General introduction and Hypotheses

*Respiratory diseases*
2.1 Introduction

Respiratory Diseases: COPD

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disorder of the lungs, becoming a global health problem with increasing morbidity and mortality (1). Recent observations indicate that COPD is the fourth cause of mortality in the USA and it is projected to be the fifth burden of morbidity world-wide in the year 2020 according to a consensus report published by the World Health Organisation (2). COPD is characterized by a slow progression of airflow limitation, which is nearly irreversible. Recently, an official definition has been formulated by the Global Initiative on Obstructive Lung Disease (GOLD); “A disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases” (2). One of the major determining factors is tobacco smoking, but it remains to be investigated to what extent other factors such as environmental and occupational exposures and genetic factors can contribute to the disease. Surprisingly, only 10-20 percent of all smokers develop COPD (1, 2).

Diagnosis of COPD should be considered in patients with symptoms of cough, sputum production and abnormal shortness of breath and a presumed history of exposure to risk factors for the disease (1, 2). The diagnosis is confirmed by spirometry with a post-bronchodilator forced expiratory volume in one second (FEV₁) < 80% of predicted value and in combination with an FEV₁/FVC (forced vital capacity) < 70% of predicted. The above lung function criteria are used in classifying the severity of the disease as stage I (mild COPD) followed by stage II (moderate COPD) with FEV₁ values 30% to 80% of predicted and stage III (severe COPD) with FEV₁ values < 30% of predicted (2). Figure 2.1 illustrates the effects of smoking on the annual decline in lung function (FEV₁) of susceptible and non-susceptible smokers and also depicts the beneficial effect of smoking cessation. These data originate from a large epidemiological study in Britain from 1977 (3), and have subsequently been confirmed in more recent (4, 5).

2.2 Structure of airways and lung parenchyma

The respiratory system is commonly divided in two separate parts: the conducting airways consisting of trachea, bronchi, bronchioles, terminal bronchioles and the respiratory part defined as respiratory bronchioles, alveolar ducts and terminal alveoli (6). The entire branching pulmonary tree consists of roughly 20 to 25 generations and a branch of the pulmonary artery accompanies each conducting airway (6). Conveniently the different anatomical sites are divided
as "central" lung tissue, representing the larger conducting airways and "peripheral" lung tissue, which includes terminal and respiratory bronchioles, and alveoli. Airways with a diameter of 2 mm or less are conveniently defined as small airways, which are considered as the most important contributors to the airflow resistance and are involved in the accelerated decline of FEV₁ in COPD (7-11). Figure 2.2 shows the important structural features of "central" and "peripheral" lung tissues in case of non-symptomatic smokers (A and B) and COPD (C and D) subjects, respectively. Conducting airways consists of an epithelial layer, its basement membrane, and the lamina propria, that consist predominantly of connective tissue and small vasculature, together forming the airway mucosa (6). The bronchial epithelium, covered with secreted mucus, protects the outer layers from first contact with the air or pathogens in the lumen. Different cell types are found in the epithelial layer, the cubical shaped ciliated cells, the secretory cells such as goblet and Clara cells which play a role in the production of mucus and the smaller basal cells which are though to be the epithelial stem cells (12). In the submucosa of the central airways irregular shaped patches of submucosal secretory glands and airways smooth muscle are found. In the adventitia of the larger airways predominately cartilage, supplying bronchial vasculature and connective tissue are observed, with a slow transition into the more peripheral areas of the lungs (13).

In peripheral tissue terminal and respiratory bronchioles as well as alveolar ducts and alveoli with accompanying arteries are present (13). Veins of several sizes are found predominantly in interstitial septa, which are rich in extracellular matrix fibres such as collagens. While branching bronchioles gradually lose their coating of secretory glands, cartilage and finally also their ASM layer (6, 13). The alveolar walls are covered with flattened respiratory epithelial cells, alveolar type I cells, which are responsible for most of the gas exchange with capillaries in close proximity, and with more cubical shaped cells, alveolar type II cells, that are progenitor for the latter cells (14-16). Furthermore a scattered population of immune cells, predominantly a low number of alveolar macrophages, T-lymphocytes and granulocytes, is found (17).

2.3 Pathology and pathogenesis of COPD

COPD consists of three distinct pathological conditions under one umbrella (Figure 2.3). These are chronic bronchitis with productive cough of more than three months and mucus hypersecretion, small airway disease with chronic obstruction and inflammation of smaller airways, and emphysema with enlargement of air spaces, destruction of lung parenchyma, loss of lung elasticity that can in turn cause collapse of respiratory airways (18).
Excessive tobacco smoking is the main cause in the pathogenesis of COPD, which can be explained by observations that inflammatory reactions are present in the entire tracheo-bronchial tree of non-obstructive smokers. Studies in the central airways indicate that the inflammatory infiltrate predominantly consists of cytotoxic CD8\(^+\) T-lymphocytes, neutrophils and macrophages in the airway wall and neutrophils in the bronchial lumen (19, 20). Moreover, in the small airways and parenchyma of young non-obstructive smokers already an inflammatory cellular infiltrate is found without any structural changes, which could pinpoint towards initial stages in the pathogenesis of the disease (11). In COPD patients the cellular infiltrate is further increased in the small airways consisting predominantly of CD8\(^+\) T-lymphocytes, neutrophils, macrophages as well as mast cells (21-23).

