure.

10.3 Airway wall remodeling and the role of growth factors

Changes to small airways and lung parenchyma

Although the investigations in the pulmonary vasculature have gained interest, the role of the changes within the airway wall and have been studied intensively during the last several decades. The conducting airways can be subdivided in central, bronchial airway as well as more peripheral or small airways. Airways with a diameter of 2 mm or less are conveniently considered as small airways (37, 38). Inflammation and structural alterations in the small airways as well as the lung parenchyma are considered as the most important contributors to the airflow limitation and the accelerated decline of FEV₁ in COPD (37, 38). Many studies, therefore, have focused on the pathological changes that take places within the airways < 2 mm in diameter and lung parenchyma (37, 39-42).
**Figure** Proposed mechanism of vascular wall thickening in COPD. Cigarette smoking imposes severe stress on the lungs both directly, via the toxic agents and reactive oxygen species in the smoke and indirectly through the activation of inflammatory cells, predominantly neutrophils (Neu), macrophages (Mφ) and T-lymphocytes (CD8⁺ T), causing tissue injury, that in turn leads to alveolar hypoxia. Moreover, pulmonary hypertension that is associated with COPD could lead to additional vessel injury via increased shear stress. Growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs) and transforming growth factor β₁ (TGF-β₁) released from inflammatory cells near sites of vascular lesions may contribute to the vascular remodeling. Hypoxic fibroblast (FB) show increased hypoxia inducible factor (HIF) 1α expression and VEGF release, inducing both recruitment and proliferation of interstitial fibroblasts, and in turn proliferation of vascular smooth muscle cells (VSMC). Hypoxia and endothelial injury cause release of VEGF and FGF-2 from endothelial cells leading to increased proliferation of endothelial and VSMC cells. During hypoxia endothelial cells show a HIF-1α dependent expression of heparan sulphate proteoglycan (HSPG) side chains. HSPGs act as co-receptors for FGF-1 and FGF-2, leading to a remarkably enhanced responsiveness of FGF-2. TGF-β₁ is involved in extracellular matrix (ECM) deposition within the vascular wall by FB and VSMC, and could initiate differentiation of EC and FB to a smooth muscle phenotype as indicated by the induction of α-SMA expression in endothelial and fibroblast. Several growth factors could play an important role in peripheral vessel remodeling during the development of COPD. Summarising, the investigated growth factors could play an important role in the pathophysiological processes that are active in peripheral vessel remodeling during the development of COPD.
Early reports showed that the specific morphologic features separating smokers from non-smokers were increases in epithelial and goblet cell metaplasia, smooth muscle mass as well as inflammation in the walls of small bronchioles and that young non-symptomatic smokers displayed early signs of inflammatory reactions in bronchiolar airways and alveolar air spaces without any apparent structural abnormalities (41, 42). Later studies further specified this increased inflammatory cell influx in COPD patients as predominantly neutrophils, macrophages, mast cells and CD8+ T-lymphocytes (43-46). Changes in the lung parenchyma also contribute to the disease. As a result of this smoke-induced ongoing inflammatory processes, the connective tissue of the lungs gets degraded by a relative excess of inflammatory-cell derived proteases and a relative depletion of anti-proteolytic defences, together referred to as the protease-antiprotease hypothesis (47).

In the light of these observations, definite progress has been made in what factors can cause damage to lung tissue. The current knowledge in the development of COPD is summarized Figure 10.2.

From recent human as well as animal studies it has become clear that COPD is characterized by breakdown of elastin but also breakdown and synthesis of collagen with scar formation by proteases including macrophage metalloelastase, neutrophil elastase and collagenases (47, 49, 50). Moreover, the main effector cells are probably resident macrophages as indicated by recent animals studies in which knockout mice for macrophage products such as macrophage metalloelastase which did not developed increased airspace sizes (emphysema) after chronic smoke exposure (51-53). Interestingly, knockout mice lacking neutrophil elastase were only 50-60% protected against smoke-induced lung injury and emphysema, which implies that neutrophils probably only partially contribute in this process (54). On the other hand, these results have to be taken with care because of possible differences between mice and men. Thus, several phenomena occurring in parallel may result in peripheral tissue destruction and remodeling in COPD. Little is known, however, about the exact role of peptide growth factors in the molecular mechanisms underlying these processes in the context of COPD.
Figure 10.2  Important mechanisms in the pathogenesis of COPD. Chronic exposure to agents from tobacco smoke leads to tissue injury and chronic inflammation in the lung parenchyma, the small and large airways and vasculature with an influx of predominantly macrophages, neutrophils and CD8^{+ve} lymphocytes. This leads to the release of many pro-inflammatory cytokines and growth factors and a misbalance in inflammatory and structural cell-derived proteases and their inhibitors (serine/cysteine proteases vs. α1-antitrypsin, secretory leukoprotease inhibitor (SLPI), and matrix metalloproteinases (MMPs) vs. tissue inhibitors of MMPs, TIMPs). Excessive breakdown of elastin and collagens in the parenchyma with destruction of alveoli as well as increased deposition of extracellular matrix within the airways pulmonary vasculature with thickening and fibrosis contribute both to airflow limitation in COPD. Based on reference (48).
Growth factors during tissue repair in COPD

