Molecular Biology of Bronchial and Vascular Remodelling

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Molecular Biology of Bronchial and Vascular Remodelling

Moleculaire biologie van bronchiale en vasculaire remodellering

Thesis

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To Nachu,
Appa and Amma

Molecular Biology of Bronchial and Vascular Remodelling

1

General Introduction

Chapter 1: General Introduction

1.1 Respiratory system

The respiratory system includes lungs, the diaphragm and muscles of the chest wall, the regulatory neural circuits, pleural spaces and the upper respiratory tract (nasopharynx, trachea and larynx). The most important function of the respiratory system is gas exchange, fulfilling the basic requirement of continuous oxygen supply to the body tissues. Gas exchange involves the transfer of oxygen from air to blood, and carbon dioxide from blood to air. Other functions include acid-base balance, sound production (phonation), participating in number of biochemical reactions that affect body functions and providing defence mechanisms to prevent the deleterious effects of environmental hazards. The respiratory system is subdivided in to two main zones based on morphology and functions. The conducting zone, which warms, moistens, and filters the incoming air, includes the nasal cavities, paranasal air sinuses, pharynx, larynx, trachea, primary bronchi, lobar bronchi, segmental bronchi, bronchioles and terminal bronchioles. The respiratory zone, where gas exchange between alveolar air and the blood stream occurs by diffusion includes the respiratory bronchioles, alveolar ducts and alveolar sacs.

1.1.1 Structure and function of the normal airways

The conducting airway is composed of an epithelial layer, basement membrane, lamina propria and cartilages (Figure 1.1). The epithelial lining is predominantly composed of pseudo stratified ciliated columnar epithelium interspersed with mucous glands and separated from lamina propria by a basement membrane. The basal cells, closely attached to the basement membrane, are considered to be progenitor cells for the airway epithelium. The epithelium serves as a barrier between the respiratory system and the external environment. It forms not only a physical barrier but also an immunologic and metabolic barrier (1). Epithelial damage, observed in asthma, may result in increased permeability and easier access of allergens, pollutants, and inflammatory and contractile agents to sub epithelial layers (2, 3). The lamina propria contains loose fibrous tissues with tiny blood and lymphatic vessels, elastic and smooth muscle fibres (under control of the autonomic nervous system, vagus nerve and sympathetic nervous system). The epithelium together with the lamina propria and the basement membrane forms the mucosa (4). The

submucosa, beneath the mucosa, contains; smooth muscle, glands and cartilage. Smooth muscle tone is important in determining airway diameter and thus affects airway resistance. Moreover, an increase in the permeability of this microvasculature and plasma exudation is considered to be important features of asthmatic airways (5-9). New vessel formation (angiogenesis) can contribute to the thickening of the airway wall. Figure 1.1 is a representation of a cross-section of normal human airway.

1.1.2 Pulmonary circulatory system

The pulmonary circulatory system is comprised of a dual blood supply; bronchial and pulmonary arteries as well as veins (10). The pulmonary arteries supply the bulk of blood flowing through the lungs. Pulmonary arteries bring relatively deoxygenated, carbon dioxide-rich blood from peripheral tissues to the lungs via the right ventricle of the heart. Pulmonary artery branches run parallel to airways to the level of the terminal bronchioles where they break off to form the capillaries in the alveolar walls. The pulmonary arteries are relatively thin-walled vessels with elastic fibres and relatively less smooth muscle than systemic arteries. The bronchial artery arises as an outgrowth from the aorta, providing oxygenated blood (11). The vessels extend longitudinally along the airways to the lung periphery as far as the terminal bronchioles. These vessels have relatively smaller lumen and thicker walls than the pulmonary arteries associated with the same bronchus. The bronchial and pulmonary circulations anastomose freely with each other throughout the tracheobronchial tree (12).

1.1.3 Factors in normal lung development

Lung development is influenced by different signalling molecules, growth factors and their receptors which mediate tissue interactions by paracrine, autocrine and endocrine mechanisms (13). This regulation of a variety of cellular functions is critical for normal lung development and homeostasis. Growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) signalling via tyrosine kinase receptors, promotes cell proliferation and differentiation, while transforming growth factor (TGF) β1 and bone morphogenetic protein (BMP) signalling by serine-threonine kinase receptors opposes these effects (14, 15). Besides their involvement in lung pattern formation, growth and cell differentiation during organogenesis, these factors have also been implicated in modulating

injury-repair responses in the adult lung (16). Disturbances in respiratory function may be due to disturbed ventilatory function, altered pulmonary circulation, or difficulty in gas exchange and they may be acute or chronic.

1.2 Chronic airway diseases

Respiratory diseases are increasing cause of morbidity and mortality for all age groups and races in the changing global environment. Today, almost one fifth of all deaths are attributable to respiratory illnesses. The International Classifications of Diseases (ICD-10) recognizes many chronic respiratory conditions including the upper respiratory tract like rhinitis and chronic inflammation as well as lower respiratory tract conditions such as bronchitis, interstitial lung diseases, cystic fibrosis, bronchiectasis besides the major chronic airway diseases; chronic obstructive pulmonary disease (COPD) and asthma.

1.2.1 Chronic obstructive pulmonary disease (COPD)

COPD is a disease state characterized by limitations in lung airflow that are not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to toxic particles or gases (17, 18). Based on World Health Organization (WHO) data from 2004, COPD ranks as the fourth leading cause of death worldwide, trailing only cardiovascular disease, pneumonia, and HIV/AIDS. In terms of morbidity (as measured in Disability Adjusted Life Years, DALYs), COPD ranks thirteenth. Annual healthcare costs are greatly affected by COPD, being estimated at 2.8×10^8 € in The Netherlands (in 2000) and over 32.1×10^9 US$ direct costs in the USA (NHLBI morbidity and mortality chart book, 2002) (19). A diagnosis of COPD should be considered in any patient who has symptoms of cough, sputum production, or dyspnoea (difficult or laboured breathing), and/or a history of exposure to risk factors for the disease. Risk factors (Table 1.1) for COPD include both host factors particular to the patient and environmental exposures, and the disease usually arises from an interaction between these two types of factors (WHO). Tobacco smoking including passive smoking, is the main risk factor associated with about 80% of COPD cases (20). Airway hyperresponsiveness, α_1-antitrypsin deficiency and certain occupational exposures are other established risk factors for the development of COPD (The Global Initiative for Chronic Obstructive Lung Disease; GOLD). The diagnosis is confirmed by spirometry, which measures lung function and capacity. Chronic cough and sputum production often precede the development of

airflow limitation by many years; although not all individuals with cough and sputum production go on to develop COPD.

1.2.2 Asthma

Asthma is a chronic inflammatory disorder characterized by difficulty in breathing due to hyperresponsive airways. The airways react by narrowing or obstructing the air movement and this causes a combination of symptoms such as wheezing, coughing, shortness of breath and chest tightness. Asthma attacks people of all ages, but often starts in childhood. Asthma varies in severity and frequency from person to person and may occur from hour to hour and day to day. Although largely avoidable, asthma tends to occur in epidemics and usually affects young people giving rise to a severe economic burden. Worldwide, the economic costs associated with asthma are estimated to exceed those of TB and HIV/AIDS combined (WHO). According to the "The Global Initiative for Asthma (GINA)", host factors include genetic predisposition to asthma or atopy, airway hyperresponsiveness, gender and ethnicity. Main environmental factors are exposure to allergens and occupational sensitizers, infections, diet, tobacco smoke and socioeconomic status. Table 1.1 summarizes the potential risk factors involved in the development of asthma and COPD.

Table 1.1: Potential risk factors for the development of COPD and Asthma

Risk Factors	COPD	Asthma
Host Factors	Genetic predisposition • α_1-antitrypsin deficiency • polymorphism Airway hyperresponsiveness Lung growth	Genetic predisposition Atopy Gender Airway hyperresponsiveness Race/Ethnicity
Environmental Factors	Tobacco smoke Indoor and outdoor air pollution Infections Socioeconomic status	Indoor and outdoor allergens Indoor and outdoor air pollution Infections Occupational sensitizers Tobacco smoke Diet and drugs Socioeconomic status

Based on GOLD (21) and GINA (22) 2005 reports.

1.3 Pathophysiology

The functional consequence of asthma and COPD is airflow limitation, which is mostly reversible in asthma and not fully reversible in COPD (23). The airflow limitation is due to cellular and structural changes; remodelling of the small airways and the lung parenchyma, promoting airway narrowing and obstruction (24). The airways in the main categories of diseases differ in nature and extent of the inflammatory cell infiltrate, degree of gland cell enlargement and goblet cell hyperplasia, bronchiolar smooth muscle mass, reticular basement membrane thickening, loss of alveolar attachments, and destructive airspace enlargement. Notwithstanding these differences, there is substantial overlap of these conditions in pathological appearance as well as in suspected mechanisms of architectural transformation (23). In a similar context, the “Dutch hypothesis", introduced the concept that the airways obstruction associated with asthma, chronic bronchitis, and even emphysema is related to different expressions of a primary abnormality in the airways, and this might result from a variation in the host response to the external stimuli (25). In asthma, CD4+ T lymphocytes, eosinophils, and mast cells are the predominant cells involved, whereas in COPD, the pulmonary inflammation consists of neutrophils, macrophages, CD8+ T cells (less prominent in severe COPD), mast cells, and eosinophils during exacerbations or in COPD patients showing reversible lung function (23, 26-28). COPD can be divided based on either clinical manifestations or pathological features into three different disorders: chronic bronchitis with airflow limitation, emphysema and chronic bronchiolitis. COPD is characterised by a progressive lung function decline, measured as an increased annual decline in forced expiratory volume in one second (FEV1) (17, 18). In emphysema, alveolar septa are deteriorated resulting in reduced gas exchange, and loss of elastic recoil of lung tissue (29). In chronic bronchitis with airflow limitation, bronchial gland enlargement and goblet cell hyperplasia leads to overproduction of mucus, resulting in mucoid airways obstruction (29). Often, COPD patients show a combination of these disorders. Pathophysiological features, in addition to decreased lung function, include chronic cough and dyspnoea. In smokers, epithelial cell metaplasia often occurs, whereas in chronic bronchitis a goblet cell metaplasia is seen. Both types of metaplasia result in an impaired ciliary function. In chronic bronchitis, the hypersecreted mucus cannot be cleared sufficiently, resulting in chronic cough and dyspnoea. After stopping smoking, the decline in lung function is minimised but not restored fully and an increased airway resistance and inflammation persist (26, 28, 30-36). It is believed that such decline in lung function is

probably due to airway fibrosis and inflammation that reduce the diameter of airway lumen. Other mechanisms which are thought to contribute to such (mal)adptation include oxidative stress and protease-antiprotease balance (37). This may be due to genetic predisposition involving polymorphisms reported in various genes such as in glutathion-S-transferase, epoxide hydrolase, matrix metalloproeteinases (MMPs)-1, 9 or 12 as well as tissue inhibitor of metalloproteinase (TIMP)-2. Additionally, an imbalance occurs due to chronic exposure to cigarette smoke and subsequent release of free radicals and proteases (elastase and MMPs) from activated inflammatory cells resulting in chronic state of tissue damage, repair and remodelling (38-40). Frequent exacerbations in COPD patients may eventually result in increased airway inflammation with eosinophils and neutrophils and higher levels of inflammatory mediators like cytokines (40).

In asthma, there is extensive infiltration of the airway with eosinophils and lymphocytes accompanied by vasodilatation, microvascular leakage and epithelial disruption (41). Inflammation of the bronchi, with associated plasma exudation, oedema, marked smooth muscle hypertrophy, mucus plugging and epithelial damage is thought to result in bronchial hyperreactivity; airflow limitation and airway hyperresponsiveness. Trophic changes, which arise because of chronic inflammation and repeated cycles of injury and repair, may eventually lead to remodelling. Therefore, the concept of airway remodelling caused by abnormal repair of the small airways and parenchyma has become more and more accepted (24).

1.4 Airway remodelling

Changes in the lung and airway anatomy (remodelling) tend to be context specific and elusive. This process is characterized by structural changes that include alterations in the composition, size, mass, number and organization of the cellular and molecular component of the airway wall (42, 43). An attempted repair process usually follows repetitive injury, secondary to the chronic inflammation during the progression of the chronic airway diseases. This repair through an autocrine or paracrine mechanism involves highly dynamic interactions and cross talks between the resident cells (myofibroblasts, ASM, epithelial, and endothelial cells) (44). Eventually these structural changes have functional consequences, as evidenced by clinical symptoms and signs. There are several components to airway remodelling, such as epithelial abnormalities, reticular basement membrane (RBM) thickening, matrix abnormalities, increased smooth muscle mass, increased vascularity,

hypersecretion and hypertrophy of mucous glands and thickening of the airway wall (42, 43). Remodelling in asthma involves fragile epithelium, significantly thicker RBM and marked smooth muscle enlargement, while in COPD it involves epithelial mucous metaplasia, airway wall fibrosis, increased ASM mass and alveolar destruction due to inflammation leading to emphysema (35). Though asthma and COPD are supposedly pathologically distinct the remodelling aspects of these diseases have overlapping features (42). Table 1.2 summarises the structural changes and functional consequences in airway remodelling.

1.4.1 Epithelial injury and repair

It is now well established that particles from cigarette smoke causes damage to the airways, particularly to the epithelial lining (45). Both non-symptomatic smokers and patients with COPD and asthma show signs of damage and repair to the epithelial surface in the form of denuded epithelial lining and squamous metaplasia (35). Epithelial changes include atrophy, squamous metaplasia and loss of ciliated epithelial cells and decrease of cilia length and function (24). The processes of normal and abnormal wound healing as a response to injury have been studied extensively (46-50). 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. This process is characterized by an influx of platelets and inflammatory cells, predominantly neutrophils, followed by macrophages and T-lymphocytes. These platelets and inflammatory cells release many growth factors and cytokines, as well as fibrin and fibronectin that act to repair wounded tissue. The environment of cytokines and growth factors, (myo)-fibroblast-derived extracellular matrix and adequate nutrient supply via angiogenesis facilitate epithelial cell proliferation and migration, leading to wound closure (50). Within the airways, the bronchial epithelium, sub-epithelial myo-fibroblasts and ASM cells are the major cell types involved in tissue repair processes. Figure 1.2 summarizes the cross talk between different cell types and eventual remodelling via bronchial epithelial repair and vessel remodelling.

Table 1.2: Structural changes and functional consequences in airway remodelling

Structural Changes	Functional Consequences
Alterations in large airways • Epithelial injury • Reticular basement membrane thickness • Goblet cell hyperplasia • Submucosal gland hypertrophy • Increased smooth muscle Alterations in small airways and lung parenchyma • Chronic inflammation • Mucous metaplasia • Increased smooth muscle mass • Subepithelial fibrosis • Matrix abnormalities Alterations in vasculature • Vascular dilatation • Microvascular leakage • Angiogenesis • Vascular remodelling	Increased Symptoms /frequency Perpetual inflammation Airway hyperresponsiveness Mucous hypersecretion Airflow limitation / obstruction Decreased airway distensibility Congestion /Oedema Swelling and stiffening of airway wall Decreased lung function Disease progression Irreversibility of airflow limitation Death

1.4.2 Matrix abnormalities

Altered ECM deposition contributing to the airway wall remodelling is an important feature of chronic airway diseases like asthma and COPD. Proteinases, such as elastase released by neutrophils and macrophages, MMPs released by macrophages, eosinophils, lymphocytes, neutrophils, and fibroblasts and cathepsins released by macrophages destruct the tissue and modulate repair. These proteinases are inhibited by antiproteinases such as α_1-antitrypsin, α_2-macroglobulin, elafin and TIMPs. The balance between proteinases and their inhibitors is critical in tissue repair and remodelling. Increased bronchial deposition of ECM proteins, such as collagen I and III, fibronectin and laminin has been demonstrated as part of the airway remodelling process in patients with COPD (51). Staining for fibronectin was increased in bronchial blood vessels and for laminin in ASM as well as the microvasculature in patients with COPD as compared to controls (51).

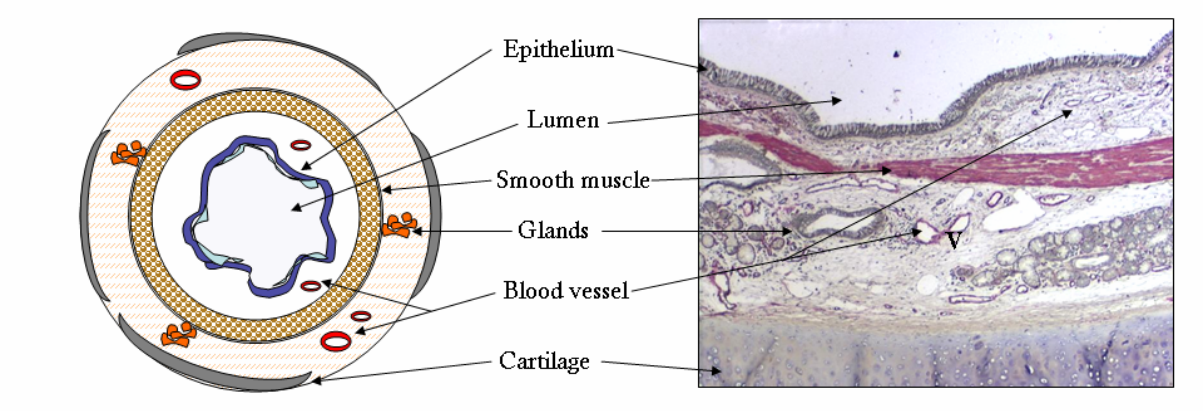

Figure 1.1: Cross-section of normal human airway

Schematic illustration (left panel) and representative photomicrograph (right panel) of a cross-section of normal human airway showing the bronchial epithelium, smooth muscle, glands, microvessels (V) and cartilage.