Cellular and structural changes in smokers with or without COPD are summarized in Table 2.1. Smokers with airflow limitation show changes in peripheral airways including inflammation, fibrosis, mucus plugging and airway smooth muscle hypertrophy (21, 22, 24-27). These factors cause deformation and narrowing of the airways and together with destruction of alveolar walls, could lead to airflow limitation. Less attention has been focused on central airways in COPD. The airway wall showed a further increase in the number of macrophages and T-lymphocytes and the airway lumen an elevated influx of neutrophils. Furthermore, changes in central airway dimensions with increased submucosal fibrosis and airway smooth muscle mass are observed in COPD patients compared to non-symptomatic subjects (8, 28).

The cellular and molecular mechanisms, which may explain the slow progression of airflow limitation, however, are not entirely clear. The currently well accepted protease-antiprotease hypothesis states that as a result of this smoke-induced ongoing inflammatory process, the connective tissue of the lungs is degraded by a relative excess of inflammatory-cell derived proteases such as neutrophil and macrophage elastases and a relative depletion of antiproteolytic defences like \(\alpha_1\)-antitrypsin or secretory leukocyte proteinase inhibitor (SLPI), (29, 30). Moreover, an unbalanced expression and release of anti- and pro-inflammatory cytokines or growth factors may play an important role.

Although definitive progress has been made in the understanding of the disease and several drugs that can diminish symptoms in COPD patients like corticosteroids, bronchodilator agents or anti-inflammatory compounds have been found, no drugs are available at present that can reduce the progression of the disease (1). The only effective therapeutic intervention currently available is smoking cessation, but the effects only account for the diminishing of future damage since the disease state is poorly reversible (31).
C

Airway Lumen

epithelium

BM
ASM
Glands
Cartilage

D

Loss of alveolar attachment

ASM
Bronchiole Lumen
V
VSM
Fibrosis
Loss of alveolar attachment
Figure 2.1 The important structural features of "central" and "peripheral" lung tissues in case of non-symptomatic smokers (A and B) and COPD (C and D) subjects, respectively. Panel A depicts the main central airway structures with the bronchial epithelium, basement membrane (BM), the arterioles, capillaries, and venules embedded in the subepithelial layer (V) as well as airway smooth muscle (ASM) and subepithelial glands (Glands) and cartilage of the airway wall. In B a small bronchiole with its epithelium lining the lumen and ASM is embedded in the surrounding alveoli. An accompanying vessel (V) is present surrounded by vascular smooth muscle (VSM). In panel C can be observed that subepithelial fibrosis (...) is present as well as an increased airway smooth muscle mass in COPD as compared to non-symptomatic subjects. Panel D shows important peripheral features of COPD, peribronchial and perivascular fibrosis, vascular wall thickening and emphysema with enlarged air spaces and loss of alveolar attachment. All sides are stained with α-smooth muscle actin (red colour).

Figure 2.2 Risk factors for COPD. Among several other possible factors involved in the development of COPD, excessive smoking is considered as the main risk factor for the disease.
COPD comprises of three distinct pathological conditions under one umbrella, chronic bronchitis, small airway disease and emphysema. AAT = α₁-antitrypsin, TNF-α = tumor necrosis factor α.

### 2.4 Tobacco-induced injury and repair

It is now well established that particles from the smoke can cause damage to the airways in particular to the epithelial lining (35). Loss of epithelium induces repair processes, which consists of many steps and involves many factors (36). The role of damage in the in COPD is less clear, since it could originate from direct effects of smoking and/or from the subsequent chronic inflammation. Yet, both non-symptomatic smokers and established COPD patients show signs of damage and repair to the epithelial surface in the form of denuded epithelial lining and also squamous metaplasia (8).

**TABLE 2.1** CELLULAR AND STRUCTURAL CHANGES PRESENT IN THE LUNGS OF NON-SYMPTOMATIC SMOKERS AND OF SMOKERS WITH ESTABLISHED COPD

<table>
<thead>
<tr>
<th>Central airways</th>
<th>Non-symptomatic smokers</th>
<th>Smokers with established COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall</td>
<td>• T-lymphocytes</td>
<td>• Further increase in macropages,</td>
</tr>
<tr>
<td></td>
<td>• Macrophages</td>
<td>• CD8&lt;sup&gt;+&lt;/sup&gt; T-lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Neutrophils in severe disease</td>
</tr>
<tr>
<td>Lumen</td>
<td>• Neutrophils</td>
<td>• Neutrophils</td>
</tr>
<tr>
<td>Peripheral airways</td>
<td>• Mononuclear cells</td>
<td>• Goblet cell metaplasia and mucus plugging</td>
</tr>
<tr>
<td></td>
<td>• Clusters of macrophages in the respiratory bronchioles</td>
<td>• Smooth muscle hypertrophy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Macrophages, mast cells, neutrophils in severe disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• CD8&lt;sup&gt;+&lt;/sup&gt; T-lymphocytes</td>
</tr>
<tr>
<td>Parenchyma</td>
<td>• No destruction</td>
<td>• Inflammation CD8&lt;sup&gt;+&lt;/sup&gt; T-lymphocytes</td>
</tr>
<tr>
<td></td>
<td>• No fibrosis</td>
<td>• Destruction centriacinar and panacinar emphysema</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Fibrosis</td>
</tr>
<tr>
<td>Pulmonary arteries</td>
<td>• Intimal thickening</td>
<td>• Endothelial dysfunction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Intimal thickening</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Medial thickening</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Adventitial inflammation CD8&lt;sup&gt;+&lt;/sup&gt; T-lymphocytes</td>
</tr>
</tbody>
</table>

Based on References (8, 20, 23, 32-34).