Growth factors such as FGF-1, FGF-2, VEGF, PDGF, TGF-β₁ as well as many others produced and secreted by various cell types including inflammatory cells, bronchiolar and alveolar epithelial and airway smooth muscle cells or released from deposited extracellular matrix stores may contribute either adverse or protective to the process of airway remodeling (40, 46, 55). Chapter 2 focused on fibroblast growth factors in the peripheral lungs and we showed that FGF-1, FGF-2 as well as their receptor FGFR-1 were expressed by bronchiolar epithelial and airway smooth muscle cells, (myo-fibroblasts) and macrophages. Also VEGF and its receptor KDR/flk-1 and flt-1 as well as TGF-β₁ and its receptor were found on bronchiolar and alveolar epithelial cells as well as airway smooth muscle cells (46). Moreover, we observed increased expression of VEGF on these cell types in COPD.

The role of growth factors in tissue remodeling is possibly ambiguous. FGF-1, FGF-2 as well as VEGF released from injured cells or deposited extracellular matrix stores are strong chemotactic agents for macrophages, mast cells and fibroblasts. Additionally, they prove to be potent mitogens for bronchiolar epithelial cells, (myo)-fibroblast and airway smooth muscle cells. FGF-2 and VEGF have been shown to be survival factors for epithelial cells as well. As indicated by a recent study from Pardo and co-workers, FGF-2 prevented toxin-induced apoptosis in pneumocyte type II cells. Cell rescue relied on de novo protein synthesis of the anti-apoptotic proteins Bcl-X(L) and Bcl-2 within 4 h of FGF-2 treatment (56). Furthermore, the protective role of TGF-β₁ is emphasized by recent observation demonstrating that smoke extracts inhibited epithelial cells repair processes by interfering with the epithelial cell proliferation, motility and TGF-β₁ release (57). These data, suggest that epithelial cells present in the airways of smokers may be altered in their ability to support repair responses, which may contribute to architectural disruptions present in the airways in COPD, associated with cigarette smoking. Thus, fibroblast growth factors, TGF-β₁ and VEGF could play a role in effectively repairing damage to the lung epithelia and underlying connective tissues and protecting against further tobacco-induced tissue injury, in order to retain the normal architecture of the lungs.

Changes to large airways

Few studies of COPD have focused attention on larger airways of more than 2 mm in diameter. The characteristic changes in the central airways of smokers with established COPD include inflammatory cellular infiltration into the airway wall and mucous gland enlargement as well as changes in airway dimension in relation to lung function of patients with COPD (58-62). This last study showed that the wall area internal to the airway smooth muscle, the lamina propria, was significantly thickened over the entire range of cartilaginous airways, which was also associated
with a reduction in \( \text{FEV}_1/\text{FVC} \) (62). Surprisingly, and in contrast to earlier reports from peripheral airways, alterations in large airway smooth muscle mass were not observed (62, 63). Therefore, those authors argued that their findings and those of others favor chronic inflammation with subepithelial fibrosis of the airways as a cause of the inner wall thickness. Bronchial microvessels in the lamina propria may contribute to the inner wall thickening by vascular wall remodeling or vascular edema, since the number of microvessels in the area 500 \( \mu \text{m} \) deep inside the airway wall appeared constant for patients with either COPD or chronic bronchitis as compared to smoking and also non-smoking controls (63).