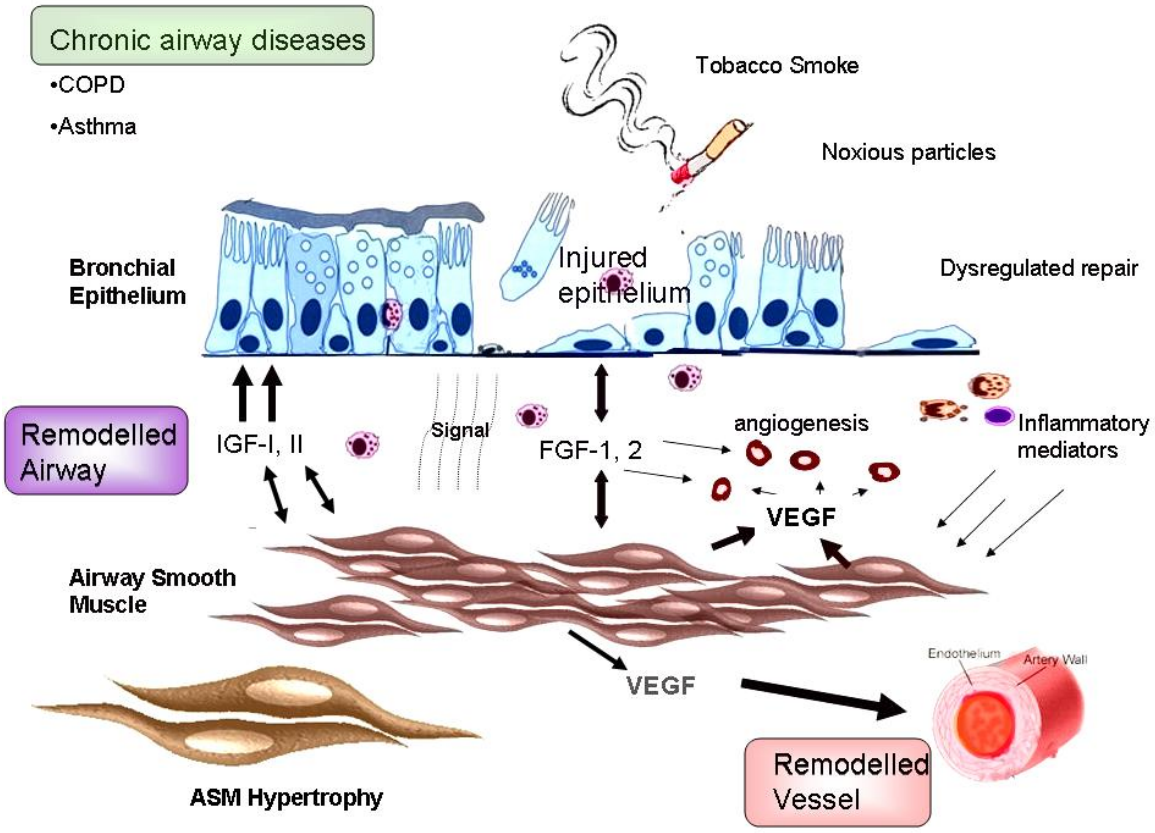

Figure 1.2: Dynamic interacting mechanisms during airway and vascular remodelling in chronic airway diseases

1.5 Bronchial vasculature in chronic airway disease

The lung is unique in its double sources of perfusion from the pulmonary and systemic circulations and it is the only organ to receive the entire cardiac output. The pulmonary vasculature seems to have much more limited capacity for angiogenesis than the bronchial vasculature (11). In regional lung injury, pulmonary blood flow decreases to the injured regions, and anastomotic bronchial blood flow and total bronchial blood flow increase (52). Increased, oxygen-rich bronchial blood perfusing during injury may serve a nutrient rather than a gas-exchange function. Acute bronchial artery dilatation observed with hypoxia is probably not due to release of dilator prostaglandins, and it is likely a direct effect of hypoxic blood on bronchial vascular smooth muscle, similar to the response of other systemic arteries (53). Similarly, the bronchial circulation has angiogenic capacity, as all systemic arteries, whereas the pulmonary circulation seems relatively inert in this regard (11). Although the pulmonary veins provide the common drainage pathway for both the pulmonary and bronchial circulations, little is known about its remodelling capabilities. Given the fact that the pulmonary veins have the capacity to narrow substantially the impact of such remodelling on lung pathology may be quite important (11). In COPD, persistent alveolar hypoxia causes pulmonary vasoconstriction and increased muscularization of small arterial branches (54). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (54). Recent observations indicate that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of $CD8^+$ T-lymphocytes and intimal thickening that correlates with collagen deposition (55, 56).

Qualitative and quantitative changes in airway blood vessel has been reported in asthma (57). Oedematous bronchial mucosa with dilated and congested blood vessels along with increase in the vascular area has been reported in severe asthma (12, 57, 58). Submucosal vascularity was increased in both the medium and small airways in asthmatic patients (59). Evidence suggests that the number and size of bronchial vessels is moderately increased in patients with asthma compared with normal individuals (12, 30, 44, 60). The function of bronchial vessel has been extended beyond traditional view of supplying nutrients, temperature regulation, humidification of airways and being a portal for immune response to that of an important contributor to airflow obstruction and airway remodelling (61, 62).

1.6 Bronchial angiogenesis and vascular remodelling

Angiogenesis and microvascular changes are documented features of chronic inflammatory diseases, including asthma and other airway diseases, of which the molecular mechanisms are not fully understood (63-66). Advances in staining methods and availability of vascular markers have improved the ability to visualize vessels in tissue specimens. The mechanisms and therapeutic implications of alterations in airway blood vessels are just beginning to be elucidated and changes in the microvasculature still represent an important gap in the understanding of the pathophysiology of asthma and other chronic inflammatory airway disease (63). During the progression of chronic airway diseases, 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 by fibrosis, analogous to arteriosclerosis in cardiovascular disease (67, 68). In COPD, persistent alveolar hypoxia causes pulmonary vasoconstriction and increased muscularization of small arterial branches (54). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (54). Patients with moderate to severe COPD display elevated pulmonary vascular pressures during exercise and pathological changes in the pulmonary circulation (35, 69). Wright et al. (69, 70) demonstrated increased wall thickness of small (<500 μm) pulmonary vessels in COPD subjects as compared to non-symptomatic smokers, which was correlated with the severity of the disease (declined FEV_1). Similar findings of vascular abnormalities in COPD have recently been reported with intimal but not medial thickening in mild COPD patients compared to non-smoking controls (55, 71). It has been postulated that emphysema actually may lead to loss of the pulmonary vascular bed and induce angiogenesis (54).

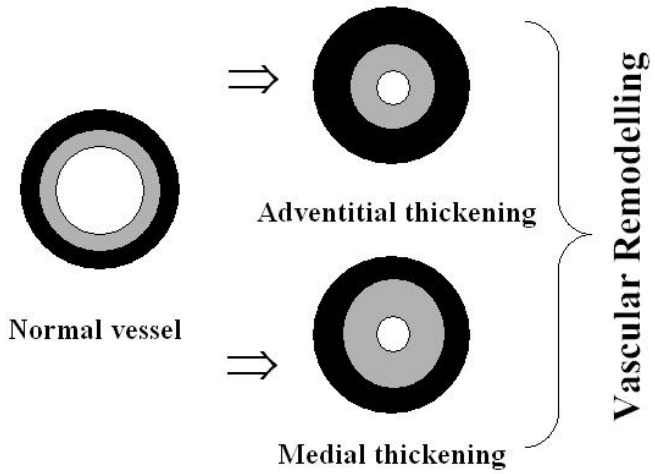

Figure 1.3: Pulmonary vascular remodelling

Vessel remodelling can occur either through adventitial (predominantly through hyperplasia/hypertrophy of fibroblasts) thickening or through the medial (mainly through vascular smooth muscle growth) thickening in different pathophysiological conditions of lungs.

Vascular abnormalities are associated with the development of COPD; conversely, advanced COPD leads to pathological changes in the pulmonary circulation (56, 69). This is likely due, in part, to alveolar hypoxia, which is well known to cause pulmonary vasoconstriction and, if the hypoxic stimulus persists, pulmonary vascular remodelling takes place (54). Several studies have commented on the importance of structural and functional abnormalities in the pulmonary vasculature of COPD patients (55, 56, 71). Hypoxic vasoconstriction is considered to represent one of the major contributing factors of pulmonary hypertension and right-sided heart failure in COPD and other chronic pulmonary diseases (69, 72). In addition, emphysema, accompanied by loss of elastic recoil, increased pulmonary pressure and destruction of part of the pulmonary microvasculature, may contribute to the increased vascular resistance observed in COPD (54, 70). Thus, several phenomena acting in concert in COPD result in pulmonary vascular remodelling. Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD. Figure 1.3 is a diagrammatic representation of two ways (medial or adventitial thickening) through which vascular remodelling could occur during chronic lung diseases.

Changes in vasculature can be due to not only hypoxia or ischemia but also different pathophysiological process such as tissue hypertrophy (increased ASM mass), wound healing (epithelial damage and repair), chronic inflammation and would reflect the environment in which it takes place. The complex molecular events of vascular remodelling could be structured into three processes. The initiation is usually a trigger in the form of hypoxia, ischemia or inflammation. The trigger is sensed by different components of the airway and a relay of signal starts within the cell and to adjacent cells. The signal induced by a variety of stimuli such as pro-inflammatory mediators, stretch, cytokines and growth factors acting in autocrine/paracrine fashion on different cellular components (ASM, VSM and epithelial cells) culminates in synthesis of factors (effectors) that potentially could take part in angiogenesis and vessel remodelling. The effectors that include angiogenic factors, vasoactive substances, growth regulators and matrix modulators influence the growth, death and migration of respective cellular elements taking part in the remodelling process along with extra cellular matrix regulation. Changes in the airway microvasculature have been described in many chronic respiratory diseases (63). In our previous reports, we postulated a role for vascular abnormalities in the pathogenesis of COPD (51, 74). Recent studies highlight distinct but interacting remodelling mechanisms in permutations of airway disease associated with asthma and COPD. Some of these

mechanisms are generously supported by *in vitro*, *in vivo*, and clinical data, whereas others are intriguing but more speculative. Figure 1.4 summarizes the potential scheme of events in angiogenesis and vascular remodelling in chronic respiratory diseases.

Table 1.3: Endogenous regulators of angiogenesis*

Angiogenesis inducers	Angiogenesis inhibitors
Heparin binding peptide growth factors VEGF, PlGF, FGF-1, FGF-2, Pleiotrophin, HIV-tat, PDGF, HGF/SF **Non-heparin binding peptide growth factors** TGF-α,β, EGF, IGF-I **Inflammatory mediators** TNF-α, IL-8, IL-3, Prostaglandin E1, E2 **Enzymes** PD-ECGF/TP, COX-2, Angiogenin **Hormones** Oestrogens, Proliferin **Oligosaccharides** Hyaluronan oligosaccharides, Gangliosides **Haematopoietic factors** Erythropoietin, G-CSF, GM-CSF **Cell adhesion molecules** VCAM-1, E-selectin **Others** Nitric oxide, Ang-1	**Protein fragments** Angiostatin , Endostatin, ααAT (fragment of antithrombin 3), Prolactin **Soluble mediators** TSP-1, Troponin I, IFN-α,γ, PEDF, IP-10, PF-4, IL-12, IL-4, VEGI, TIMP-1, -2, PAI-1, Retinoic acid, Ang-2, 2-methoxyoestradiol **Tumour suppressor genes** P53, VHL

*VEGF: vascular endothelial growth factor; PlGF: Placental growth factor; FGF: fibroblast growth factor; HIV-tat: HIV-transactivating regulatory protein; PDGF: platelet-derived growth factor; HGF/SF: hepatocyte growth factor/scatter factor; TGF: transforming growth factor; EGF: epidermal growth factor; IGF: insulin-like growth factor; TNF: tumour necrosis factor; IL: interleukin; PD-ECGF/TP: platelet-derived endothelial cell growth factor/thymidine phosphorylase; COX: cyclo oxygenase; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; VCAM: vascular cell adhesion molecule; Ang: angiopoietin; TSP-1: thrombospondin-1; IFN: interferon; PEDF: pigment-epithelium derived factor ; IP-10: interferon-gamma-inducible 10 kD protein; PF4: platelet factor 4; VEGI: Vascular endothelial growth inhibitor; TIMP: tissue inhibitor of metalloproteinases; PAI: plasminogen activator inhibitor; VHL: von Hippel-Lindau. *Based on reference (75)*

1.6.1 Mechanisms of angiogenesis

Diseased or injured tissues produce and release angiogenic growth factors (proteins) that diffuse into the nearby tissues and bind to specific receptors located on the endothelial cells (EC) of nearby pre-existing blood vessels. The activated endothelial cells send signals from

the cell's surface to the nucleus. The endothelial cell's machinery begins to produce new molecules including enzymes that dissolve tiny holes in the sheath-like covering (basement membrane) surrounding all existing blood vessels. The endothelial cells begin to divide (proliferate), and they migrate out through the dissolved holes of the existing vessel towards the diseased tissue. Sprouting endothelial cells roll up to form a blood vessel tube. A variety of adhesion molecules help the vessel to sprout forward. Matrix metalloproteinases (MMPs) dissolve the tissue and remoulds it around the vessel. Finally, newly formed blood vessel tubes are stabilized by specialized muscle cells (smooth muscle cells, pericytes) that provide structural support. The switch to the angiogenic phenotype involves a change in the local equilibrium between positive and negative regulators (Table 1.3) of the growth of microvessels.

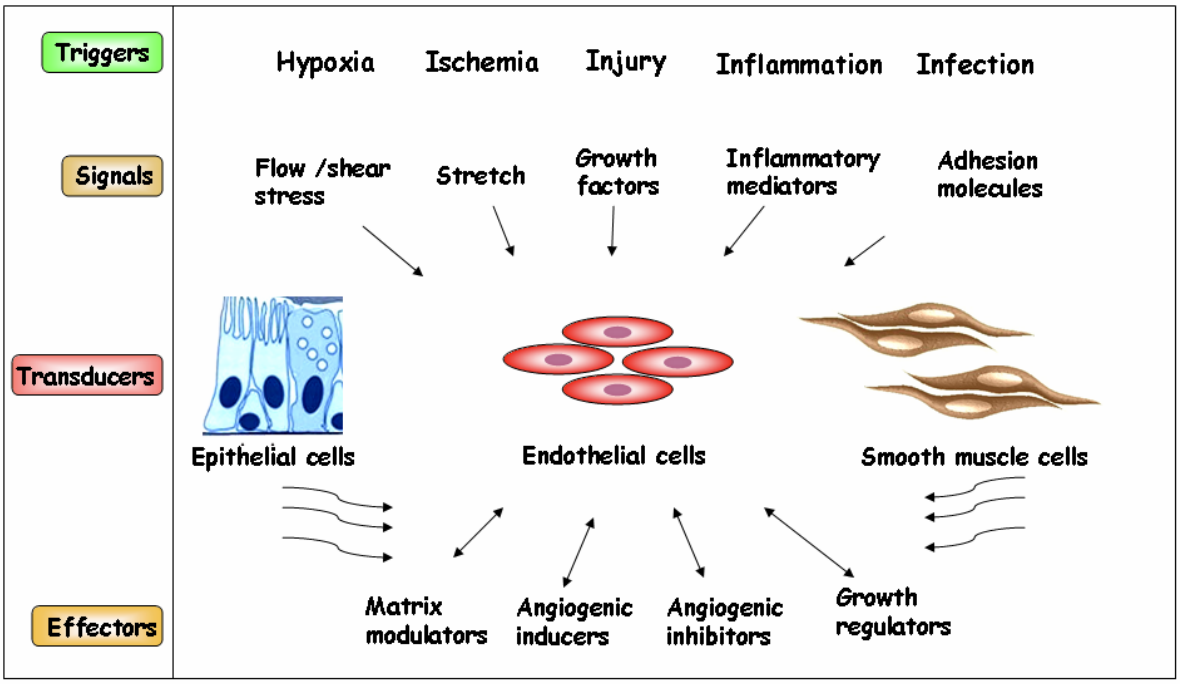

Figure 1.4: Potential scheme of events in angiogenesis and vascular remodelling in chronic respiratory diseases

Note the stages of trigger, signal transmission, activation of endothelial cells and initiation of the angiogenic process. Adapted from reference (73).