In general, wound healing involves a series of cellular and molecular events which initiates after injury of the epithelial lining and disruption of the underlying vasculature with an increased influx
of platelets and inflammatory cells, in the primary stages predominantly neutrophils, followed by macrophages and T-lymphocytes (41). These platelets and inflammatory cells are capable of releasing many growth factors and cytokines, and molecules like fibrin and fibronectin to close and hold together the wounded tissue (41). Currently, neutrophils are believed to act as first-line of defense against invading micro-organisms and the elimination of other foreign material by the release of anti-microbiologic peptides like defencins, reactive oxygen species (ROS) and proteinases, but they are also responsible for so called “friendly-fire” leading to damage on viable surrounding tissues (29). The next cell to appear is the macrophage, which is the key-orchestrator of tissue repair processes (41, 42).

Cytokines and growth factors released by surrounding epithelial cells, macrophages and T-lymphocytes attract (myo-) fibroblast to the wounded area which start to release additional growth factors and cytokines, especially TGF-β1, responsible for the synthesis and consecutive deposition of extracellular matrix (ECM) products such as collagen subtypes I, III V, VIII, and proteoglycans (37, 40, 43). Neo-vascularization and angiogenesis is initiated by the release of angiogenic growth factors like vascular endothelial growth factor (VEGF) by macrophages and other immune cells in response to a hypoxic environment. VEGF stimulates endothelial cell proliferation, migration and new tube formation (44). Taken together this environment of a rich cocktail of growth stimulatory cytokines and growth factors, (myo-)fibroblast derived new extracellular matrix networks and adequate capillaries facilitate proliferating epithelial cells to migrate which leads to closure of the wound (41). Controlling inflammation and (myo-) fibroblast growth is as important as initiating above events, thus minimizing additional damage and abnormal wound healing with scarring and excessive fibrogenesis (41).

*Deregulated repair processes*

Ongoing chronic inflammation with repetitive cycles of tissue damage and repair can lead to severe scarring abnormalities, predominantly by excessive deposition of ECM products by myo-fibroblasts. Within the airways, the bronchial epithelium, sub-epithelial myo-fibroblasts, airway smooth muscle cells are major cell types involved in tissue repair processes and excessive stimulation can lead to airway wall remodeling with subepithelial fibrosis (8). Although it is becoming clear that many cytokines and growth factors are involved. Among these are the pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukine-8 (IL-8) and IL-1β. These cytokines play an important role in chemotaxis of neutrophils and macrophages to the airway wall and lumen, and are also involved in bronchial epithelial survival and repair (36, 43, 45-47). TNF-α is a multi-functional cytokine, which can be induced in epithelial cells and inflammatory cells by cigarette smoke (48). It can induce neutrophil degranulation, release of proteolytic enzymes and mucus cell metaplasia with mucus hypersecretion (48). Furthermore,
TNF-α has the ability to induce many additional products among them TNF-α itself, IL-8, IL-1β. Indeed, increased levels of TNF-α and IL-8 are found in sputum, bronchial alveolar lavage (BAL) fluid, bronchial epithelium and airway of COPD subjects as compared to non-symptomatic smokers (48).

Furthermore, a variety of growth factors including platelet–derived growth factor-BB (PDGF-BB) and vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1) and fibroblast growth factors (FGFs) that are released from the epithelium, neutrophils, macrophages and myo-fibroblast may contribute to the pathogenesis of COPD (49-52). Although many other cytokines and growth factors can contribute, some of the important cellular and molecular events in the epithelial repair process and possible mechanism leading to sub-epithelial fibrosis in COPD are summarized in Figure 2.5. The major sources, target cells and effects for several growth factors implicated in chronic lungs diseases are listed in Table 2.2. Taken together, growth factors could therefore be important players in airway remodeling in the development of COPD.

2.5 The Role of cytokines and growth factors in COPD

*Fibroblast growth factors*

The fibroblast growth factor family is implicated in a wide variety of patho-physiological conditions including systemic hypertension, ischemic heart disease and interstitial lung fibrosis and may as well be involved in chronic inflammation, fibrosis and tissue repair during airway remodeling in COPD (53-56). The fibroblast growth factor family currently consists of at least 23 members of which FGF-1 (acidic FGF) and FGF-2 (basic FGF) were the first discovered and are the most important ones, which share approximately 53 % sequence homology (57). FGFs play a role in morphogenesis, angiogenesis, tissue and ECM remodeling during normal development and disease states in almost every organ (57-61). In the lungs, FGF-1 and FGF-2 are produced by many cell type including airway epithelium, alveolar macrophages and mast cells, (myo-)fibroblast, airway smooth muscle cells (38, 62-64). Next to FGF-1 and FGF-2, two important members FGF-7 (keratinocyte growth factor) and FGF-10 are predominantly involved in development and maturation of the lungs (60). Their cellular responses are very divers ranging from proliferation, migration, differentiation, cell viability as well as either stimulation or inhibition of ECM production. Target cells of FGF-1 and FGF-2 include epithelial cells, fibroblasts on which they act as potent mitogen as well as inducers of ECM synthesis (38, 67). Although both FGFs have mitogenic effects on epithelial cells, fibroblasts and on cells of smooth muscle origin, FGF-1 has been associated with higher proliferation of epithelial cell lineage, while FGF-2 is generally more potent than FGF-1 on cells of mesenchymal origin like fibroblast and
smooth muscle cells. Basic FGF induces vascular smooth muscle cells and endothelial cell proliferation, and is therefore also considered as a potent factor in angiogenesis (68, 69).
Figure 2.3 A scheme of cytokine and growth factor actions in human airways. On triggering, eg, with tobacco smoke, epithelial cells are damaged, epithelial cells and resident macrophages produce inflammatory mediators such as tumor necrosis factor (TNF)-α interleukin (IL)-1β, IL-8. In turn, inflammatory mediators-stimulate migration of monocytes/macrophages, neutrophils, CD8 positive T-lymphocytes to the airway. Both TNF-α and IL-8 can cause degranulation of neutrophils with production and release of serine-proteinases, metalloproteinases (MMPs) as well as free radicals that can cause matrix and epithelial damage. In turn, TNF-α and released growth factors like vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF-1 and FGF-2) orchestrate epithelial repair. Ongoing inflammation and tissue breakdown trigger the release of growth factors like transforming growth factor-β1 (TGF-β1) inducing ECM production by myo-fibroblasts. Repetitive tissue damage and repair can lead to excessive ECM deposition and subepithelial fibrosis. Neu = neutrophil; Mφ = macrophage CD8+ve T = CD8 positive T-lymphocytes. Based on Refs. (1, 48).