*Extracellular matrix and subepithelial fibrosis in COPD*

Chapter 4 in this thesis described the role of fibroblast growth factors 1 and 2 as well as their receptor FGFR-1 in the bronchial airways during COPD. In COPD patients we found increased expression of FGF-1 in the bronchial epithelium, whereas FGF-2 was elevated in bronchial airway smooth muscle cells and FGFR-1 was more intense on both cell types. The central airways were also immunohistochemically positive for VEGF and its receptors KDR/flk-1 and Flt-1 and in COPD displayed increased expression for VEGF but not for its receptors in the bronchial epithelium, ASM cells, and the macrophages and microvessels in the lamina propria and adventitia of the bronchial airways.

Within the bronchial airways, collagen subtypes I and III, the most abundant ones, and fibronectin and laminin are found beneath the epithelial lining, throughout the interstitial spaces and in between most cells types and within the blood vessels of airway wall (64-66). Collagens and fibronectin are bound to cells through specific binding sites or receptors, the integrins, which are heterodimeric transmembrane receptors, consisting one \( \alpha \) and \( \beta \) chain, which specially bind different ECM molecules (64, 65). Collagen IV and laminins are the main constituents of epithelial or endothelial basement membranes, which connects these cells, functioning as outward cellular lining of the airways or of blood vessels, with collagen subtypes I, III and VI from within the underlying interstitial spaces (67, 68).

In the light of damage and repair of the bronchial epithelium and the surface epithelial basement membrane (SEBM) as well as airway remodeling and fibrosis in underlying subepithelial regions including the lamina propria, airway smooth muscle, and adventitial layers, we also investigated the expression and deposition of various extracellular matrix molecules in the central airways of COPD patients. We found within the surface epithelial basement membrane that the deposition of total collagen as well as subtypes collagen I, III and IV, fibronectin and laminin was increased at sites of epithelial denudation, irrespective of the disease state. Furthermore, COPD patients showed a significant elevation of the deposition of fibronectin, collagen I and III but not collagen.
IV or laminin as compared to non-COPD patients at the SEBM with or without the presence of epithelial damage (chapter 5). Moreover, in COPD patients collagen I and III but not fibronectin, laminin and collagen IV were upregulated within the lamina propria and adventitia of the bronchial airways with accumulation of macrophages, fibroblast and α-SMA positive myofibroblasts (chapter 4 and 5). These results pinpoint towards ongoing bronchial subepithelial as well as adventitial fibrosis and airway remodeling in COPD. Within the bronchial airways, extracellular matrix is mainly produced by epithelial cells, (myo-)fibroblasts and airway smooth muscle cells. Bronchial epithelial cells and subepithelial fibroblast are rich sources of fibroproliferative cytokines and growth factors as well as extracellular matrix products (69). TGF-β1 is able to induce production of fibronectin and the release of VEGF in an autocrine manner in bronchial epithelial cells (70, 71). Interestingly, bronchial epithelial cell and fibroblast interactions with regard to extracellular matrix production were observed with cell culture. Conditioned media of bronchial epithelial cell were shown to induce macromolecule release accompanied by increased steady-state fibronectin and collagen I alpha mRNA levels (72). TGF-β1 neutralizing antibody blocked this increase in extracellular matrix production, suggesting that TGF-β1 produced by the epithelial cells may drive fibroblast matrix production (72). The increased deposition of collagen I and III within the interstitial matrix in the lamina propria and adventitial spaces could be produced by (myo-)fibroblast present in the bronchial airways.

The role of fibroblast growth factors on ECM molecule production appeared to be more variable among different cell types within the airways. In human epithelial cells FGF-1 has been shown to induce collagen I and III (73). Our results of increased FGF-1 expression together with its receptor FGFR-1 in the bronchial epithelium of COPD patients could contribute to the elevated deposition of fibronectin, collagens I and III in the SEBM at sites with intact and especially at areas with denudation of the bronchial epithelium in COPD (chapter 5). It has been shown that TGF-β1 is also able to induce FGF-2 from airway epithelial cells and that FGF-2 to induce collagen IV in human epithelial cells (73, 74). We found expression of FGF-2 expression on bronchial epithelial cells but observed no difference in FGF-2 expression as well as collagen IV deposition in COPD patients as compared to con-COPD patients.