1.7 Role of growth factors

Growth factors are small to large proteins that are divided in families based on their structural or functional homologies. The main families include the epidermal growth factor (EGF) family, transforming growth factor (TGF)-β family, fibroblast growth factors (FGFs), insulin-like growth factor (IGF) family, platelet-derived growth factor (PDGF)

family, vascular endothelial growth factor (VEGF) family and hepatocyte growth factor (HGF). Growth factors are involved in a variety of processes including cellular proliferation, differentiation, migration, metabolism and gene expression. In airway epithelium of asthmatics, expression of EGFR but not of other c-erbB members or of EGF family members was enhanced as compared to epithelium from healthy control subjects (76, 77). As only the expression of EGFR was increased in airway epithelial cells in a wound repair model, it has been suggested that EGFR is involved in epithelial repair in asthmatics, while the altered expression also affects airway remodelling in asthma (78). In the airway epithelium of patients with COPD, higher expression of EGFR, heregulin and EGF is reported as compared to control subjects (79) indicating that these EGF-like growth factors may be involved in pulmonary tissue remodelling in COPD.

1.7.1 Growth factors in tissue repair and remodelling

Several lines of evidence confirm the wound repair potential of growth factors, such as the PDGFs, FGFs, EGF, TGF-α, VEGF, PlGF, angiopoietins, IGFs, nerve growth factor (NGF), TGF-β, connective tissue growth factors (CTGF) and other chemokines, cytokines and leptins (77, 80-82). IGF/IGFR-I system and FGF/FGFR-1 system as explored in detail in Chapter 2 and 3 respectively have the potential to enhance tissue repair and remodelling.

Insulin like growth factors

Endocrinologic disturbances and the levels of circulating of IGF-I have been linked with the disease processes, such as airway and vascular remodelling in patients with chronic airway disease (83). IGF-I and IGF-II are single chain polypeptides, which play a pivotal role in regulating cell proliferation, differentiation and apoptosis. The mitogenic effects of IGFs are primarily mediated through the type I IGF receptor (IGFR-I), which is a tetrameric tyrosine kinase receptor composed of two α-chains and two β-chains joined by disulfide linkages (84). Insulin receptor and IGFR-I have 60% homology between them thus enabling the IGFs and insulin to cross-bind to each other's receptor, though weakly (84). IGF-I has been reported to promote growth during post-natal life in various organs while IGF-II stimulates growth mainly pre-natally (85). Recent reports suggest that, precise delivery on the need of the hour, of IGF-I to a site-specific location may hasten, improve and facilitate the tissue recovery after an injury (86).

Fibroblast growth factor system

The FGF family consists of 23 members in man and their functional receptors are designated FGFR1 to FGFR5 (87, 88). These FGFs are mitogenic factors involved in development, tissue homeostasis and repair processes, while FGF-1 and FGF-2 are also angiogenic factors (89). Differences in pulmonary expression of FGF-1, FGF-2 and FGFR1 were found between non-smokers, or smokers with or without COPD (51, 90). With regard to FGF-2, airway expression is higher in bronchial tissue from asthmatics as compared to healthy control subjects (91). In COPD, expression levels of FGF-1, FGF-2 and FGFR1 were observed to be increased in vascular and in epithelial compartments in lungs of patients with COPD as compared to subjects without COPD (74, 92). FGF-1 can stimulate tissue remodelling by increasing collagenase expression and down-regulation of collagen I expression in lung fibroblasts (93). These data support a role for the FGF-FGFR axis in tissue remodelling in COPD.

1.7.2 Angiogenic growth factors

Some of the heparin binding peptide growth factors (VEGFs, FGFs) and non-heparin binding peptide growth factors (TGFs, IGFs) are known angiogenic inducers (Table 1.3). Amongst the above mentioned and known factors, vascular endothelial growth factor (VEGF) family is the most potent and studied, direct angiogenic factor. Moreover, cytokines (IL-6, oncostatin-M) and growth factors (IGF, TGF), which may be called indirect angiogenic factors, take part in angiogenesis via production of VEGF-like molecules.

Vascular endothelial growth factor system

The VEGF family consists of seven members (VEGF-A to VEGF-F and placenta growth factor PlGF), which bind to their respective cellular receptors VEGFR-1 (or Flt-1), VEGFR-2 (or KDR or Flk-1), and VEGFR-3 (or Flt-4). VEGF is predominantly known as a paracrine angiogenic factor stimulating mitogenesis, migration and permeabilisation of the vascular endothelium. Several studies support the idea that VEGF and its receptors contribute to tissue remodelling and disease severity in chronic lung diseases including asthma (92, 94). In patients with COPD, higher pulmonary VEGF expression was found in bronchial and alveolar epithelial and vascular smooth muscle as well as alveolar macrophages, whereas higher VEGFR-1 and VEGFR-2 expression was found in the

endothelium as compared to patients without COPD (74). Over expression of VEGF in mice caused pulmonary emphysema, aberrant structure of capillary endothelium, and pulmonary inflammation with macrophages (95). Also transgenic over expression of PlGF caused apoptosis of pneumocytes, emphysema and a reduced endothelial cell number and VEGF expression (96). Although not conclusive, data from these animal studies further support a role of VEGFs and their receptors in tissue and vascular remodelling as seen in patients with COPD. In addition, subjects with COPD showed an increased pulmonary vascular remodelling and thicker vessel walls (51). In contrast, in subjects with emphysema, pulmonary VEGF and VEGFR2 expression is lower as compared to subjects without COPD. Concomitant with the decreased expression, endothelial cell death and decreased endothelial proliferation occurred (97). Supporting evidence for the role of VEGFR2 in emphysema was provided in an animal model in which VEGFR2 was blocked resulting in pulmonary endothelial cell apoptosis and emphysema (98). Table 1.4 lists the expression patterns of some of the growth factors in COPD.

Table 1.4: Expression of growth factors and cytokines in COPD*

Molecule	*Product*	*Compartment*	*Expression/ Subtype*	*Key references*
EGF	protein	lung tissue	↑ (CB)	Vignola et al. (99)
HER1	protein	lung tissue	↑ (COPD)	de Boer et al. (79)
HER3	protein	lung tissue	↑ (COPD)	O'Donnell et al. (100)
FGF-1	protein	lung tissue	↑ (COPD)	Kranenburg et al. (51, 90)
FGF-2	protein	lung tissue	↑ (COPD)	Kranenburg et al. (51, 90)
FGFR1	protein	lung tissue	↑ (COPD)	Kranenburg et al. (51, 90)
TGFβ1	protein	lung tissue	↑ (COPD)	de Boer (101); Takizawa et al. (102)
	mRNA	lung tissue	↑ (COPD)	de Boer (101);Takizawa et al. (102)
VEGF	protein	lung tissue	↓ (emphys)	Kasahara (97); Santos et al. (103)
	protein	lung tissue	↑ (COPD)	Kranenburg (74); Santos et al. (103)
		sp	↓ (emphys)	Kanazawa et al. (94)
		sp	↑ COPD)	Kanazawa et al. (94)
VEGFR1	protein	lung tissue	↑ (COPD)	Kranenburg et al. (74)
VEGFR2	protein	lung tissue	↓ (emphys)	Kasahara (97)
			↑ (COPD)	Kranenburg et al. (74)

*sp = induced sputum; CB = chronic bronchitis; emphys = severe emphysema; EGF= epidermal growth factor; HER- EGF receptor; HER= EGF receptor; FGF=fibroblast growth factor; TGFb1= transforming growth factor-β1; VEGF=vascular endothelial growth factor; VEGFR= VEGF receptor. *Based on reference (104)*

1.8 Airway smooth muscle and beyond

Airway smooth muscle research has gone a long way now after the acknowledgment that the ASM is not just a structural component in the chronic airway diseases. Traditional view that ASM is a merely passive participant in inflammation and contracts and relaxes in response to inflammatory mediators and bronco dilators respectively has now changed (105). It is now recognized that ASM, the most important cell type in airway remodelling, can be a rich source of biologically active chemokines, cytokines, matrix proteins, matrix metalloproteinases and their tissue inhibitors, adhesion molecules, bronchoprotective factors, surface receptors, lipid mediators, enzymes and growth factors; in short ASM is a biological factory (105-111). Table 1.5 depicts the immunomodulatory molecules expressed by ASM cells in response to a variety of stimuli.

Since the advent of human ASM cells in culture, the synthetic and highly proliferative properties of ASM cells have been discovered as well as the effects of various mitogens and the signal transduction pathways leading to ASM proliferation have been studied intensively (112). Mitogenic effects in ASM cells 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) (112, 113). Table 1.6 summarizes the known ASM cell mitogens.

The synthetic response of ASM cells can vary both qualitatively and quantitatively in health and disease. Moreover, the synthesized contractile, inflammatory, growth and angiogenic mediators are readily available not only to act in a paracrine fashion on the nearby cells but also via an autocrine mechanism could influence further the heterogeneity of ASM phenotype expression (107). Mitzner (114), Seow and Fredberg (115) have discussed the possibility that the potential functions of airway smooth muscle are not essential to normal lung physiology. However, the muscle is influenced by its dynamic microenvironment in the diseased state. Figure 1.5 shows the various phenotypes exhibited by the ASM under the influence of mediators and an altered environment. Moreover, the potential interaction between ASM cells and resident cells and infiltrating inflammatory cells (Figure 1.6) may indeed play a pivotal role in the vicious cycle of disease progression.

Table 1.5: Immunomodulatory molecules expressed by airway smooth muscle cells*

Chemokines	Cytokines	Growth Factors	Surface molecules/ receptors	ECM	Lipid mediators/ enzymes
RANTES IL-8 MCP-1,2,3 Eotaxin	IL-1β, 2, 5, 6, 10, 11, 13 GM-CSF IFN-β LIF NGF	PDGF-BB FGF-1,2 IGF-1 TGF-β_1, β_2 SCF CTGF VEGF	VCAM-1 ICAM-1 $\alpha_9\beta_1$ $\alpha_5\beta_1$ $\alpha_v\beta_6$ CD40, 44, 80, 86 IL-2R,4Rα6R, 12R, 13Rα$_1$, α_2 IFNγR FGF-1/2R VEGF-1/2R	Fibronectin Laminin Pro-collagen Perlecan Tenascin-C MMPs-1,2,3,9,10,12 TIMP-1,2 Betaig-h3 Chondroitin Thrombospondin Verisican Decorin	PGE2 PGD2 PGF2α NOS TxA2

**Adapted from References (105-107, 109, 111)*

Table 1.6: Airway smooth muscle cell mitogens

Growth Factor	Inflammatory mediators: Cytokine /Cell derived	Contractile agonist
Intrinsic protein tyrosine kinase (RTK)	*Cell surface glycoprotein receptors*	*G protein-coupled receptors (GPCRs)*
PI3K, ERK, protein kinase C- dependant and reactive oxygen-dependent pathways		
Epidermal Growth factor Insulin-like growth factors Platelet-derived growth factor Basic fibroblast growth factor	Interleukin-1β Tumour necrosis factor-α Lysosomal hydrolases (β-hexosaminidases and β-glucuronidase) α-thrombin tryptase sphingosine 1-phosphate	histamine endothelin-1 Substance P phenylephrine serotonin thromboxane A_2 leukotriene D4 Mechanical Stretch

1.8.1 Cellular crosstalk with ASM

It is now well established that the ASM cells interact with both infiltrating cells such as mast cells, neutrophils, eosinophils, macrophages and resident cells such as fibroblasts, epithelial cells and endothelial cells (Figure 1.6). ASM cells have been shown to secrete regulated on activation, normal T cells expressed and secreted (RANTES), eotaxin, IL-8, monocyte chemotactic protein (MCP) 1, MCP-2, MCP-3, thymus and activation-regulated

chemokine, and GM-CSF (105, 108). In addition to secreting chemokines that recruit leukocytes into the airways, ASM promote leukocyte retention and activation through the expression of cell adhesion molecules (108). Thus, the intertwined relationship between the infiltrating cells and ASM cells helps to perpetuate the inflammatory processes.

Evidences propose the cross talk between the damaged/activated epithelial cells and ASM cells in diseases such as asthma and COPD (35, 42). We hypothesized that growth factors such as FGF and IGF expressed by ASM cells (90, 116, 117) most likely in response to epithelial injury would enhance the repair potential of the self-healing epithelium. *In vivo* and *in vitro* data indicate that smooth muscle cells, and their cross-talk with myo-fibroblasts and inflammatory cells via growth factors and cytokines, are major actors in airway remodelling due to a variety of pathophysiological conditions (90, 93, 118-120).

Attention has also been given recently to the bidirectional relationship between ASM cells and the ECM (121). In addition to increased synthesis of ECM proteins, ASM cells can be involved in down regulation of matrix metalloproteinases (MMPs) and up regulation of tissue inhibitors of metalloproteinases (TIMPs) thus eventually contributing to the alteration in ECM. In turn, ECM proteins promote the survival, proliferation, cytokine synthesis, migration and contraction of human airway smooth muscle cells. Thus, the intertwined relationship of ASM and ECM and their response to stimuli like chronic inflammation in diseases like asthma and COPD contribute to the remodelling seen in airways of patients with these diseases (121).

More recently, ASM cells and its interaction with endothelial cells in the perspective of angiogenesis in chronic respiratory diseases have come under limelight. We (122) and others (123-125) have shown that sensitized ASM cells secrete VEGF which is readily available for acting on neighbouring endothelial cells. Enhanced expression of VEGF in ASM and VSM has been reported in diseases such as COPD (74, 103), as investigated in Chapter 4 suggesting that ASM cells have an important role in vascular remodelling and angiogenesis in chronic airway diseases. Thus, it is more likely that the active contribution of these cells (ASM) make to the development and perpetuation of chronic airway diseases may be in fact greater than what it has been recognised. Several *in vitro* models have paved the way to decipher these molecular interactions.

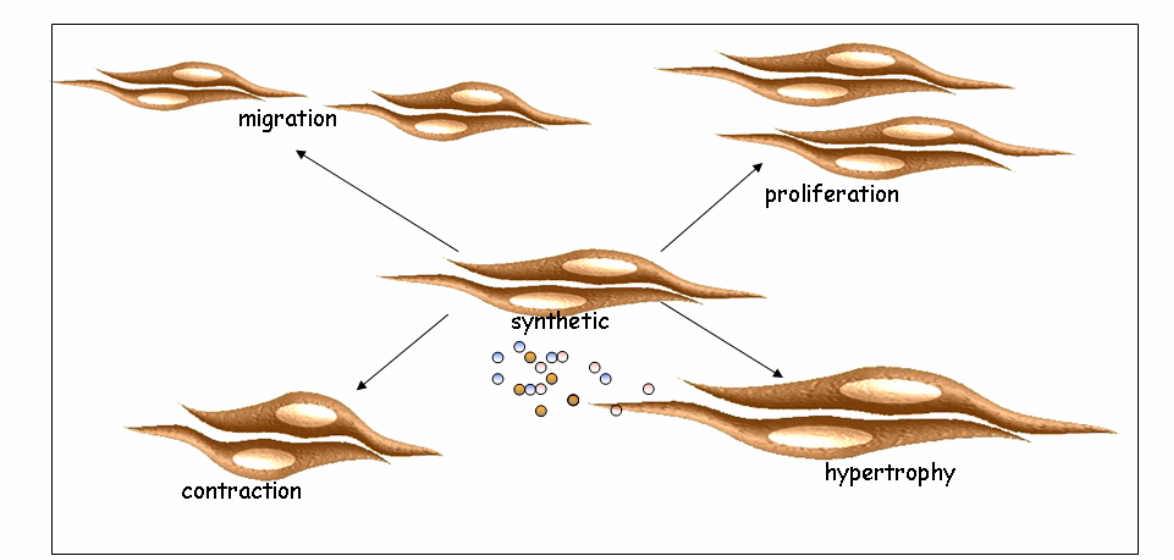

Figure 1.5: Multifaceted airway smooth muscle cell

Upon the influence of variety of mediators the airway smooth muscle cell exhibit versatile phenotypes such as proliferative, migratory, contractile and synthetic phenotype.