Table 2.2

<table>
<thead>
<tr>
<th>Major growth factors in airway remodelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factor</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>FGF-1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>FGF-2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>VEGF</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>TGF-8</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>PDGF</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IGF-2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Transforming growth factor beta (TGF-β), Fibroblast growth factor (FGF), Vascular endothelial growth factor (VEGF), Platelet-derived growth factor (PDGF), Insulin-like growth factors (IGF), Airway and Vascular smooth muscle (ASM and VSM), Extracellular matrix (ECM). References (37, 38, 64-66).

Figure 2.4 FGF receptor signaling. Multiple levels of regulation of FGF-mediated cellular responses exist. A: Selection of the ligand: 23 different FGF-ligands can bind to the FGF receptors. Predominantly FGF-1, FGF-2, FGF-7 and FGF-10 are expressed in the embryonic, postnatal and normal or pathological adult lungs. B: Selection of the FGF receptor and coreceptors: there are four FGF tyrosine kinase receptors FGFR-1 to FGFR-4 and heparan
sulphate proteoglycans (HSPGs) co-receptors such as membrane-associated Syndecans (1-4), Glypicans (1-6) and Perlecans. The expression of different receptors and co-receptors can influence the cellular responses to the FGFs. C: Selection of multiple signaling pathways: many intracellular signaling pathways have been described. Abbreviation; TK = tyrosine kinase domain; Ig = Immunoglobulin-like domain; AB = acid box, ligand binding site; TM = trans membrane domain; HS = heparan sulphate chains; CP = HSPG core protein. Based on Refs. (58, 61).

Fibroblast growth factors exert their biological effects via binding to four high-affinity, transmembrane tyrosine-kinase receptors designated FGFR-1 through FGFR-4 (58). Distinct FGF subtypes bind with different affinity to the various FGF receptors. Alternative splicing and regulated protein trafficking further modulate the intra-cellular events and resultant response initiated by FGF ligand-receptor interaction (58). Additional regulatory binding sites for FGFs consist of heparan-sulphate proteoglycans (HSPGs) that appear to be macromolecular receptors which can modulate the effects of FGFs, both stimulatory and inhibitory depending on the heparan-sulphate side chain as well as the proteoglycan core protein (70). HSPGs are part of the ECM and are located on the surface of most cell membranes closely linked with the high affinity tyrosine-kinase receptors (70). Figure 2.6 schematically summarizes the interactions of FGFs with their tyrosine-kinase receptors and HSPGs. Unlike the other members of the FGF family, the acidic and basic FGF lack cytoplasmic sequences for extracellular export. In this regard, the growth factors could be released during cell lysis and the HSPGs could act as a reservoir of growth factor that can be released in an enzymatic regulated manner during ECM breakdown (71).

Additionally, fibroblast growth factor family members are implicated in pathological conditions with tissue remodeling and lung fibrosis (55, 62, 72). Barrios and coworkers (55) showed FGF-1 and FGFR-1 expression in experimentally induced pulmonary fibrosis. Becerril and colleagues found that FGF-1 overexpression in the lung fibroblasts results in down-regulation of collagen synthesis and up-regulation of collagenases, which may protect against fibrosis (72). In a recent study production of FGF-2 from mast cells and the expression of FGFR-1 (Flg) and FGFR-2 (Bek) protein were positively linked to idiopathic pulmonary fibrosis (62). FGF-2 and also PDGF have been implicated in the pathogenesis of obliteratorive bronchiolitis after transplantation (73).

In the normal pulmonary vasculature, FGF-1, FGF-2 and FGFR-1 are constitutively expressed in the media (vascular smooth muscle cells) of pulmonary vessels and FGF-2 is also found in endothelial cells (63). Singh and colleagues demonstrated that increased expression of FGF-2 in vascular smooth muscle and endothelium precedes arterial enlargement in response to increased arterial blood flow in vivo (54). Furthermore, Bryant et al recently found that administration of FGF-2 could be protective against a decrease in vessel luminal area and wall thickening in response to altered blood flow and that this inhibitory effect could be blocked by anti-FGF-2
neutralizing antibodies (74). Taken together, FGFs could therefore be important players in airway and vascular remodeling in the development of COPD.