Summarizing, the elevated expression of FGF-1 and its receptor FGFR-1, the increased expression of VEGF and the presence of KDR/flk-1 and flt-1 on bronchial epithelial cells and the increased deposition of ECM molecules in COPD, suggests a mechanism of ongoing repair processes at sites of tobacco induced epithelial damage, triggering and perpetuating subepithelial fibrosis in COPD.
Evidence is emerging that (myo-)fibroblast and/or airway smooth muscle cells from diseases including asthma and idiopathic pulmonary fibrosis are phenotypically different compared to isolated cells from control patients (75-80). We found increased FGF-2 and FGFR-1 as well as VEGF in ASM cells of COPD patients, which could also contribute to smooth muscle mass increase and ECM deposition during airway remodeling in COPD (chapter 3 and 4). Moreover, in chapter 6 we showed that isolated ASM cells in vitro, treated with TGF-β1 or FGF-1 but not FGF-2, displayed increased mRNA levels of pro-collagen subtypes III and I. Furthermore, in chapter 6 we described that active TGF-β1 is released from FGF-2 and to a lesser extent FGF-1 stimulated ASM cells with a maximum at 2-4 hours of incubation. Moreover, FGF-2 and also FGF-1 but interestingly not TGF-β1 induced proliferation of isolated ASM cells in vitro.

Normal mature ASM cells exist in vivo predominantly in a non-proliferative state with a fully differentiated contractile phenotype and expression of contractile makers (78, 81). The isolation and culturing in vitro on a serum-enriched medium with the exposure to many cytokines and growth factors causes the transition to a more proliferative phenotype, mimicking the events during chronic inflammation in vivo (78, 81). Serum deprivation restores the expression of most contractile markers. Intermediate forms may exist including a more “synthetic” phenotype with partly impaired proliferation, the synthesis of extracellular matrix components such as pro-collagen subtype I and the expression of some of the contractile elements like α-smooth muscle actin (α-SMA), together resembling a (myo-)fibroblast phenotype (77, 82-84). A recent study using isolated ASM cells demonstrated that ASM cells-derived TGF-β1 localized extracellular and that plasmin regulated the secretion of a biologically active form of TGF-β1 by ASM cells as well as the release of extracellular TGF-β1. The biologically active TGF-β1 induced ASM cells to synthesize collagen I in an autocrine as well as manner α-smooth muscle actin (α-SMA), (85).
During development of experimental lung fibrosis upregulation of FGF-1 expression is observed in fibroblast (86). As indicated by a recent follow-up study, however FGF-1 reduced the expression and synthesis of type I collagen and increased the collagenase protein expression were found in cultured human lung fibroblasts (87). Their findings demonstrated that FGF-1 might have a protective role in avoiding collagen accumulation during lung ECM remodeling. Also FGF-2 has been found to decrease mRNA expression and synthesis of the pro alpha-chains for types I and III collagen and to induce interstitial collagenase (MMP-1), which is required for degradation of collagen types I and III in vascular smooth muscle cells (88). Furthermore, FGF-2 completely disassembled the smooth muscle alpha-actin-containing stress fiber network and increased proliferation and migration of VSM cells (89).

Although an exact mechanism remains unclear, a link between TGF-β1 induced ECM production and the role of FGFs increased proliferation has been proposed by recent investigations. Inactive TGF-β1 is bound to latency-associated peptide (LAP) and this TGF-β1 is bound to latent binding protein-1 (LTBP-1) and in turn to the extracellular matrix, servings as a reservoir for active TGF-β1 (90). Release of bioactive TGF-β1 by macrophages, ASM cells or from the ECM-bound reservoirs may occur by simultaneously released serine proteases of which plasmin is one of the most important (85, 91). Thanickal and colleagues showed in human lung fibroblasts that FGF-2 release increased after TGF-β1 stimulation and that FGFR-1 (Flg) and FGFR-2 (Bek) were upregulated by TGF-β1 incubation, mediating enhanced mitogenic responses to FGFs (92, 93). This suggests an autocrine loop for both factors.

We further show the opposite, the induction of TGF-β1 by FGF-2 in human ASM cells, which has only been shown earlier to our knowledge in a cell line of glial origin and neonatal cultured astrocytes (94, 95). Since this induction was too rapid for de novo transcription and translation, we hypothesize that this release originates from intracellular or cell-bound latent TGF-β1 stores. The role of FGF-2 stimulated TGF-β1 induction in ASM cells is unclear. FGF-2, however, is known to induce plasminogen activator inhibitor-1 (PAI-1), blocking the cleavage from tissue-type and urokinase-type plasminogen activators (tPA and uPA), and thereby the formation of plasmin and thus of bioactive TGF-β1 (96, 97). It could, therefore, be that the decrease in bioactive TGF-β1 is counteracted by its own induction by FGF-2. Taken together, these findings could pinpoint towards a dual mechanism to regulate pro-collagen I synthesis by actions of TGF-β1 or FGF-2 on the level of plasmin. And in general, these growth factors could be involved in phenotypic switches between a proliferative/synthetic state versus a more contractile state.
Nuclear localization of FGF-2 in airway and vascular smooth muscle