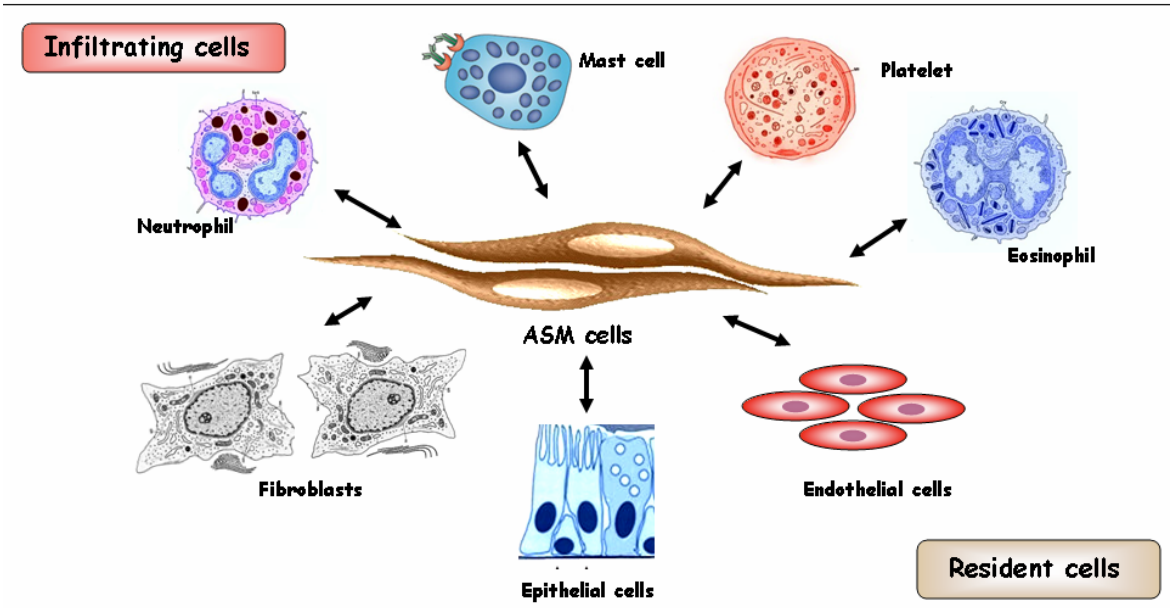

Figure 1.6: Cellular crosstalk between airway smooth muscle cells with infiltrating cells and other resident cells in the airways

1.9 In vitro models of bronchial and vascular remodelling

Recent advances in the field of molecular and cellular biology have enabled us to develop methodologies on which cells could be cultured and then manipulated to express phenotypic surface markers, to produce immunomodulatory cytokines and represent the molecular events in a given situation. Excellent models (22, 126, 127) have been developed over the years which are simple and reproducible; important tools to reveal the molecular events and to deduce mechanisms of action. Research has now massively adopted the low-cost, *in vitro*, molecular biology techniques in lieu of the idiosyncratic and expensive *in vivo* models. Currently, *in vitro* models and experiments are a vital and highly productive research tool.

1.9.1 Epithelial wound/repair model

Three different wound models have been described: (A) straight mechanical scratch model, (B) crosshatch model and (C) circular wound model (Figure 1.7). The mechanical scratch model and crosshatch model involved scraping the cells using a pipette tip. However, taking into consideration the possible damage caused to the plate surface by the plastic pipette tips in the other models and the precision of taking images of the same wound area the circular wound repair model as reported previously has been explored in this thesis (126). Images were collected using a digital camera after regular time intervals and analyzed using ImageJ 1.32j software (National institutes of health, Bethesda, USA) for percentage wound closure at various time points. This model represents a simple over view of how the repair process eventually takes place in a cell system with relevant factors and inhibitors.

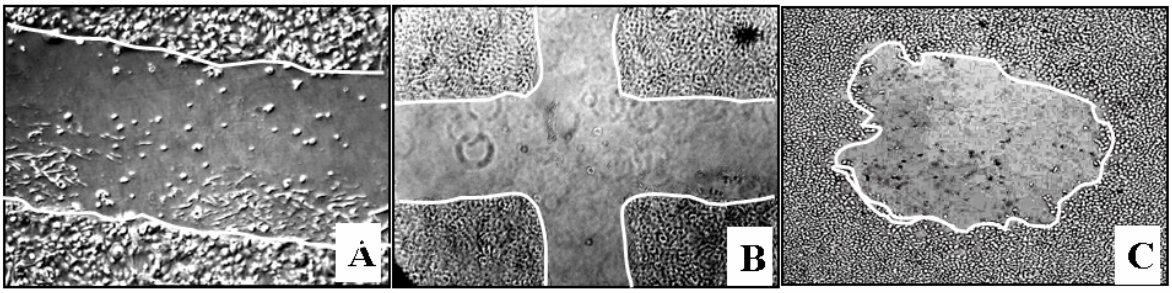

Figure 1.7: Examples of different in vitro wound repair models

Representative examples of different wound repair models experimented. Panel A represents a straight mechanical scratch model. Original magnification: x20. Panel B represents a crosshatch model, Original magnification: x10 and Panel C represents the circular wound model, Original magnification: x4.

1.9.2 Human ASM and endothelial cell based model of angiogenesis

Airway smooth muscle cells in culture have been used as a successful model over the past few years. They have provided new insights into the nature of smooth muscle function in disease and led to the recognition of multiple phenotypes: contractile, proliferative and synthetic (128). We have extended this model further in order to explore the angiogenic potential of airway smooth muscle derived soluble factors. Isolated and cultured primary human ASM cells and pulmonary artery endothelial cells have been characterized morphologically (hill and valley appearance: ASM, cobblestone appearance: endothelial cells) and immunohistochemically with respective specific markers for purity. The conditioned media from stimulated or sensitised ASM cells, routinely collected and stored appropriately (-20 ̊C), were then used to stimulate the endothelial cells for different time and experimental conditions. The proliferative potential of the angiogenic molecules secreted in the conditioned medium by the sensitized ASM cells were assessed and was used as an indicator for angiogenesis (129, 130). Respective inhibitors of the ASM derived factors confirm their angiogenic potential.

1.9.3 In vitro cyclical stretch model

ASM cells, *in vivo*, undergo cycles of stretching and shortening during respiration. However, in disease conditions like asthma and COPD due to airway narrowing and airflow limitation there is enhance stretch and a state of hypercontractility. Stretching cells in culture simulates the *in vivo* environment causing dramatic morphologic and biochemical responses. Many *in vitro* stretch models, using rat bronchial ring (131), bovine tracheas (132) or human ASM cells (127, 133-135), have been developed in order to mimic this pathophysiologic state. This model applies a defined, controlled, static or cyclic deformation to cultured cells. The pattern of cyclical stretch and the strength of the applied strain could be varied and fine-tuned in this model according to the disease condition using a computer-controlled vacuum. Human ASM cells are plated onto type I collagen-coated wells made up of a flexible silastic membrane bottom. The cells are subjected to cyclic strain at 1 Hz for different time intervals, using the Flexcer cell unit, FX 2000); Figure 1.8. Depending upon the culture plates the mechanical stretch may be uniaxial or biaxial (radial and circumferential)**.**

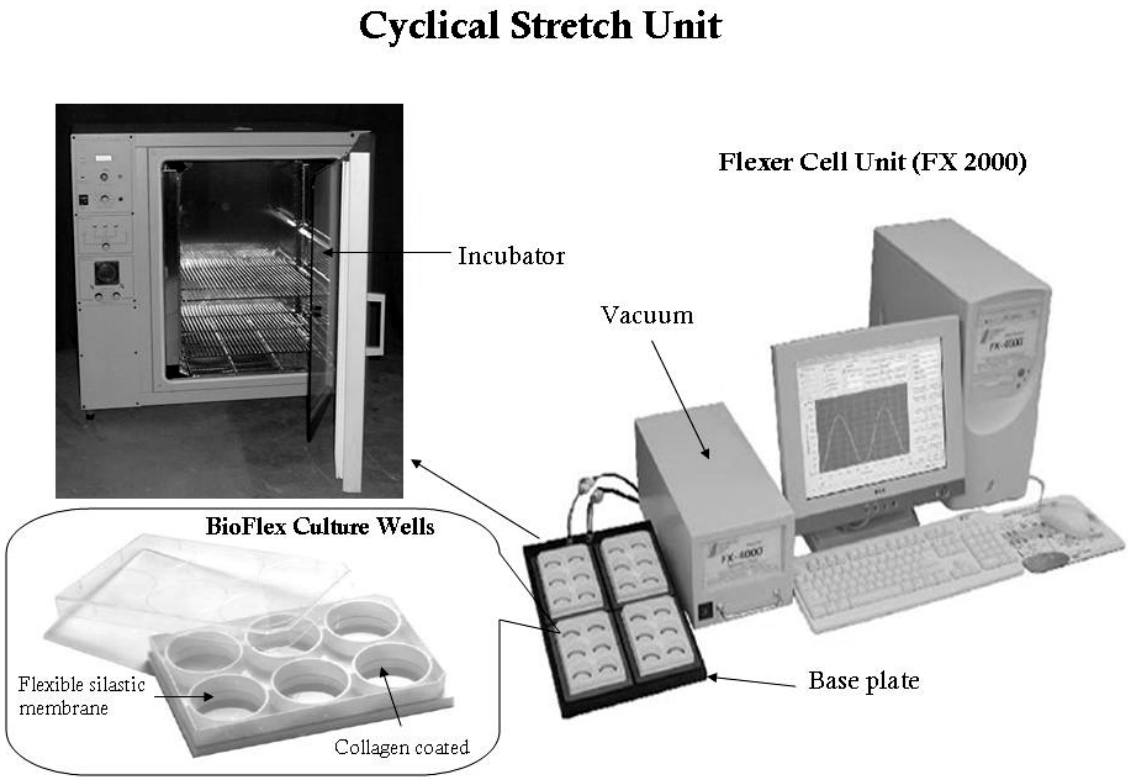

Figure 1.8: Mechanical stretch apparatus

Representative photograph of the mechanical stretch apparatus; Flexer cell unit (FX 2000) with attached base plate and a computer controlled vacuum system. Zoomed panel below shows the "BioFlex" 6 well, cell culture plate, coated with collagen-I and having a flexible bottom made up of silastic membrane, placed on the base plate inside the incubator under experimental conditions.

The available evidence (131, 136, 137) suggests that upon *in vitro* mechanical stretch, ASM cells respond with increased proliferation, cell reorientation, protein production, reorientation of stress fibres and hence contribute to airway remodelling. In this thesis, we have focused on the effect of cyclical mechanical stretch on angiogenic molecule secretion by the ASM cells *in vitro*.

1.10 Aims of the thesis

Increasingly, it has become clear that ASM cells are not only structural and contractile components of airways, rather they can express and release a large number of pro-inflammatory and mitogenic factors (e.g. IL-1β, TNF-α, IL-6, IL-8, TGF-β1, FGF, PDGF and VEGF) that may play a pivotal role in regulating the airway and vascular remodelling. Thus we hypothesized that proliferating ASM cells produce angiogenic factors upon sensitization with mediators of Asthma and COPD and that the up-regulated

pulmonary expression of various growth factors could contribute via autocrine/paracrine pathways to epithelial repair, bronchial angiogenesis and vascular remodelling during chronic lung diseases. The central aim of the thesis is to investigate the molecular mechanisms involved in bronchial and vascular remodelling in chronic airway diseases. Employing the techniques of cell culture, immunohistochemistry, pharmacology and molecular biology, in this thesis we explored the following questions:

1. Do IGF-I/IGFR-I and FGF/FGFR-1 systems play a pivotal role in epithelial repair in COPD (Chapter 2 and 3)?

2. What is the expression pattern of various angiogenic molecules (VEGF, Flt-1 and KDR/Flk-1) in COPD and other chronic airway diseases (Chapter 4, 5)?

3. Do ASM cells express and release angiogenic molecule, VEGF in response to pro-inflammatory cytokines (IL-1β and TNF-α; Chapter 6), vasoactive peptides (ANG-II and ET-1; Chapter 7) and if the secreted VEGF is biologically active as assessed by in vitro angiogenesis?

4. Does nitric oxide play a role in pro-inflammatory cytokine (IL-1β) induced VEGF synthesis by ASM cells (Chapter 8)?

5. Whether cyclic mechanical stretch leads to the expression and secretion of different pro-angiogenic molecules in human ASM cells (Chapter 9)?

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Part 1

Bronchial Remodelling

2

Role of Insulin-Like Growth Factor System in Airway Epithelial Repair

Based on: "Alagappan VK, Willems-Widyastuti A, Kranenburg AR, Verhoosel RM, de Boer WI, Hiemstra PS and Sharma HS. Bronchial Expression of Insulin-Like Growth Factor-I and IGFR-1 in COPD: Role in Epithelial Repair" – Submitted.

Chapter 2: Role of Insulin-Like Growth Factor-I and IGFR-I in Airway Epithelial Repair

Summary
Airway epithelial injury and repair and associated remodelling processes are common features in chronic obstructive pulmonary disease (COPD). Insulin-like growth factors (IGF-I and II), exerting their mitogenic action primarily via IGF-I receptor (IGFR-I), could play a role in bronchial re-epithelialisation and airway remodelling. We investigated the bronchial expression pattern of IGF-I and IGFR-I in COPD patients in relation to lung function data and further assessed the mitogenic potential of IGFs in an *in vitro* wound injury repair model. Serial sections of lung tissue obtained from COPD (FEV1 <75%; n=14) and non-COPD (FEV1 >85%; n=15) patients were immunohistochemically stained for IGF-I and IGFR-I and the expression levels were correlated with lung function data. [^{3}H]thymidine incorporation and cell counts assessed the mitogenic effects of IGFs, whereas mechanically injured confluent monolayer of NCI-H292 cells constituted the *in vitro* wound repair model. Enhanced expression of IGF-I was localized in airway (ASM, $p \leq 0.001$) and vascular smooth muscle (VSM, $p \leq 0.02$) cells in patients with COPD as compared to controls and inversely correlated with respective lung function data (FEV1/FVC). IGFR-I was localized in epithelial cells and its expression levels remained unaltered between two groups. IGF-I and IGF-II induced cell proliferation (2-5 folds) and growth (2-4 folds) of ASM as well as NCI-H292 cells. Repair experiments demonstrated accelerated wound closure after 72 h of IGFs treatment. Taken together, our results suggest that IGF-I/IGFR-I system play a pivotal role in epithelial repair processes and hence contribute to the airway remodelling in COPD.

2.1 Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by reduced expiratory flow and airway inflammation. This airflow limitation may be irreversible due to the narrowed airway lumen and alveolar destruction, while the release of multiple mediators and increased number of alveolar macrophages and neutrophils contribute to the airway inflammation. Inflammation in COPD could further be perpetuated by the presence of high level of oxidative stress due to cigarette smoke which is an important etiological factor (1). The bronchial epithelium and airway smooth muscle are two major cell types involved in the airway remodelling (2). Cellular events in these airways reveal selective bronchial epithelial cell damage and intense inflammation (3). Studies have shown that repetitive injury and repair of the bronchial epithelium involves cell proliferation and migration that lead to the tissue remodelling and subsequent irreversible changes in the airways (4-7). The repair of the damaged epithelium is crucial for restoration of barrier function and reduction of inflammation of which the molecular mechanisms are poorly understood. A variety of

growth factors including insulin like growth factors (IGFs) could play a role in bronchial re-epithelialisation and airway remodelling during chronic airway diseases (8, 9).

Both IGF-I and IGF-II are single chain polypeptides, which play a pivotal role in regulating cell proliferation, differentiation and apoptosis. The mitogenic effects of IGFs are primarily mediated through the type I IGF receptor (IGFR-I), which is a tetrameric tyrosine kinase receptor composed of two α-chains and two β-chains joined by disulfide linkages (10). Insulin receptor and IGFR-I have 60% homology between them thus enabling the IGFs and insulin to cross-bind to each other's receptor, though weakly (10). Endocrinologic disturbances and the levels of circulating of IGF-I have been linked with the disease processes like airway and vascular remodelling in patients with chronic airway disease (11). In order to assess the role of IGFs/IGFR-I system in the pathogenesis of COPD, we examined the expression pattern of IGF-I and IGFR-I in lung tissue specimens obtained from patients with or without COPD and investigated the mitogenic effects of both IGF-I and IGF-II on cultured human airway epithelial and smooth muscle cells. In addition, we examined the wound repair potential of IGFs in a cultured airway epithelial cell model.

2.2 Materials and methods

2.2.1 Selection of specimens

The lung tissue specimens were taken from the bronchial airways, at a distance from the tumour in patients with or without COPD. Based on lung function data (Table 2.1), subjects were assigned to the COPD (n=14) and non-COPD (15) groups (12, 13). All lung tissues were fixed in 10% neutral buffered formalin for approximately 24 h after which the tissues were further processed for embedding in paraffin and immunohistochemical staining. The patients in these two groups participated in a larger research project, part of which has been published previously (14, 15).

All patients were free of symptoms of upper respiratory tract infection and they did not receive antibiotics preoperatively. Except three in the COPD group, patients did not receive glucocorticosteroids in the period of three months prior to operation. All selected lung tissues were checked histologically using the following exclusion criteria: (i) presence of tumour in the lung tissue specimen submitted for the study, (ii) presence of poststenotic pneumonia in the specimen, (iii) fibrosis of lung parenchyma, and (iv) obstruction of the

main bronchus of the resection specimen by tumour, regardless of the histology of the specimen intended for this study (12, 13).

2.2.2 Pulmonary Function Tests

All pulmonary function tests were performed within 3 months prior to surgery. FEV_1 and forced vital capacity (FVC) were measured by spirometry, total lung capacity and residual volume with the closed circuit helium dilution test and the K_{co} using the single breath-holding technique, as described by Quanjer and co-workers (16).

Table 2:1 Parameters used to classify subjects as COPD and Non-COPD

Criteria	COPD	NON-COPD
FEV_1	<75%	>85%
FEV_1/FVC ratio	<75%,	>85%
Reversibility of FEV_1	<12%	<12%
Kco	<80%	>80%

FEV1: Forced one-second expiratory volume calculated as % of predicted value before bronchodilatation, FEV1/Forced Vital Capacity (FVC) ratio <75%, reversibility in FEV1 calculated as % of predicted after 400 µg inhaled salbutamol (12, 13), and Kco is transfer factor for carbon monoxide (diffusion capacity) per litre alveolar volume calculated as % of predicted value. In order to exclude accompanying lung disease leading to a restrictive function disorder, it was required that the total lung capacity (TLC) of each subject included in the study was over 80% of the predicted value (16).