**Vascular endothelial growth factor**

A variety of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and FGF-2 released from various cell types of airway as well as vascular walls have the potential to contribute to the pathogenesis 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 (75). Like FGFs, VEGFs are heparin-binding proteins and acting via their high affinity, transmembrane receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), (75). The receptors belong to the family of tyrosine kinases and are predominantly expressed by endothelial, VSM cells and epithelial cells (75). Recent studies indicate that VEGF is expressed in the lung by bronchiolar, submucosal 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 (76-78).

VEGF promotes an array of responses in the endothelium including endothelial cell proliferation and angiogenesis with new vessel tube formation in vivo (75, 79). Moreover, the expression of VEGF can be induced under a variety of pathophysiological conditions, including pulmonary hypoxia and pulmonary hypertension with increased shear stress (76, 79). Hypoxia and pulmonary hypertension are pathological features often seen in advanced COPD patients and increased VEGF expression under 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 (8).

The role of VEGF and its receptors in the lungs of COPD patients remains unclear. Vascular endothelial growth factor (VEGF) and its receptor 2 (VEGFR-2) are involved in proper maintenance, differentiation, and function of endothelial as well as epithelial cells. Voelkel and co-workers demonstrated that VEGFR-2 blockade in combination with chronic hypobaric hypoxia destroyed lung capillaries by inducing endothelial cell apoptosis and at the same time caused precapillary pulmonary arteries occlusion by proliferated endothelial cells (79-82). Furthermore, they observed that emphysematous patients have decreased levels of VEGF messenger RNA and protein as well as decreased expression of VEGF receptor 2, KDR/flk-1(83). In this recent study, decreased VEGF and KDR/flk-1 expression was associated with endothelial and also epithelial cells death in alveolar septa due to a decrease of endothelial cell maintenance factors which may be part of the pathogenesis of emphysema (83). Thus, the expression of VEGF may be protective...
against signals leading to apoptosis such as toxic agents from tobacco smoke. Alternatively, abundance of VEGF and receptor mRNAs (Flt-1 and KDR/Flik) decreased in endothelial cells during hyperoxia, possibly secondary to the loss of endothelial cells by apoptosis. This also indicated that VEGF functions as a survival factor in the normal adult rat lung, and its loss during hyperoxia contributes to the pathophysiology of oxygen-induced lung damage (84). Although the role of VEGF in the vascular biology is thoroughly studied, it has become clear that VEGF and receptors 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 (79, 81, 85). Taken together, VEGF and its receptors could therefore also be important players in airway and vascular remodeling in the development of COPD.

*Transforming growth factors*

TGF-β and receptor expression in lungs have been associated with asthma, chronic bronchitis, idiopathic pulmonary fibrosis (86, 87). In patients with chronic bronchitis or COPD TGF-β₁ mRNA and protein are observed in bronchial and bronchiolar epithelium, macrophages, mast cells and pulmonary vessels and increased TGF-β₁ protein levels are found in the epithelium of COPD patients as compared to smoking controls (50, 88).

TGF-β is a multifunctional polypeptide growth factor, which is involved in inflammation and connective tissue synthesis. TGF-β belongs to a large superfamily currently including more than 30 members, which also include bone morphogenetic proteins, inhibins and activins (37). Three different mammalian isoforms exist (TGF-β₁ to -β₃) of which the TGF-β₁ isoform is the most potent and binds to at least three high affinity receptors (TGFβR I-III), (37). TGF-β is released as a biologically inactive precursor consisting of a dimer with the N-terminal pro-region, latency-associated peptide (LAP), to which inactive TGF-β₁ is bound. Furthermore, latent TGF-β₁ complex can bind to latent binding protein-1 (LTBP-1) that in turn binds to the extracellular matrix which serves at a reservoir for active TGF-β₁ (89). In addition, the release of latent TGF-β₁ from the extracellular matrix is a consequence of cleavage of LTBP. Activation and release of TGF-β₁, dimer is achieved in vivo by enzymatic cleavage from intracellular and extracellular latent TGF-β₁ stores by serine proteases as well as various metalloproteinases (37, 90).

Its actions highly depend on the target cell-type or situation present. The TGF-β superfamily is important in cell development and differentiation and proliferative regeneration (37). In epithelial and endothelial cells TGF-β₁ is usually associated with terminal differentiation, growth inhibition and even apoptosis. During wound healing TGF-β₁ is involved in regeneration (37). In (myo-) fibroblasts, smooth muscle cells and other cells of mesenchymal origin stimulation of
proliferation, synthesis of ECM proteins including collagens, elastin, proteoglycans and fibronectin are induced by TGF-β₁, (37, 91, 92).