In chapter 4 we show in COPD increased nuclear FGF-2, but not FGF-1, expression in airway smooth muscle cells by interactively counting of them using video image analysis. In chapters 2 and 4 we found that vascular smooth muscle cells also displayed this nuclear localization pattern. Currently, the role of FGF-2 in the nucleus has been partly clarified, as has been reviewed in two recent reviews (98, 99). The FGF-2 gene can produce at least five different isotypes: the conventional 18 kDa extracellular FGF-2, as well as four high molecular weight (HMW) forms (22, 22.5, 24 and 34 kDa). All four HMW isoforms, are able to translocate to the nucleus upon activation of different cells and in the nucleus, FGF-2 can act as modulator of ribosomal gene transcription (98, 99).

From several investigations it is becoming clear that the primary role of translocation of HMW FGF-2 isoforms to the nucleus is involved mechanisms of responding to cellular injury. Pro-inflammatory cytokines and growth factors such as interleukin-1 β (IL-1β), tumor necrosis factor α (TNF-α) and epidermal growth factor (EGF) were shown to selectively increase the expression of HMW-isoforms (22 and 24-kDa) but not of the conventional 18-kDa isoform, followed by nuclear translocation in cultured connective tissue cells (100). Also the FGF receptors can be translocated to the nucleus, as was evidenced by recent studies of Stachowiak and coworkers, showing increased expression and nuclear accumulation of basic fibroblast growth factor and the receptor FGFR-1 in primary cultured astrocytes following ischemic insults and in adrenal medulla cells after angiotensin II treatment (101-103). In fibroblast cell lines, overexpression of nuclear 24 kDa HMW FGF-2 is associated with increased resistance against toxic drugs and radiation induced DNA injury (104, 105). Additionally, cellular debris at sites of injury contains nucleic acid fragments released from dead cells and growth factors such as FGF-2. In viable but damaged surrounding cells, re-uptake followed by nuclear translocation of FGF-2 coupled to DNA fragments can occur, which could be important events in early wound repair processes (106). Furthermore, Singh and colleagues also have shown that increased nuclear expression of 24 kDa HMW FGF-2 in vascular smooth muscle and endothelium precedes arterial enlargement in response to increased arterial blood flow in vivo (107).

Although the function of FGF-2 in the ASM cell nucleus in COPD patients remains unclear, from the pattern we observed we believe that the positive staining in the nuclei was not due to an artifact but representative of specific localization of the appropriate antigen by the antibody used. We hypothesize that pro-inflammatory cytokines that may be involved in perpetuation of chronic inflammation in COPD patients and the proliferation of airway smooth muscle (ASM) cells may rely on nuclear FGF-2 effects. Angiotensin II (Ang II), IL-1β and TNF-α, potent cytokines for a
wide variety of cells including (myo-)fibroblasts and ASM cells, could be implicated in the expression and release of other fibro-proliferative messengers like TGF-β, and IL-6 by ASM cells (108-110). As indicated by recent studies, increased nuclear expression of 24 kDa HMW FGF-2 in ASM cells could be involved in the expression of cytokines like IL-6 by inducing gene transcription pathways (111-113). Taken together, these observations suggest that nuclear FGF-2 expression could be transcriptionally involved in a variety of compensatory mechanisms in response to cellular injury, which could indicate a novel FGF-2 and FGFR-1 signal transduction mechanism in COPD. The exact role of nuclear FGF-2 expression in COPD remains, however, to be elucidated.

10.4 Concluding remarks

Taken together, the investigated growth factors could play an important role in the pathophysiological processes that are active in airways as well as lung parenchyma during the development of COPD. The results presented in this thesis lead to the following conclusions:

- The protein expression of growth factors FGF-1, FGF-2 and their receptor FGFR-1 is increased in the pulmonary vasculature, which could be linked to the structural vascular abnormalities observed in COPD patients.
- The expression of the angiogenic growth factor VEGF-A and its receptors KDR/Flk-1 and Flt-1 are upregulated in the peripheral vasculature and airways of COPD patients, implicating VEGF-A and receptors in vascular and airway remodeling.
- COPD patients display more intense protein expression of FGF-1, FGF-2 as well as VEGF-A in the bronchial epithelium, airway smooth muscle cells, microvasculature and macrophages in the central airways, indicating their involvement in epithelial repair processes and the initiation and perpetuation central airway wall remodeling.
- The deposition of extracellular matrix components collagens I and III, fibronectin and laminin was increased in the bronchial airways of COPD patients as compared to non-COPD controls, contributing to bronchial airway wall thickening in COPD.
- ASM cells may contribute to bronchial wall thickening, indicated by their ability to produce the ECM markers collagen I, III and fibronectin in response to FGF-1 or TGF-β1, as well as their proliferative response to FGF-1 and FGF-2 in vitro.
10.5 Implications for future research

The studies in this thesis indicate that growth factors (FGF-1, FGF-2, VEGF and TGF-β1) expressed on various cell types and released from various sites in the lungs during chronic exposure to toxic agents from tobacco smoke, are important mediators in COPD. A rapidly growing number of cellular and molecular biomarkers with a large amount of possible interactions is implicated in the disease, reviewed by reference (48).

First of all, COPD is a complex disease affecting all three compartments of the lungs in a variable manner in individual patients, the lung parenchyma (emphysema), small airways (small airways disease) and the large airways (chronic bronchitis). The balance of inflammatory and structural cell-derived proteases as well as their inhibitors is also important in COPD (48). Excessive breakdown of elastin and collagens in the parenchyma with destruction of alveoli as well as increased deposition of extracellular matrix within the airways with thickening and fibrosis contribute both to airflow limitation in COPD. Thus, although evident progress has been made in the understanding of the disease, several important questions remain to be answered.

What is the individual contribution of different cells to the pathogenesis of COPD? In others words which of the already known cell types, intercellular mediators as well as intracellular messengers are involved in initiating and perpetuating the most important events of the three disease states in the lung parenchyma (emphysema), small airways (small airways disease) and the large airways (chronic bronchitis). Most likely several different mediators are involved in chronic inflammation, tissue damage and fibrosis. As reviewed recently, interesting targets for COPD treatment include anti-inflammatory drugs, antioxidants and anti-remodeling agents (48). However, new drugs for the treatment of COPD are needed and the identification of an association between peptide-growth factors such as FGF-1, FGF-2 and VEGF and the pathology of COPD could lead to new interventions either by promoting repair processes or preventing the formation of fibro-proliferative lesions.

Also of clinical importance for the progression of the disease, are mild and severe COPD differential stages of the same disease or totally different pathologies? The number of neutrophils, macrophages and CD8\(^{\text{w}}\) T-lymphocytes in the peripheral airways correlated with the severity of airflow limitation (44, 114). Furthermore, our observations that the cellular expression of several growth factors in the airways is correlated with the functional determinant of airflow limitation (FEV\(_1\)) emphasize their contribution to the disease. However, above observations do not rule out either possibility. If the progression from mild to severe COPD involves differential stages of the same disease, the question of reversibility of the disease is emphasized. Smoking cessation is obviously considered as beneficial, but further studies are necessary to investigate what the
consequences are for the pathologic lesions such as the chronic inflammation and fibro-proliferative abnormalities in the airways of clinical COPD patients (115).

What is the role of the blood vessels in the pathogenesis of COPD and their possible contribution in the treatment of the disease? Although structural abnormalities in the blood vessels of COPD patients have been observed several decades ago, their importance has been re-emphasized by several recent studies. We observed that the vessels of COPD patients have increased expression of peptide-growth factors including FGF-1, FGF-2 and VEGF. Therefore, these peptide growth factors could be protective against tobacco-induced injury and may prove attractive therapeutic agents in the reversibility of the disease in the future.

The most intriguing question for the understanding of COPD is why only a minority of 10% of all smokers actually develops the disease, given the fact that the amount of exposure to tobacco smoke is comparable between cases and non-symptomatic smokers. Clearly, some people are more susceptible than others are, for the same amount tobacco smoked. Several genetic predispositions are identified, including associations between COPD and polymorphisms, in first of all α1-antitrypsin, tumor necrosis factor-α and surfactant protein B genes (2). The associations above pinpoint towards differences in protection to alveolar destruction, in inflammatory mediator profile and in variations in lining fluid, respectively. The goal is to find which other heritable factors may contribute to the increased risk of development and progression of COPD. It would be interesting to investigate whether or not genetic polymorphisms can be found in genes that are involved in the initiation of repair processes and perpetuation towards pulmonary fibrosis, like peptide growth factors and their receptors.

10.6 References


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