2.2.3 Immunohistochemistry

Sections of paraffin-embedded lung tissue were cut at 4 µm, mounted on Super Frost Plus® microscopic slides (Meinzl-Gläser, Braunschweig, Germany) and processed for immunohistochemistry. Serial sections were used to detect the expression of IGF-I and IGFR-I employing immunohistochemistry. Sections were deparaffinised and rehydrated prior to incubation with specific purified mouse monoclonal antibodies raised against human IGF-I and IGFR-I (Neomarkers, Fremont, CA, USA), as described previously (17). The optimal dilutions for all antibodies were identified by examining the intensity of staining obtained with a series of dilutions: the optimum concentration resulted in specific and easily visible signal on paraffin sections of control specimens. To block non-specific second antibody binding, sections were pre-incubated with 5% bovine serum albumin in phosphate

buffered saline (5% BSA/PBS, pH = 7.4). Subsequently, sections were incubated overnight at 4 °C with primary antibodies diluted appropriately (IGF-I, 1:50 and IGFR-I, 1:1000). IGF-I immunostaining was performed after antigen retrieval by boiling in citrate buffer (10 mM citrate buffer, pH = 6.0) for 10 min in a microwave oven while IGFR-I was done using pronase pretreatment for 10 min. Incubation for 30 min with post antibody blocking for power vision plus (Immunovision technologies, The Netherlands) and 30 min with poly HRP-GAM/ R IgG (Immunovision technologies, The Netherlands) were used to enhance the detection sensitivity. Colour was developed by staining with 3,3-diaminobenzidine (Sigma, St Louis, USA) dissolved in PBS containing 0.03% H_2O_2. Sections were subsequently, counter-stained with Mayer's haematoxylin. Slides were mounted and studied light-microscopically. Negative controls consisted of omission of the primary antibody.

2.2.4 Quantification of staining

To assess the expression levels of IGF-I and IGFR-I, a visual scoring method on immunostained tissue sections was applied. Prior to screening, sections were coded so that the observers were unaware of the clinical details of the case under study. Expression of IGF-I and IGFR-I was analyzed semi-quantitatively, using a visual scoring method with grades ranging from 0 to 3 (0 = no staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining) as previously described (12, 18, 19). The entire slide of a tissue block was investigated and scored at the same magnification. The staining intensity of IGF-I and IGFR-I was scored by three independent observers in bronchial airways as well as alveolar parenchyma in epithelial, smooth muscle cells and in macrophages. Inter observer errors were tested by correlating the expression scores using Pearson's analysis and found a high correlation ranging from 0.7 to 1. In the bronchial airways staining for IGF-I and IGFR-I was assessed in the bronchial epithelium, airway smooth muscle (ASM) and vascular smooth muscle cells and macrophages in the bronchial airway wall. Similarly, in peripheral lung tissues the staining of IGF-I and IGF-I receptor was analyzed in bronchiolar an alveolar epithelium, bronchiolar ASM and VSM cells, and alveolar macrophages.

2.2.5 Isolation and culture of human airway smooth muscle cells

Human airway smooth muscle cells were isolated and cultured as described previously (20, 21). Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus. After removal of the epithelium, parts of smooth muscle was

dissected free of adherent connective and peripheral lung tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (HBSS; Life Technologies BV, Breda, The Netherlands) containing bovine serum albumin (BSA, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (3.3 U/ml; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) at 37°C in a humidified incubator containing 5% CO_2 / 95% air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies BV, Breda, The Netherlands) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Bio-Whithaker BV, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml) (Life Technologies BV, Breda, The Netherlands). Cells were subsequently seeded at $2x10^5$ cells per 35 mm dish and maintained in culture by replacing the medium every 48 h. After 10-14 days in culture, ASM cells grew to confluence and were then detached by trypsinization (0.5% trypsin; 0.02% EDTA; Life Technologies BV, Breda, The Netherlands) and subcultured into 25 cm^2 and 75 cm^2 tissue culture flasks. Immunocytochemical staining of confluent serum-deprived primary cultures of human ASM cells, using monoclonal antibodies to smooth muscle α-actin and smooth muscle-myosin heavy chain (SM1 and SM2) (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) (20, 21), demonstrated that the cultures were essentially free (>95%) of other contaminating cell types.

2.2.6 Culture of NCI-H292 cells

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were cultured in RPMI 1640 medium containing 10% foetal bovine serum (FBS; Bio-Whitaker BV, Verviers, Belgium) supplemented with sodium pyruvate (2 mM), gentamicin (50 μg/ml), L-glutamine (4 mM), HEPES (10 μM), penicillin (5 U/ml), streptomycin (5 μg/ml) at 37 °C in a 5% CO_2-humidified atmosphere. Cells were maintained in culture by replacing the medium every 48 h and when confluent were further passaged using trypsin/EDTA solutions into 75cm^2 tissue culture flasks. Near confluent cells were used for all the experiments.

2.2.7 Proliferation assays

[^{3}H]thymidine uptake: Serum deprived NCI-H292 and ASM cells (quadruplo in 96 wells plates) were incubated with 100 μl serum-free medium alone (negative control) or

supplemented with 10% FBS (positive control), 5 ng/ml human recombinant IGF-I, which has been reported to induce near maximal proliferation in NCI-H292 cells (4), or 5 ng/ml IGF-II. The NCI-H292 cells were stimulated for 8, 16 and 24 h while the ASM cells were stimulated for 8, 24 and 48 h. Five hours prior to the end of the treatment 10 µl of [^{3}H]thymidine (1 µCi/10 µl in HBSS; Amersham, Roosendaal, the Netherlands) was added to the wells, at a final concentration of 1 µCi/110 µl per well. The medium was removed after the time points and the cells were washed twice with cold PBS. The cells were detached with 50 µl trypsin for 10 min after which 50 µl PBS was added. Cells were frozen overnight at –20°C and subsequently harvested on glass fibre filters using a Filtermate 196 cell harvester (Packard, Meridan, USA). Activity was counted using a Microplate Scintillation ß-counter (Topcount, Packard, Meridan, USA) and measured radioactivity was expressed as counts per min (CPM). The mean CPM of quadruple wells was expressed as ratio as compared to control cells in serum free medium (fold-induction).

Cell counts: In a parallel series of experiments, 200.000 cells were seeded in 24 well plates. Serum deprived NCI-H292 and ASM cells (in quadruplo) were stimulated for 24 and 48 h with IGF-I, or IGF-II or controls and cell numbers were determined using automated cell counting (Casy®1, Schärfe system GmbH, Reutingen, Germany). After stimulation, the cells were trypsinized with 100 µl Trypsin/EDTA for 10 min. Cells in suspension were added to 10 ml of Casy®1 isotonic solution (6.38 g/l NaCl, 0.2 g/l Na-tetraborate, 1.0 g/l Boric acid and 0.2 g/l EDTA). Cell numbers, volumes and diameters were measured and analyzed using Casey®1 system software.

2.2.8 Epithelial injury and repair model

Circular wound repair model as described previously (22) was employed where three circular wounds (3 mm in diameter) were scraped in confluent monolayer of overnight serum deprived NCI-H292 cells. The wounded monolayers were incubated in serum-free medium alone or supplemented with IGF-I or IGF-II (5 or 10 ng/ml) or 10%FBS (positive control). Images were collected using a digital camera after 0, 24, 48 and 72 h and analyzed using ImageJ 1.32j software (National Institutes of Health, USA) by determining the percentage wound closure as compared with the time point of starting the stimulation (t = 0, Figure 2.1).

2.2.9 Statistical analysis

Data were statistically analysed using the unpaired, two-tailed Students' t-test as well as the non-parametric Mann-Whitney test when the distribution was not normal. Data were expressed as mean±SEM. Differences with $p \leq 0.05$ were considered statistically significant. Spearman's correlation between FEV_1/FVC values and expression of IGF-I and IGFR-I was analyzed using the SPSS 11.0 statistical package.

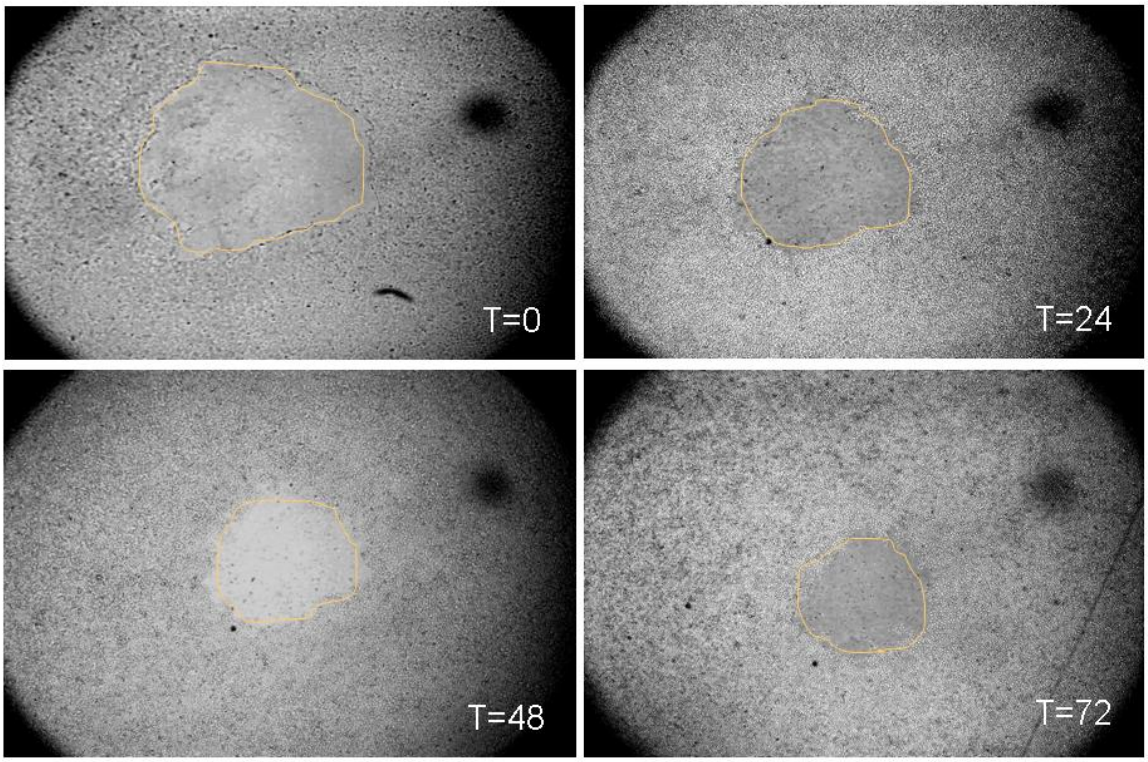

Figure 2.1: Measurement of wound closure using image analysis software

Representative example of the images taken of the wounded epithelial monolayer (NCI-H292) at different time points (t=0, 24, 48, 72 h). ImageJ 1.32j software (National institutes of health, USA) was used to analyze the wound repair potential of the growth factors by calculating the remaining wound area at different time points and comparing with the wound area at t=0.

2.3 Results

2.3.1 Clinical parameters

The clinical and lung function characteristics of all subjects included in the study are listed in Table 2.2. All patients included in this study participated in a larger project (14). The COPD group demonstrated lower FEV_1 and FEV_1/FVC values, ($p \leq 0.001$), whereas there was no significant difference between the two groups in age and smoking status (pack-years).

Table 2:2 A summary of the clinical characteristics of subjects with and without chronic obstructive pulmonary disease

Group	Sex (M/F)	Age	PY	FEV_1 (% Pred)	FEV_1/FVC (%)	Steroid treatment
Non-COPD	12/3	59.3±14.8	34.2 ±24.7	97 ± 6.9	100 ±8.64	None
COPD	13/1	63.5±8.8	39.7±14.5	57± 13.2	62.4 ± 8.75	3
p-value		0.5	0.5	≤0.001	≤0.001	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; Forced expiratory volume in 1 s (FEV1) and Forced vital capacity (FVC) are given as percentages of the predicted values (% Pred.) before bronchodilatation. M = Male; F = Female. PY = number of pack years. Data shown represent means ± SD. The patients in these two groups participated in a larger project, part of which has been published previously (14, 23).

2.3.2 Expression of IGF-I and IGFR-I

Representative examples of IGF-I and IGFR-I staining in non-COPD (Panel A, C) and COPD (Panel B, D) specimens are depicted in Figure 2.2 and 2.3 respectively. Immunoreactive IGF-I was localized (brown colour; DAB) in bronchial ASM and in VSM cells of the vessel wall and its level were significantly enhanced (Figure 2.4; Panels A and B) in COPD as compared to the non-COPD patients. Unpaired two tailed t test (ASM staining scores: 1.38±0.25 vs. 0.39±0.13, $p \leq 0.001$ and VSM staining scores: 1.08 ±0.25 vs. 0.40±0.12 $p \leq 0.02$) as well as non-parametric Mann-Whitney (ASM: $p \leq 0.001$ and VSM: $p \leq 0.01$) revealed significant levels for difference in staining scores of IGF-I respectively. Furthermore, IGF-I was localized in peripheral sections in ASM, VSM and in inflammatory cells (Figure 2.2; Panels C and D) though there was no significant difference in the expression pattern between the groups.

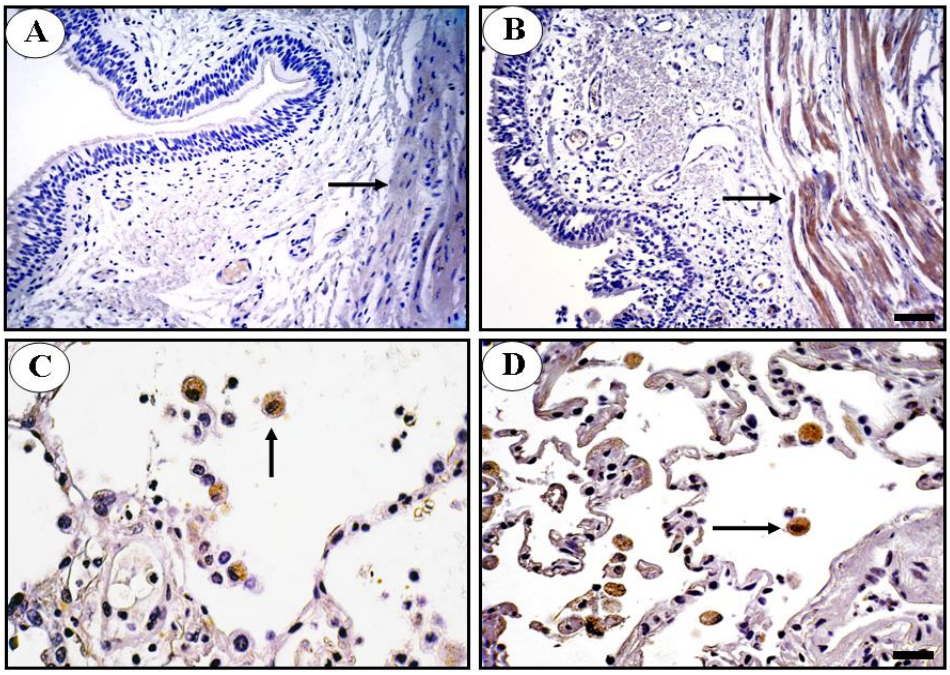

Figure 2.2: Expression of IGF-I in central and peripheral lung sections

Photomicrographs of lung tissue sections from patients without COPD (A and C) and with COPD (B and D). Panel A and B represents IGF-I staining in airway smooth muscle (ASM) in central lung sections, original magnification: x200. Panel C and D is represents IGF-I staining in macrophages in peripheral lung sections, original magnification: x400. Colour is developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown colour) and counterstained with Mayer's haematoxylin. Arrows indicate sites of positivity for IGF-I. Scale bar = 50 μm.

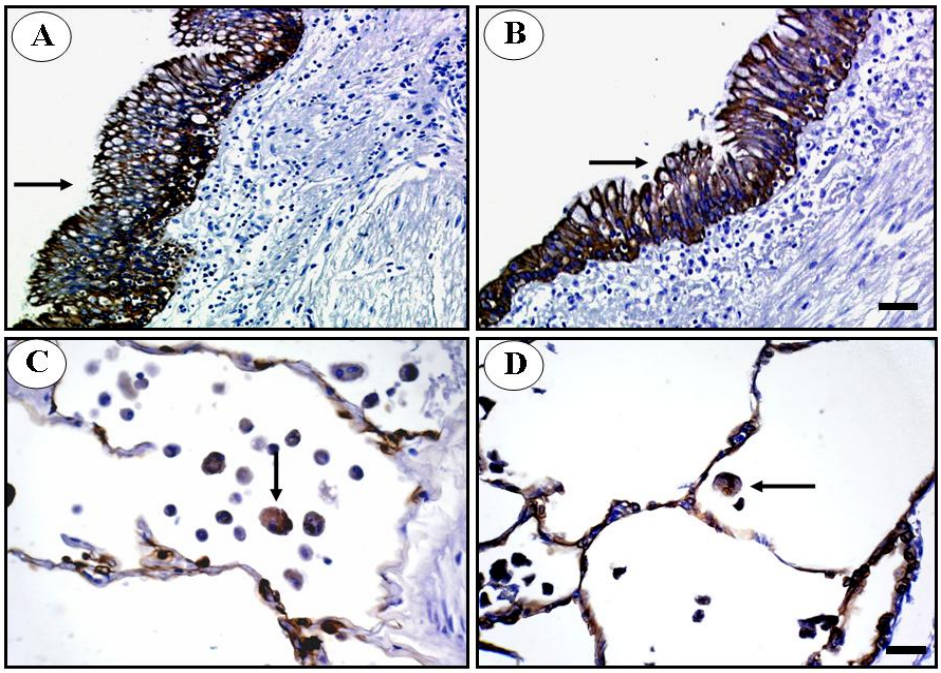

Figure 2.3: Expression of IGFR-I in central and peripheral lung sections

Photomicrographs of lung tissue sections from patients without COPD (A and C) and with COPD (B and D). Panel A and B represents IGFR-I staining (brown) in bronchial epithelium in central lung sections, original magnification: x200. Panel C and D represents IGFR-1 staining in macrophages in peripheral lung sections, original magnification: x400. Scale bar = 50 μm.