2.6 Extracellular matrix biology in COPD

*Extracellular matrix proteins in the lungs*

The extracellular spaces within tissues and cells are filled with organized extracellular matrix (ECM) proteins that are important for structural integrity, strength as well as elasticity of tissues. The major components of the ECM consists of fibrous proteins like collagens, elastin and fibrillin, proteoglycans such as syndecans, glypecan, perlecan and decorin as well as adhesion molecules like fibronectin and laminins. It has been become clear that the ECM molecules play important roles in cell signaling and cellular activities. Fibronectin and laminin well as some collagens are bound to cells through specific binding sites or receptors, the integrins, of which more than 20 different subtypes are identified. These integrin receptors are heterodimeric transmembrane receptors, consisting one α and β chain, which specially bind different ECM molecules (65, 93). Furthermore, fibronectin, on the other hand, has specialized domains for different collagens, so that the various components of the ECM are tightly interconnected with each other and with cells. Currently, more than 20 collagen subtypes are identified, of which the subtypes I and III are the most abundant forms, found throughout the interstitial spaces and in between cells of many tissues (65, 93, 94). Within the lungs, these collagen subtypes are deposited in the interstitium of airway wall, beneath the epithelial lining, and within the blood vessels and alveolar septa (95, 96). Collagen IV and laminin are the main constituents of cellular basement membranes, connecting epithelial or endothelial cells, functioning as outward cellular linings, with collagen subtypes I, III and VI inserting within the underlying interstitium (97, 98).

Mature processed collagen molecules aggregate to form larger triple-stranded helical fibrous structures and help to form the ECM with other components (65, 93). Therefore, normal structural type I and III collagen production and deposition in the ECM to make normal physiological connective tissue is highly regulated by cytokines and growth factors like TGF-β, TNF-α and FGF-2 and their transcriptional as well as post-translational modulatory steps (65, 93). Abnormalities in any of regulatory step may cause defective and accumulation of collagen in ECM, which in turn causes pulmonary fibrosis (94).

*ECM production and fibrosis*

Stimulation of ECM production by TGF-β₁ appears to be normal for either mesenchymal or epithelial cell origin. In fibroblasts and smooth muscle cells TGF-β₁ also promotes expression of
actin, myosin, smooth muscle actin and cell adhesion molecules such as integrins, including one specific and important combination α5β1 integrin receptor, also known as the collagen receptor. Moreover, TGF-β1 down-regulates the expression of matrix degrading enzymes (matrix metalloproteinases), specifically MMP-1, MMP-3 and induces the expression of protease inhibitors, such as tissue inhibitor of matrix metalloproteinase (TIMPs), (37). Taken together, the observations above inextricably link the processes of ECM guided degradation and migration, ECM production and scarring contraction of myo-fibroblasts to the functions of TGF-β1. As indicated by early reports and many follow-up studies the most important concepts in the onset and continuation of fibrosis is the presence of TGF-β1 at areas with injury to the epithelium and underlying basement membranes (86, 87).

**ECM breakdown**

Historically, the role of compounds from neutrophils and also macrophages have been implicated in the pathogenesis of COPD, based on the relation between α1-antitrypsin deficiency and the predisposition for the development of emphysema in a rare number of patients (42, 99, 100). α1-antitrypsin or secretory leukocyte proteinase inhibitor (SLPI), inhibits neutrophil serine-proteinases, especially elastase, which can cleave elastin, thus causing damage to alveoli and eventually emphysema (29, 30). Neutrophils store high amounts of serine proteinases and in addition at least two MMPs, MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B), Zn2+ ion catalyzed enzymes of which currently more than 20 members have been identified (99, 101). These MMPs can cause degradation of most components of the extracellular matrix upon neutrophil activation (100). Furthermore, MMPs can be secreted by macrophages. The release and action of MMPs are strictly regulated by for instance growth factors and cytokines and especially by enzymes called tissue inhibitors of metalloproteinases (TIMPs), of which currently four members have been identified (102). Furthermore, MMPs can mediate the release and activation of ECM-bound (e.g. TGF-β1, FGFs, EGF, IGF-1 and TNF-α) or cell membrane-bound (IL-6 and TNF-α) growth factors and cytokines, thereby promoting ongoing inflammation and tissue remodeling (100). However the opposite, degradative inactivation of IL-1β has also been described (100). All of these actions can contribute to pathologic tissue remodeling including inflammation and cellular proliferation as well as ECM breakdown and deposition during COPD.

Indeed, subjects of emphysema showed increased levels of MMP-1 (interstitial collagenase 1), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in macrophages, alveolar type II cells and fibroblasts as compared to non-emphysematous controls (99). A similar approach, using bronchial tissue from COPD subjects, immunolocalized several MMPs, including MMP-1, MMP-2, MMP-9 and also MMP-8 and MMP-13 (Interstitial collagenases 2 and 3), and found increased expression
of MMP-1 and MMP-2 levels in bronchial epithelial cells, luminal and interstitial macrophages (103). Furthermore, MMP-9 and its inhibitor TIMP-1 were upregulated in sputum of chronic bronchitis patients (104).

Taken together, these MMPs can degrade collagen and elastin, the major components the extracellular matrix, thereby implicating macrophages and neutrophils as important contributors to excessive tissue breakdown and injury in COPD and predisposing regenerative tissue towards deregulated repair with fibrosis.

2.7 Angiogenesis and vascular remodeling in COPD

COPD patients with moderate to severe disease display elevated pulmonary vascular pressures during exercise and pathological changes in the pulmonary circulation (8, 105). Wright et al. (105, 106) demonstrated increased wall thickness of small (< 500 μm) pulmonary vessels in COPD subjects as compared to non-smoking smokers, which was correlated with the severity of the disease (as indicated by a decline in FEV₁). Additionally, COPD patients with mild to moderate COPD showed intimal thickening and severe subjects of the disease also developed medial thickening.

In COPD, alveolar hypoxia can cause pulmonary vasoconstriction and, if the hypoxic stimulus persists, pulmonary vascular remodeling, of which increased muscularization of small arterial branches is the most striking feature (18). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (18). Intimal thickening and emergence of smooth muscle cells within the intima of small pulmonary arterial branches has been attributed to a chronic inflammatory process accompanied with fibrosis in part similar to arteriosclerosis in cardiovascular disease (107, 108).

Recently, Peinado et al. showed also intimal but not medial thickening in the vasculature of mild COPD patients compared to non-smoking controls (109). Furthermore, observations from the same group indicated that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of inflammatory cells, predominantly CD8⁺ T-lymphocytes and displayed VSM heterogeneity in relation to desmin as well as intimal thickening that was correlated to the amount of total collagen deposition (110, 111). The infiltration of the vascular wall with inflammatory cells may contribute to vascular wall thickening. Finally, loss of the pulmonary vascular bed by emphysema has been suggested to lead to the formation of new vessels (18). Thus, several
phenomena acting in concert in COPD result in pulmonary vascular remodeling. Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

**Angiogenesis**

Mature endothelial cells are quiescence cells with an extremely low proliferative index. Smoke induced injury with hypoxia, however, induce VEGF-A mRNA expression via hypoxia inducible transcription factors (HIF 1 to 3), (75, 112). This initiates angiogenesis by increasing endothelial permeability and stimulates endothelial cells to secrete several proteinases, such as MMPs including collagens and elastin degrading MMP-1, MMP-2, MMP-3 and MMP-9, and heparinase acting on proteoglycans (44). This, in turn, leads to ECM breakdown and the liberation of additional growth factors, predominantly VEGF-A itself as well as FGF-2 and insulin-like growth factor-1 (IGF-1) sequestered in within the surrounding matrix (44, 75). Proliferating endothelial cells migrate to distant sites in wounded or inflamed tissue, which is predominantly guided by actions of VEGF and FGF-2 in close contact with the collagen and heparan-sulphate proteoglycan matrix, thus resulting in new tube formation (70).

**Vascular remodeling**

Of great importance is the recruitment of a stable vascular smooth muscle coating to newly formed vessels. This is initiated by VEGF in combination with angiopoietins produced by endothelial cells, of which currently four ligands are known (ANG-1 to ANG-4) that bind to two receptors expressed by endothelial cells, tie-1 and tie-2 (113). Binding ANG-1 to tie-2 receptor induces endothelial cells to recruit fibroblasts or VSM, whereas ANG-2 binding to tie-2 repels this event (113). TGF-β1 and TGF-βR2 are involved in vessel maturation by inhibiting endothelial cell proliferation and inducing smooth muscle differentiation and stimulating of ECM deposition by VSM cells and fibroblast, thereby solidifying the vessel wall (44).

Pathological arteriogenesis involves hypoxia, tissue ischemia, increased sheer stress, which can inflicts damage to endothelial and VSM cells (44). Inflammatory cells such as monocytes, macrophages and CD8\(^{+}\) T-lymphocytes infiltrate the vessel wall constitutively. Inflammation can cause additional damage to the vessel wall. Growth factors such as FGF-2, PDGF and TGF-β1 are released by endothelial and VSM cells in response to inflammatory mediators. Eventually deregulated repair can lead to fibrotic tissue deposition and vascular remodeling (44). Taken together, vascular remodeling and angiogenesis in peripheral, as well as in central airways could also be associated with the pathogenesis of COPD.
2.8 ASM cells in airway remodeling

Concepts of the contribution of human airway smooth muscle (ASM) cells to pathophysiological events during chronic airway diseases like asthma and COPD have drastically changed. Historically, ASM cells were considered as structural cells implicated in the regulation of producing immediate airway narrowing or widening merely by contraction and relaxation. However, the ASM cell can participate in inflammatory responses, release many chemotactic cytokines and growth factors, present necessary receptors and adhesion molecules and produce ECM components as well as ECM degrading proteases (114-116).

**Heterogeneity and phenotypic plasticity in ASM cells**

Recent studies indicate that ASM cells are apparently functionally and structurally divers and that heterogeneity and plasticity in phenotypes exists, which equip ASM cells with the potential to regulate airway lumen diameter both transiently, via reversible contraction, as well as chronically via remodeling by muscle hypertrophy (117). Phenotypic plasticity was first described in differentiated, cultured vascular smooth muscle cells derived from the medial layer of large elastic arteries (118). Mature vascular and also airway smooth muscle cells acquire an “immature” synthetic phenotype when incubated in serum-enriched culture, exhibiting a high proliferative index and loss of contractile elements and their associated proteins, defined as modulation (118, 119).

For example, pro-inflammatory mediators such as TNF-α and IL-1β were potent inducers of interleukin (IL)-8 release by ASM cells and together they synergistically augmented IL-8 release. IL-8 is a C-X-C chemokine that potently chemoattracts and activates neutrophils. Therefore, in addition to its contractile responses, airway smooth muscle cells have synthetic and secretory potential with the release of IL-8 and subsequent recruitment and activation of neutrophils in the airways.