Table 2:3 Comparing means: Independent Samples Test

		Sig. (p value)	Mean Difference	Std. Error Difference	95% Confidence Interval	
					Lower	Upper
IGF-I /ASM	Equal variances assumed	000	1.10	0.26	0.57	1.63
	Equal variances not assumed	0.001	1.10	0.27	0.54	1.66
IGF-I/VSM	Equal variances assumed	0.005	0.79	0.26	0.26	1.32
	Equal variances not assumed	0.009	0.79	0.26	0.23	1.35
IGFR-I/BE	Equal variances assumed	0.990	000	0.21	-0.44	0.43
	Equal variances not assumed	0.990	000	0.21	-0.44	0.43
IGFR-I/IC	Equal variances assumed	0.034	0.63	0.28	0.05	1.21
	Equal variances not assumed	0.047	0.63	0.29	0.01	1.25

Table depicts the mean difference between staining scores between the two groups (COPD and non-COPD) in bronchial airway sections. Definition of abbreviations: IGF-I/ASM: IGF-I in airway smooth muscle; IGF-I/VSM: IGF-I in vascular smooth muscle; IGFR-I/BE: IGFR-I in bronchial epithelium; IGFR-I/IC: IGFR-I in inflammatory cells.

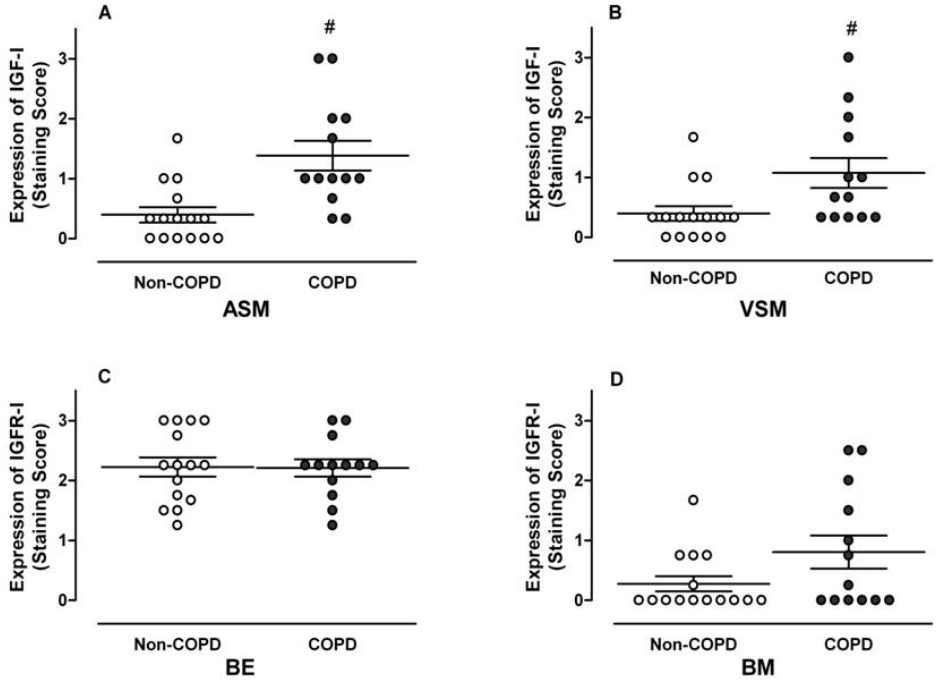

Figure 2.4: Expression of IGF-1and IGFR-I in COPD and non-COPD

Graphic representation of IGF-I expression in (A) airway smooth muscle (ASM) and (B) vascular smooth muscle (VSM) and IGFR-I in (C) bronchial epithelium (BE) and (D) macrophages (BM). Open and closed circles represent patients without and with COPD respectively. Lines represent mean±SEM values. # indicates a significant difference ($p \leq 0.05$, Student's t-test) as compared to non-COPD subjects.

IGFR-I was localized in bronchial epithelial cells, glandular epithelial cells and macrophages (Figure 2.3). The expression of IGFR-I was not enhanced (Figure 2.3; Panels C and D) in COPD patients as compared to the non-COPD group in the bronchial and glandular epithelial cells as well as in the inflammatory cells (macrophages). Similarly, in parenchymal lung sections, IGFR-I was localized in bronchiolar epithelium, alveolar epithelium and macrophages (Figure 2.3, Panels C and D), with no significant differences between the groups.

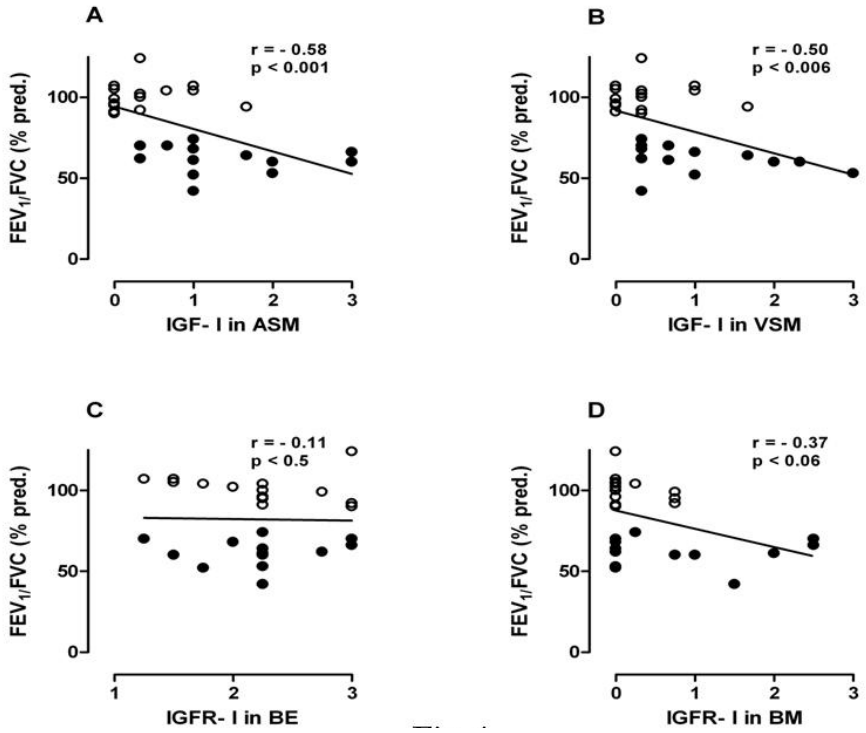

Figure 2.5: Correlation of IGF-I and IGFR-I expression with lung function

Correlation with FEV1 / FVC (% predicted) of IGF-I protein expression in (A) bronchial airway smooth muscle (ASM) and (B) bronchial vascular smooth muscle (VSM) and of IGF-I receptor in (C) bronchial epithelium (BE) and (D) macrophages (BM). Open and closed circles represent patients without and with COPD respectively. Correlation was assessed for the combined patient groups (non-COPD and COPD). Correlation coefficient (r) was obtained using linear regression (Spearman's) analysis. Differences with $p \leq 0.05$ were considered statistically significant.

2.3.3 Correlation with clinical data

We examined the relation between the lung function (FEV_1/FVC values) of patients in both groups and the expression (staining scores) of IGF-I and IGFR-I in the investigated areas with non-parametric Spearman's correlation analysis. Within the bronchial airways, FEV_1/FVC values were inversely correlated with IGF-I staining sores in bronchial ASM cells ($r = -0.58$; $p \leq 0.001$, Figure 2.5A) and VSM cells ($r = -0.50$; $p \leq 0.006$, Figure 2.5B) if

all subjects were analyzed together. However, FEV_1/FVC values did not show any correlation with IGFR-I expression in the bronchiolar epithelium ($r = -0.11$; $p \leq 0.5$, Figure 2.5C) and in bronchial macrophages ($r = -0.37$; $p \leq 0.06$, Figure 2.5D) from the total group.

2.3.4 Role of IGFs in ASM cell proliferation and growth

[^{3}H]thymidine incorporation assay was performed to assess the effect of IGF-I or IGF-II on DNA biosynthesis in human ASM cells. Figure 2.5A shows the concentration response proliferation of ASM cells to incubation with IGF-I or IGF-II (0, 1, 5, 10 ng /ml). Cell proliferation was near maximal at a concentration of 5 ng/ml for both the IGFs. Subsequently, all further experiments were done with 5 ng/ml of IGF-I or IGF-II. Cell counts done with insulin in the medium showed increased cell numbers after 24 and 48 h compared to the controls however, the fold change was not significant (Figure 2.5 B). In parallel, experiments were done with and without insulin in the medium to rule out its influence in the proliferation. Interestingly, there was significant fold induction (Figure 2.5 D) in cell number after stimulation with IGF-I (2.8 fold vs. control, $p \leq 0.001$) or IGF-II (3.02 fold vs. control, $p \leq 0.001$) without insulin supplement in the medium after 48 h. Moreover, there was significant difference in cell count between the controls with and without insulin ($3.613 \pm 0.67 \times 10^4$ vs. $1.87 \pm 0.14 \times 10^4$ cells, $p \leq 0.001$).

Similar experiments with ASM cells showed significant fold induction in DNA biosynthesis after 24 (IGF-I: 3.1 fold vs. control, $p \leq 0.004$) or IGF-II (5.4 fold vs. control, $p \leq 0.05$) and after 48 h (IGF-I: 2.67 fold vs. control, $p \leq 0.001$) or IGF-II (3.67 fold vs. control, $p \leq 0.02$) as assessed by [^{3}H]thymidine incorporation assay (Figure 2.5 C).

2.3.5 Role of IGFs in NCI-H292 cell proliferation and growth

Similar sets of experiments, performed to assess the cell proliferation in NCI-H292 by [^{3}H]thymidine incorporation assay at 8, 16 and 24 h (Figure 2.6 A) and cell count at 24 and 48 h (Figure 2.6 B) after stimulation with both the IGFs revealed a significant fold induction of cell proliferation as compared to their respective controls. NCI-H292 cells showed significantly increased DNA biosynthesis (IGF-I: 2.83 ± 0.30 fold increase, $p \leq 0.0001$ and IGF-II: 2.09 ± 0.19 fold increase, $p \leq 0.0002$) after 24 h and significantly higher cell numbers

after 48 h of stimulation. (IGF-I: 3.17 ± 0.79 fold increase, p≤0.03 and IGF-II: 2.52 ± 0.48 fold increase, p≤0.03).

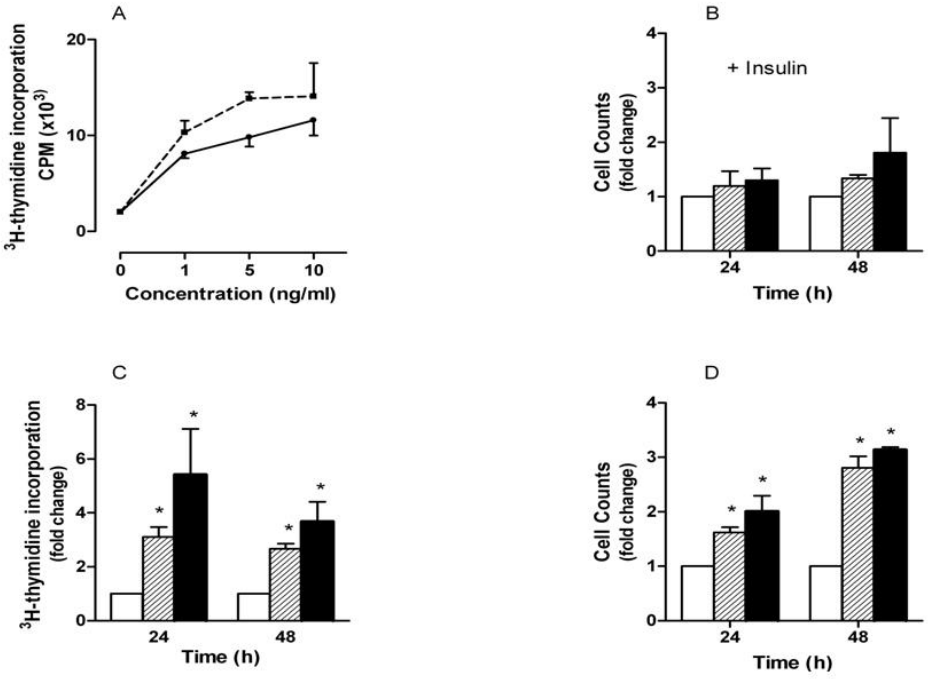

Figure 2.6: ASM cell proliferation in response to IGF stimulation

*A graphic representation of human airway smooth muscle (ASM) cell proliferation in relation to IGF-I or IGF-II and role of insulin in the medium. Panel A: Dose-dependent increases in [3H]thymidine uptake in ASM cells after 24 h stimulation with increasing concentrations (0, 1, 5, 10 ng/ml) of IGF-I (straight line) or IGF-II (dotted line). Panel B: Fold induction in ASM cells relative to control (open bar) after stimulation with 5 ng/ml IGF-I (striped bars) or IGF-II (closed bar) after 24 and 48 h with insulin in the medium as supplement. Panel C: Time course of [3H]thymidine uptake in ASM cells after stimulation with 5 ng/ml of IGF-I (striped bar) or IGF-II (closed bar). Panel D: Fold induction in ASM cells relative to control (open bar) after stimulation with 5 ng/ml IGF-I (striped bar) or IGF-II (closed bar) after 24 and 48 h without insulin in the medium as supplement. Data is represented as mean fold increase in relation to control from three independent experiments performed in quadruplicate. Values are mean±SEM and *P≤0.05 versus the control group.*

2.3.6 Enhanced epithelia wound closure by IGFs

To investigate the effects of the mitogenic IGF-I or IGF-II on epithelial wound closure, mechanically wounded NCI-H292 cell monolayers were incubated with medium alone (negative control) or with IGF-I (5 or 10 ng/ml), IGF-II (5 or 10 ng/ml) or FCS, and the wound area was measured at various time points (24, 48, and 72 h). Wounded monolayers incubated with 5 ng/ml of IGF-I or IGF-II did not show any significant wound closure as compared to negative control after 24, 48 or 72 h (Figure 2.7A). However, parallel experiments with 10 ng/ml of IGFs showed enhancement in wound closure (IGF-I: 47% vs.

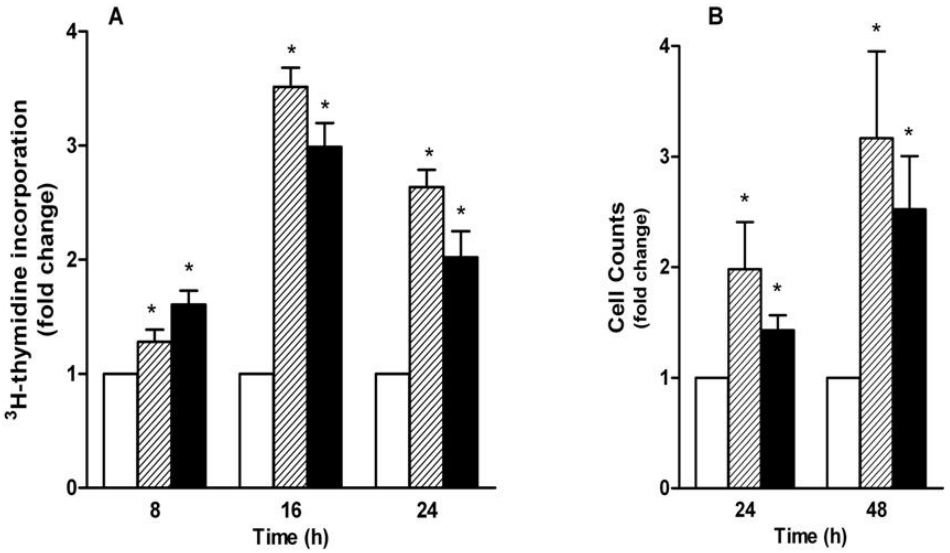

Figure 2.7: H292 cell proliferation in response to IGF stimulation

*A graphic representation of IGF-I or IGF-II stimulation on DNA biosynthesis and cell counts in NCI-H292 cells. Panel A: Time course of [3H]thymidine uptake (fold change) in NCI-H292 cells after stimulation with 5 ng/ml of IGF-I (striped bar) or IGF-II (closed bar) in relation to control cells (open bar). Panel B: Fold induction in ASM cell counts relative to control (open bar) after stimulation with 5 ng/ml IGF-I (striped bar) or IGF-II (closed bar) at 24 and 48 h. Data are represented as mean fold change counts per min (CPM) and cell number values in relation to respective control from 3 independent experiments performed in quadruplicate. Values are mean±SEM and *P≤0.05 versus the control group.*

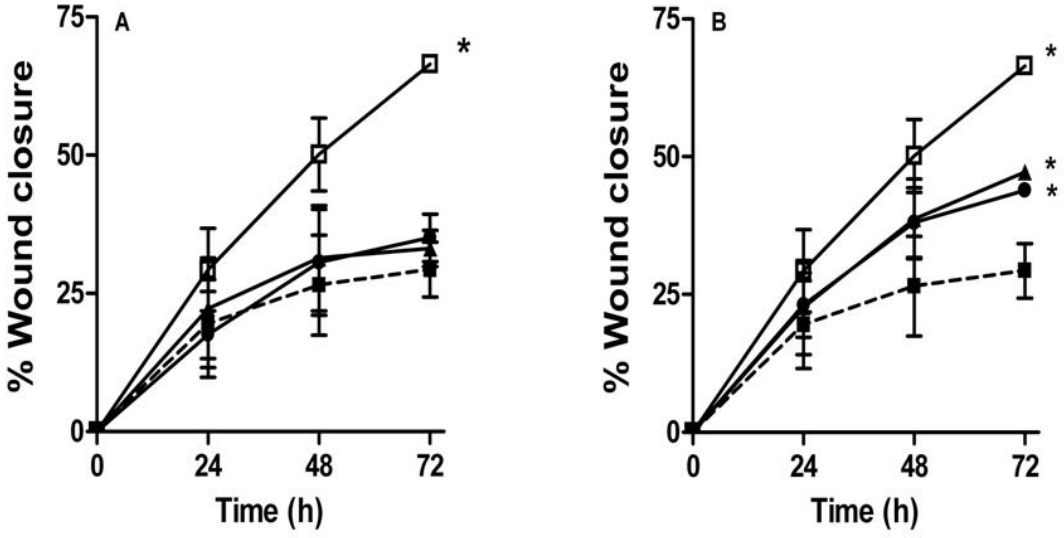

Figure 2.8: Dose- and time-dependent enhancement of airway epithelial wound closure by IGF-I or IGF-II

*Mechanically wounded NCI-H292 monolayers were incubated with medium alone (closed square) or supplemented with 5 or 10 ng/ml of IGF-I (closed triangle) or IGF-II (closed circle) or 10% FCS (open square) and the percentage of closed wound area compared to t=0 h was measured after 24, 48 and 72 h. Panel A depicts the time-dependent wound closure with IGF-I or IGF-II at 5 ng/ml concentration and Panel B represents wound closure with IGF-I or IGF-II at 10 ng/ml concentration. Data represent mean±SEM of three separate experiments, each performed in triplicate. * P≤0.05 versus serum-free medium-treated cells.*

29%, P≤0.02 and IGF-II: 44% vs. 29%, P≤0.05) after 72 h as compared with monolayers incubated with medium alone (Figure 2.7B). In FCS-treated monolayers, 67% of the wound area was closed after 72 h.

2.4 Discussion

The result of our current study contributes to our insight into the role of IGFs in repair of injured airway epithelium and eventually airway remodelling in chronic airway diseases such as COPD. Enhanced localization of IGF-I in ASM and VSM compartment of the bronchial airway in COPD patients along with the localization of the IGFR-I in the epithelium and in inflammatory cells (macrophages) point towards the possible role of IGFs in the whole milieu. Additionally, the results from our cross-sectional study indicate that the expression of IGF-I in the bronchial airways increases as the lung function values (FEV1/ FVC) decreases. Moreover we demonstrated the mitogenic effects of the IGFs on epithelial cells and also its wound repair potential in an *in vitro* model. Taken together, our findings clearly indicate a role for IGF-I/IGFR-I system in the well-acknowledged remodelling processes of the airways in COPD patients.

In the central airway sections, IGF-I protein was localized in the airway and vascular smooth muscle cells and the expression was enhanced in COPD patients as compared to respective controls. Similarly, in parenchymal sections we could localize IGF-I to ASM, VSM and inflammatory cells particularly macrophages as recognized morphologically. We did not observe any IGF-I staining in the bronchial epithelium in our study in both the groups (COPD and non-COPD patients). In agreement with other studies reporting IGF-I localization to alveolar epithelium and macrophages in adult with fibrotic lung and acute respiratory distress syndrome (24-28), we also observed IGF-I staining in alveolar epithelium although only in a few sections with no significant difference. Evidence suggests differential localization and expression pattern of IGF-I and IGF-II in foetus, infants and adults (24-26, 29). In the study of Chetty et al. (29), it has been reported that with advancing gestational age the presence of IGF-I decreased in the mesenchyme and cuboidal epithelium. However, IGF-I staining is markedly higher in neonates with bronchopulmonary dysplasia and respiratory distress syndrome. This indicates that there may be an re-emergence of foetal pattern of IGF-I expression during intense repair after lung injury as also reported in hyperoxic rat lung injury (30). Additional support for the involvement of IGF-1 comes from our observation that levels of IGF-I were inversely correlated with lung function, suggesting activation of repair

machinery that is also used during foetal development. Similarly, in our previous study (23) we found up-regulated FGF-1, FGF-2 and FGFR-1 expression in bronchial epithelium indicating that such compensatory mechanisms are also active in COPD. Whether this increased expression of these growth factors in COPD also contributes to squamous metaplasia and goblet cell hyperplasia in COPD remains to be determined.

The localization of IGF-I receptor in the bronchial epithelium and macrophages in lungs of our patient groups is in agreement with earlier reports which describe a similar staining pattern in human normal adult lung as well as expression in human epithelial cell lines (9, 31). However, these studies found increased staining for total IGFR-I in fibroproliferative acute respiratory distress syndrome (9, 24) and squamous cell carcinoma (31) while we did not find any significant difference in expression between COPD and non-COPD. Interestingly, FGF-2 has been shown to enhance IGFR-I expression (24) while IGF-I by itself decreases the expression of receptor by auto regulation (32, 33). This may explain the absence of any correlation of IGFR-I expression level with lung function data suggesting the possibility that IGF-I levels would be high in the vicinity (central airways) in COPD patients and hence decrease the receptor expression. In support, the expression of IGFR-I is not enhanced in COPD patients as compared to the non-COPD group. Both, IGF-I and IGFR-I were not discernible (localized) when other compartments like endothelium, fibroblast etc were looked at.

Correlation of lung function data with staining pattern revealed a significant relationship with IGF-I expression in the smooth muscle compartment (ASM and VSM) in the central airways. Exclusion of the patients, three in number, who were taking steroids peri-operatively, did not alter the data significantly. Also subgroup analysis with patients with and without COPD indicates that the correlation was indeed related to the severity of disease and not just to the mere presence of the disease.

Epithelial injury is normally followed by a complex repair process that comprises subsequent epithelial migration, proliferation, and differentiation (22). The primary aim is to rapidly restore the denuded epithelium and this process has been reported to be mediated via growth factors such as Transforming Growth Factors-β (TGF-β), Keratinocyte Growth Factor (KGF), Epidermal Growth Factor (EGF) and their receptors (3, 7, 34). It is

suggested that there may be an abnormal repair response in chronic airway disease leading to persistent activation and chronic secretion of cytokines and growth factors (8). This can drive the remodelling process via alteration or activation of the adjacent fibroblasts/myofibroblasts, bronchial and vascular smooth muscles and mucus-secreting glands. Our results add up to the available evidence that indeed IGFs have the potential to induce repair similar to EGFs (35, 36).

At present, our knowledge of airway and vascular remodelling during the development of COPD is far from complete. IGF-I has also been reported to contribute to vascularisation in developing lung through maintenance of endothelial cell population through its action as a survival factor (37). Inflammation and subsequent local production of many growth factors, including IGFs, play an essential role in the epithelial repair in response to tissue injury. The increased bronchial IGF-I expression in COPD patients and its receptor localization in epithelium in addition to the wound repair potential of IGF-I advocate for its crucial role in tissue repair mechanism during smoke-induced airway injury. Considering all data in this context, we conclude that ASM cells derived IGF-I act in a paracrine fashion on the IGFR-I positive epithelial cells resulting in their proliferation and thus contributing to airway remodelling in COPD.

Acknowledgement

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3

Role of Fibroblast Growth Factor-Receptor System in Bronchial Epithelial Repair

Based on "Alagappan VK, Kranenburg AR, Aarbiou J, de Boer WI, Hiemstra PS and Sharma HS. Expression and Autocrine Role of Fibroblast Growth Factor-Receptor System in Bronchial Epithelial Repair" – Submitted.

Chapter 3: Role of Fibroblast Growth Factor-Receptor System in Bronchial Epithelial Repair

Summary
Repeated injury/repair of the bronchial epithelium leading to aberrant airway remodelling is hallmark of chronic respiratory diseases. Previously, we demonstrated enhanced bronchial epithelial expression of FGF-1, FGF-2 and FGFR-1 in COPD patients. In this study, we explore the proliferative activity of bronchial epithelial cells in COPD in relation to lung function. Moreover, the mitogenic activities, signalling mechanisms and wound closure potentials of FGF-1 and FGF-2 were assessed using NCI-H292 cells. Proliferation marker, Ki-67 Labelling Index values showed significant doubling in COPD patients as compared to non-COPD and inversely correlated with FEV_1 values ($r = -0.43$; $p<0.03$). Both FGF-1 and FGF-2 increased proliferation (3-7 folds) after 24h and cell counts (2 folds) after 48 h. Analysis of the injured epithelial monolayer's showed enhanced wound closure with FGF-1 (67%, $P <0.01$) or FGF-2 (61%, $P<0.01$) as compared to controls (38%) after 72 h. Specific inhibitors of ERK1/2 (U0126) and FGFR-1(SU5402) completely blocked FGF-1 or FGF-2 induced ERK1/2 activation after 15 min. U0126 and SU5402 significantly blocked both FGF-1 (6% and 23% Vs 67%) and FGF-2 (9% and 19 % Vs 61%) enhanced wound closure. Similarly, the phosphatidylinositol-3-kinase inhibitor, LY294002 blocked epithelial repair in FGF-1(28%) and FGF-2 (22%) treated cells. Taken together, our results suggest a potential role for the FGF/FGFR-1 system in bronchial epithelial repair and airway remodelling in patients with chronic airway diseases.

3.1 Introduction

Airway epithelial injury/repair and associated remodelling processes are common features in chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) (1, 2). While there is genetic predisposition to COPD, we also know that smoking is a major determining factor that routinely leads to inflammation and damage, and those at risk lack the capacity to repair this damage (3). One of the key pathological features involves an influx of neutrophils, macrophages and T-lymphocytes as a result of tobacco-induced injury to the bronchial epithelial lining (1, 4). The bronchial epithelium consists of several cell types including basal, parabasal and ciliated cells, as well as Clara, some neuroendocrine and Goblet cells (5, 6). Recent studies indicate that basal and parabasal cells are capable of cell division and are proposed as epithelial cell progenitor cells for ciliated cells, and these ciliated cells are sensitive for toxins from cigarette smoke (4, 5). Attraction to and transmigration through the bronchial epithelial layer of predominantly neutrophils under influence of various cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-8 (IL-8) may lead to additional damage to the bronchial epithelium by the release of granule components from neutrophils including human neutrophils proteases (7-9).

In addition to the bronchial epithelial cells, (myo-) fibroblasts are the other major cell type involved in the airway wall remodelling and thickening, which is thought to be in part a result of hyperplasia and hypertrophy of (myo-) fibroblasts and airway smooth muscle associated with an increased deposition of extra cellular matrix (1). Growth factors that are released from these cells and that contribute to pathogenesis of airway remodelling include platelet–derived growth factor-B (PDGF-B), epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and tumour necrosis factor (TNF)-α (10-12). Co-culture studies with bronchial epithelial cells and myo-fibroblasts support the relationship between epithelial injury and enhanced airway remodelling as evidenced by increased epithelial cell proliferation and collagen expression due to interaction with several growth factors such as basic fibroblast growth factor (FGF-2), insulin-like growth factor-1, PDGF-B, TGF-β, endothelin-1 and EGF (13, 14). Evidence suggests that members of EGF and FGF family contribute to chronic inflammatory and tissue repair processes including chronic respiratory diseases (15-18). Given their function, FGFs may well play a pivotal role in regulating the airway wall remodelling in COPD as well. Increased expression of FGF-1 and FGFR-1 has been shown during the development of lung fibrosis (19) and FGF-2 has been implicated in the pathogenesis of obliterative bronchiolitis in lung transplants (20). We have previously demonstrated increased bronchial expression of fibroblast growth factors and their receptor in COPD patients (21). In that study, we observed a highly significant correlation of FGF-1/FGFR-1 co-localization in bronchial epithelium and FGF-2/FGFR-1 in ASM cells. These findings indicate that FGF-1 and FGF-2 are differentially expressed and may regulate locally different events in the corresponding tissues.

In order to further establish the role of the FGF receptorFGFR-1 and its ligands in the pathogenesis of COPD we examined the proliferative activity in the bronchial epithelium using the cell-cycle marker Ki-67. We investigated the proliferative potential as well as the wound repair potential of FGF-1 and FGF-2 *in vitro* using a cultured bronchial epithelial cell line (NCl-H292 cells). Finally, we explored partly, the signal transduction route of FGFR-1 by assessing the phosphorylation status of the downstream signalling protein Extracellular Regulated Kinase 1/2 (ERK1/2). Specific inhibitors of FGFR-1, ERK1/2 and phosphatidylinositol-3-kinase (PI3K) were used to decipher the role of these pathways in FGFs induced wound repair.

3.2 Materials and methods

3.2.1 Selection of specimens

Anonymized bronchial tissue of current and ex-smokers, who underwent surgery for lung cancer (lobectomy or pneumonectomy), was obtained from the Pathology departments of the Leiden University Medical Center (LUMC, Leiden, The Netherlands) and Southern Hospital (Rotterdam, The Netherlands). We examined routine formalin-fixed paraffin embedded lung tissue specimens from bronchial airways, taken distant from the tumour, in patients with or without COPD. COPD (n=12) and non-COPD (n=14) subjects were selected based on lung function parameters (22). Some of the tissue samples which belong to a larger database were also used in previous studies (21, 22).

COPD group: Twelve subjects were assigned to this group on the basis of the following parameters: forced expiratory volume in one-second (FEV_1) <75% of predicted value (23) before bronchodilatation, FEV_1/Forced Vital Capacity (FVC) ratio <75%, a reversibility in FEV_1 ≤12% of predicted after 400 μg inhaled salbutamol, and CO diffusion capacity (K_{co}) ≤80% of predicted value.

Non-COPD group: Fourteen subjects were assigned to this group based on the following data; a FEV_1 >85% of predicted before bronchodilatation, FEV_1/FVC ratio >85%, and reversibility in FEV_1 ≤12% of predicted after 400 μg salbutamol inhalation. In order to exclude accompanying lung disease leading to a restrictive lung function disorder, it was required that the total lung capacity (TLC) of each subject included in the study was over 80% of the predicted value. Clinical data of all patients were examined for possible co-morbidity and medication usage. All patients were free of symptoms of upper respiratory tract infection and none received antibiotics perioperatively. After the selection based on lung function, all the lung tissues were checked histologically using the following exclusion criteria (12, 22, 24): (i) presence of tumour in the lung tissue specimen submitted for the study, (ii) presence of poststenotic pneumonia in the specimen, (iii) fibrosis of lung parenchyma, and (iv) obstruction of the main bronchus of the resection specimen by tumour, regardless of the histology of the specimen intended for this study.

Pulmonary function tests

All pulmonary function tests were performed within 3 months prior to surgery. FEV_1 and FVC were measured by spirometry, total lung capacity and residual volume with the closed circuit helium dilution test and the K_{co} using the single breath-holding technique, as described by Quanjer and co-workers (23).