The reversion of primary cultured smooth muscle cells to a contractile state also occurs after cultures grow to confluence or undergo serum starvation (maturation), which is marked by an increase in myofilaments and contractile apparatus-associated protein content (120). Recently, intermediate subtypes have been identified. The results from a recent study showed that IL-4 and IL-13 increased alpha-smooth muscle actin expression in myo-fibroblasts and thus that IL-4 and IL-13 are capable of inducing the phenotypic modulation of human lung fibroblast to myo-fibroblasts (121). This can influence the interaction of myo-fibroblast with the surrounding collagen matrix, modulating there contractile properties as indicated by a study investigating the contraction of these cells embedded in collagen I type gel matrix in response the cytokines (122).
Additionally, a distinct subset of ASM cells has been identified with a fully contractile phenotype, elongated morphology, abundant contractile apparatus proteins such as smooth muscle α and γ-actin, smooth muscle myosin heavy chain, SM22 and α1-integrin, reacquisition of pharmacological responsiveness to acetylcholine (116, 120). Additionally, contractile myocytes show a time-dependent subcellular reorganization of the contractile apparatus in response to changes in muscle length defined as mechanical plasticity (117). Figure 1.7 summarizes the important features of ASM cell heterogeneity, phenotypic and mechanic plasticity.

**Proliferation of ASM cells**

Since culture of human ASM cells was possible and since the discovery of the synthetic, highly proliferative ASM cells, the effect of mitogens and the signal transduction pathways leading to proliferation have studied intensively (114). The effects of mitogens for ASMC are mediated through at least two distinct receptor systems: Receptor tyrosine-kinase (e.g. platelet derived growth factor, epidermal growth factor as well as acidic and basic FGF) and G protein-coupled receptors (e.g. thrombin), (114, 123). Also the effects of TGF-β on ASM cell proliferation and ECM production have been thoroughly studied.

Black and colleagues found that 24 hours of incubation with TGF-β1 decreased DNA synthesis, whereas 48 and also 72 hours increased DNA synthesis and proliferation in cultured bovine ASM cells (125). Interestingly TGF-β1 inhibited 10% FBS induced DNA synthesis in sparsely seeded bovine ASM cells, whereas DNA synthesis was increased after 48 hours of TGF-β1 treatment in the presence of only BSA in confluent grown cells (126). Taken together, these studies demonstrated that TGF-β and TGF-β receptors are present on ASM cells and that TGF-β1 modulates the effects on proliferation with a condition-dependent nature (125-129).

**ASM and extracellular matrix**

Khalil et al. demonstrated that the release of biological active TGF-β1 under influence of plasmin can induce ASM cells to synthesize pro-collagen I in an autocrine manner (130, 131). Furthermore, human ASM cells produce many other ECM components including collagen types I, III, IV and V, fibronectin, laminins, elastin and HSPGs (e.g. perlecan and syndecan) (132). In addition, ASM cells can secrete MMP-1 (interstitial collagenase 1), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) as well as TIMP-1 (124, 132).
Figure 2.7 Schematic representation showing the association of phenotypic and mechanical plasticity on airway smooth muscle. Phenotypic plasticity results from reversible modulation and maturation of airway smooth muscle cells (ASMC) between a synthetic and contractile state associated with differential gene expression. Mature ASM cells acquire an “immature” synthetic phenotype when incubated in serum-enriched culture, exhibiting a high proliferative index and loss of contractile elements such as smooth muscle α and γ-actin (α, γ-SMA), smooth muscle myosin heavy chain (smMHC), SM22 and α₁-integrin, and loss of pharmacological responsiveness to acetylcholine via muscarinic M3 receptor. ASM cells can produce growth factors, extracellular matrix products as well as matrix degrading enzymes (matrix metalloproteinases, MMPs) and their inhibitors (TIMPs). Integrins are involved in the interaction of ASM cells with the binding to collagen I in the ECM. The binding of α₁β₁ to collagen I results in an almost complete arrest of collagen synthesis, whereas the binding to α₁β₁ integrin leads to induction of growth factors like TGF-β, MMP-1 as well as collagen gene expression (synthetic phenotype). Mechanical plasticity occurs in contractile myocytes as the result of time-dependent subcellular reorganization of the contractile apparatus in response to changes in muscle length. Adapted from Refs. (114, 117, 124).
Hirst and colleagues showed that cell-matrix interactions, in addition to growth factors, could have important effects on ASM cell proliferation and phenotype. In this study the authors showed that ASM cells cultured on collagen I or fibronectin matrix have increased proliferation whereas ASM cells grown on laminin proliferate more slowly yet express contractile proteins. Evidence for airway SMC heterogeneity and plasticity in vivo is indicated by observations in asthma and also COPD of accumulation of synthetic myocytes (myo-fibroblasts) in the submucosal region of the bronchial wall as well as significant increased airway smooth muscle mass possibly through hypertrophy and hyperplasia (28, 133, 134). Taken together, the ASM cell can be considered as an important cell type in the progression of airway remodeling in COPD.

2.9 Hypotheses

Chronic obstructive pulmonary disease is characterized by airflow limitation that is irreversible and without smoking cessation usually progressive. The disease is associated with an abnormal inflammatory response of the lungs to noxious particles and gases. Exposure to particles from the tobacco smoke inflicts damage onto a variety of structures at several levels in the lungs from conducting larger airways, respiratory airways to alveolar regions as well as in the pulmonary and bronchiolar vasculature. Although subtle local differences may exist in the lungs the common feature of pathological processes in COPD is; chronic challenge lead to repetitive cycles of tissue injury with inflammation and repair, which may result in tissue remodeling and structural abnormalities that, in turn, can cause airflow limitation. Although definite progress has been made in the descriptions of pathological alterations in COPD at the histology level, the underlying molecular events remain largely unknown. We, therefore, hypothesized that the observed structural alteration may arise from alterations in gene expression in the affected cell populations. We investigated role of growth factors and extracellular matrix in the development of airway and vascular wall structural changes in COPD at the molecular level.

2.10 References