3.2.2 Immunohistochemistry

Sections of paraffin-embedded lung tissue were cut at 4 μm, mounted on Super Frost Plus® microscopic slides (Meinzl-Gläser, Braunschweig, Germany) and processed for immunohistochemistry. Serial sections were deparaffinised, rehydrated and blocked for endogenous peroxidase prior to incubation with specific purified mouse monoclonal antibodies. To examine proliferation of cells in the airways, a monoclonal antibody against Ki-67 (Dako Corporation, Glostrup, Denmark) was used. Ki-67 immunostaining was performed after antigen retrieval by boiling in citrate buffer (10 mM citrate buffer, pH 6.0) for 10 minutes in a microwave. To block non-specific second antibody binding, sections were preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (5% BSA/PBS, pH = 7.4). Subsequently, sections were incubated for 1 hour at room temperature with Ki-67 primary antibody at an appropriate dilution (1:400 v/v). To enhance sensitivity of detection, sections were incubated for 30 min30 minutes with secondary biotinylated anti-immunoglobulins (Multilink®, 1:75 dilutions, Biogenex, San Ramon, USA) and tertiary complex of streptavidin conjugated with horseradish peroxidase was used. Staining for FGF-1 and FGFR1 (kind gift from Dr. J. Walters, Oxford, UK) and FGF-2 (Transduction Laboratories, Lexington, USA) in the bronchial epithelium were done as described previously (21, 22). Colour was developed using 3,3-diaminobenzidine or New Fuchsin as chromogen. Slides were counterstained with Mayer's haematoxylin. Human breast carcinoma and placental tissue served as positive controls. Omission of primary antibodies served as negative control. The optimal dilutions for all antibodies were identified by examining the intensity of staining obtained with a series of dilutions: the optimal concentration resulted in specific and easily visible signal on paraffin sections of positive control specimens. Slides were mounted and evaluated by light-microscopy.

Quantitative analyses of immunostaining

Digital images from each subject (pixel size: 736x574) were taken using a 3CCD camera (Hitachi) coupled to a light microscope (Leica, Rijswijk, The Netherlands) at a magnification of 400x. Four randomly photographed sites of the airway section of each patient were analyzed using Leica Qwin system version 3.0 image analysis software (Leica B.V., Rijswijk, The Netherlands). The nuclear localization of Ki-67 in epithelial cells was assessed by interactive computerized counting of individual nuclei. Proliferation was

expressed as (number of stained nuclei/number of total nuclei) x 100% (labelling index, LI). The distribution of total nuclei divided by measured area was also assessed. Data are given as mean±SEM.

3.2.3 Culture of NCl-H292 cells

NCl-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line (purchased from American Type Culture Collection; ATCC, Manassas, VA), were cultured in 1640 medium containing 10% foetal bovine serum (FBS; Bio-Whitaker BV, Verviers, Belgium) supplemented with sodium pyruvate (2 mM), gentamicin (50 µg/ml), L-glutamine (4 mM), HEPES (10 µM), penicillin (5 U/ml), streptomycin (5 µg/ml) at 37 °C in a 5% CO_2-humidified atmosphere. Cells were maintained in culture by replacing the medium every h and when confluent were further passaged using trypsin/EDTA solution into 75 cm^2 tissue culture flasks. Semi-confluent cultures were used for the experiments.

3.2.4 Proliferation assays

[^{3}H]Thymidine uptake: Serum deprived (48 h) NCI-H292 cells (quadruplo in 96 wells plates from three different flasks) were incubated with 100 µl serum-free-medium alone (control medium serving as negative control) or supplemented with 10% FBS (positive control), 10 ng/ml human recombinant FGF-1 (Promega, Madison, USA) or FGF-2 (Sigma-Aldrich BV, Zwijndrecht, The Netherlands). The optimal concentrations for FGFs were based on pilot experiments and from our previous study (21). Three independent experiments, using different batches of cells, were performed in which cells were treated for 8, 16 or 24 h. Five hours prior to the end of the treatment 10 µl of [^{3}H]thymidine (1 µCi/10 µl in HBSS; Amersham, Roosendaal, the Netherlands) was added to the wells, at a final concentration of 1 µCi/110 µl per well. The medium was removed after 8, 16 and 24 h and the cells were washed twice with cold PBS. The cells were detached with 50 µl trypsin for 10 min after which 50 µl PBS was added. Cells were frozen overnight at -20°C and subsequently harvested on glass fibre filters using a Filtermate 196 cell harvester (Packard, Meridan, USA). Activity was counted using a Microplate Scintillation ß-counter (Topcount, Packard, Meridan, USA) and measured radioactivity was expressed as counts per min (CPM). The mean CPM of quadruple wells and subsequently three different experiments were expressed as ratio as compared to control cells in serum free medium (fold-induction).

Cell counts: In a parallel series of experiments, 200,000 cells were seeded in 24-well plates. Serum deprived NCI-H292 cells (in quadruplo) were stimulated for 24 and 48 h with FGF-1, or FGF-2 or controls and cell numbers were determined using automated cell counting (Casy®1, Schärfe system GmbH, Reutingen, Germany). After stimulation, the cells were trypsinized with 100 µl Trypsin/EDTA for 10 min. Cells in suspension were added to 10 ml of Casy®1 isotonic solution (6.38 g/l NaCl, 0.2 g/l Na-tetraborate, 1.0 g/l Boric acid and 0.2 g/l EDTA). Cell numbers, volumes and diameters were measured and analyzed using Casey®1 system software.

3.2.5 Wound repair model

In order to assess the wound repair potential of the FGFs, an *in vitro* model as described previously (25) was used. Briefly, NCI-H292 cells were cultured to confluence in 6-well tissue culture plates. After overnight serum deprivation, three circular wounds (3 mm in diameter) were scraped in each well using a sharpened silicone tube attached to a Pasteur pipette and a microscope. After washing with PBS and allowing the cultures to recover for 1 h in serum-free medium, the wounded monolayers were incubated in serum-free medium alone or supplemented with FGF-1 or FGF-2 (10 ng/ml) or 10% FBS (positive control). To study the role of selected signalling pathways, wounded monolayers were incubated with U0126 (20 µM; Promega, Madison, WI), LY294002 (10 µM; Stratagene, La Jolla, CA) or SU5402 (20 µM; Calbiochem, UK) 1 h before addition of FGFs. Possible cytotoxic effects of the inhibitors at concentrations used in these experiments were excluded by pilot studies and literature review. Images were collected using a digital camera after 0, 24, 48 and 72 h and analyzed using ImageJ 1.32j software (National institutes of health, USA) by determining the percentage wound closure at various time points as compared with the time point of starting the stimulation (t = 0).

3.2.6 ERK1/2 phosphorylation in NCI-H292 cells

NCI-H292 cells were cultured to near confluence and subsequently serum-deprived overnight. After incubation of cells with serum-free medium alone or supplemented with FGF-1 (10 or 100 ng/ml), FGF-2 (10 or 100 ng/ml) or 20 ng/ml transforming growth factor-α (TGFα; Sigma-Aldrich) for 15 or 30 min, cells were washed and lysed in 0.5% (v/v) Triton X-100, 0.1 M Tris-HCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_3VO_4 and complete protease inhibitor cocktail (Roche, Basel, Switzerland) on ice.

The effects of the inhibitors U0261 and SU5402 on FGFs induced signalling pathway were assessed by pre-incubating NCI-H292 cells with these inhibitors for 1 h using concentrations as described for wound closure experiments. Cell lysate protein samples (8 µg) were separated on a 10% acrylamide gel using standard SDS-PAGE procedures and transferred to polyvinylidene difluoride membranes using the Mini-transblot system (Biorad). The membranes were then pre-incubated with 0.5% casein and 0.05% Tween-20 in PBS for at least 1 h, followed by incubation with rabbit polyclonal antibodies directed to phospho-specific or native ERK1/2 (New England Biolabs, Beverly, MA) overnight at 4 °C. After incubation with horseradish peroxidase conjugated goat anti-rabbit (BD Transduction Laboratories, Franklin Lake, NJ, USA) polyclonal antibodies, immunoreactivity was visualized using electrochemiluminescent detection reagent (Amersham).

3.2.7 Statistical analysis

Data were analyzed for statistical significance using the unpaired, two-tailed Students' t-test as well as Pearson's correlation analysis wherever appropriate. At $p \leq 0.05$ differences were considered statistically significant.

3.3 Results

3.3.1 Cell proliferation in bronchial epithelium

Bronchial airways were stained for the cell cycle and proliferation marker, Ki-67 and Figure 3.1A and 3.1B represents examples of the staining patterns in non-COPD and COPD subjects. Ki-67 immunoreactivity was mainly observed in the nucleus of basal and parabasal epithelial cells, and also in some inflammatory cells. Within the bronchial epithelium, the total number of bronchial epithelial cell nuclei and Ki-67 positive nuclei were measured and expressed as the labelling index (LI). We observed a significant doubling of the LI in COPD patients (LI = 6.0%) as compared to non-COPD (LI = 3.0 %) (Figure 3.1C).

3.3.2 Correlation with clinical data

We examined the relation between the lung function (FEV1values) of patients in both groups and the expression (staining scores) of proliferation marker, Ki-67 in the bronchial epithelial cells in central airways. FEV_1 values were inversely correlated with the Labelling

Index values of Ki-67 ($r = -0.43$; $p<0.03$, Figure 3.2) when all subjects were analyzed together. However, the Ki-67 values did not shown any significant correlation to the FGF-1, FGF-2 or FGFR-1 staining score in the bronchial epithelial cells.

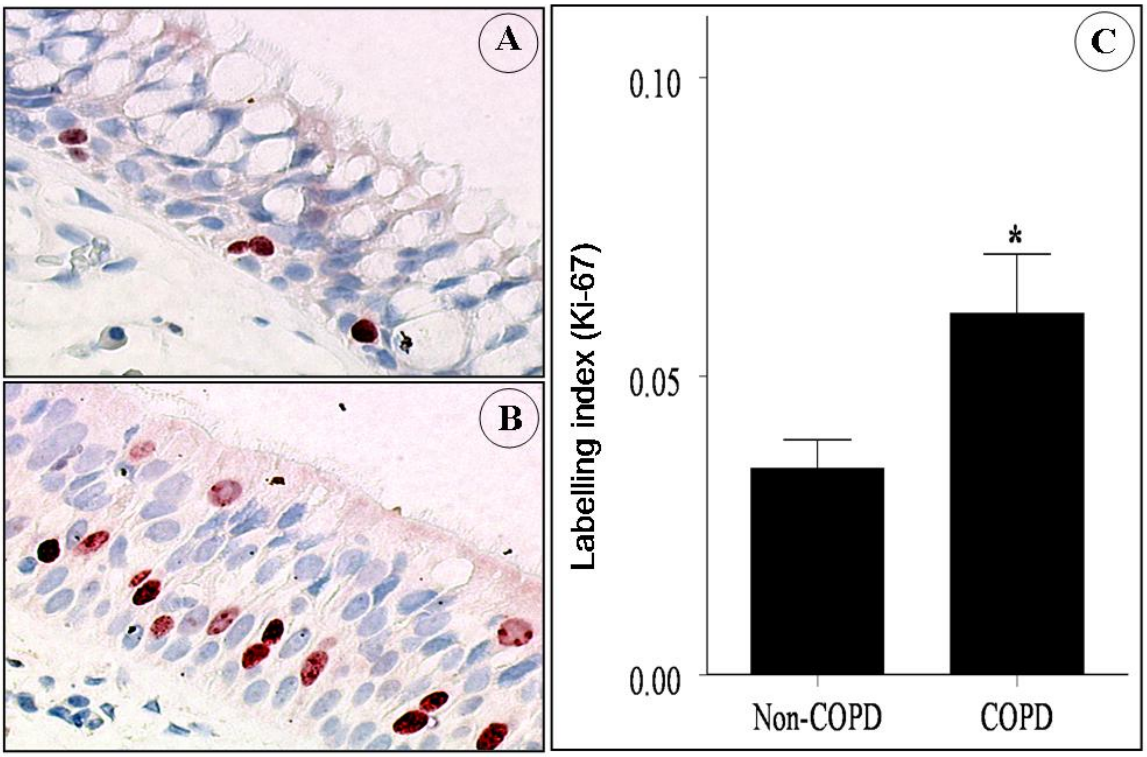

Figure 3.1: Immunohistochemical localization of Ki-67 in bronchial epithelium

*Photomicrographs of central bronchial tissue sections from patients without COPD (**A**) and with COPD (**B**) showing brown nuclear staining for cell proliferation marker, Ki-67. Original magnification: x200. (**C**) Graphic representation of Labelling index (L.I.) of Ki-67 staining in bronchial epithelium in COPD and non-COPD groups. At P≤0.05 differences were considered as significant.*

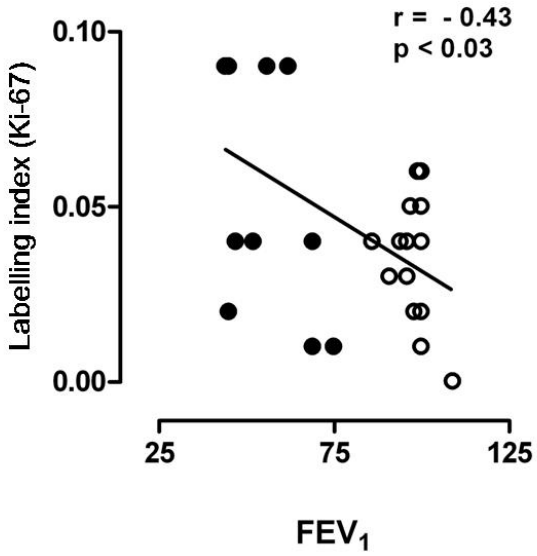

Figure 3.2: Correlation analysis of Ki-67 expression in epithelial cells and lung function (FEV1)

Graph represents correlation analysis between Labelling Index of Ki-67 expression in bronchial epithelial cells and FEV_1. Open and closed circles represent patients without and with COPD respectively. Correlation coefficient (r) was obtained using linear regression (Pearson's) analysis and P value. At P≤0.05 differences were considered as significant.

3.3.3 Localisation of FGF-1, FGF-2 and FGFR-1 in bronchial epithelium

Immunohistochemical staining reveals localisation of FGF-1, FGF-2 and FGFR-1 in the bronchial epithelium in lung sections obtained from patients with (Figure 3.3) and without COPD. We have reported earlier (21) the enhanced expression of these proteins in bronchial sections of COPD patients as compared to patients without COPD.

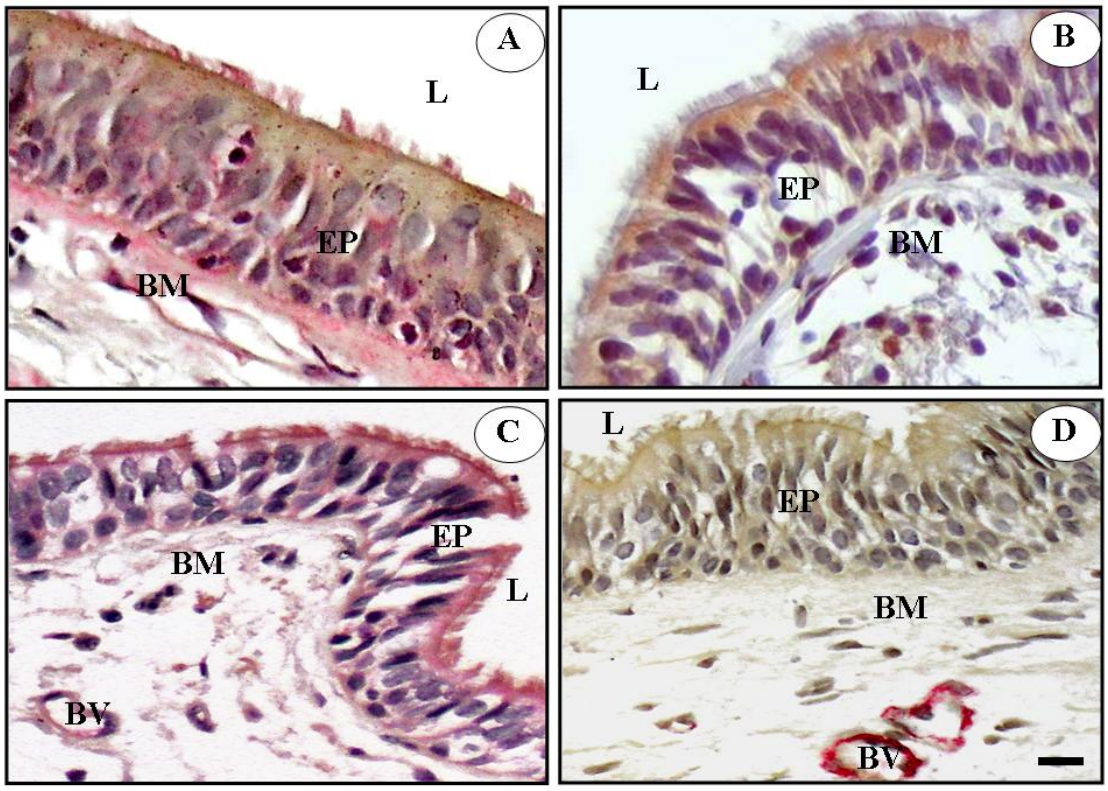

Figure 3.3: Immunohistochemical localization of FGF-1, FGF-2 and FGFR-1 in bronchial epithelium.

*Photomicrographs of human central bronchial tissue sections showing localization of (**A**) FGF-1 (red new-fuchsin), (**B**) FGF-2 (brown), (**C**) FGFR-1 (red new-fuchsin) and (**D**) α-smooth muscle actin (α-SMA, red new-fuchsin). Original magnification: x200. Scale bar = 50 µm. Arrows indicate positive staining for respective ligands.*

3.3.4 Mitogenic effects of FGFs in cultured NCI-H292 cells

Repair is a combination of proliferation, migration and cell death. Using bronchial epithelial NCI-H292 cells as a model system we further explored FGF-mediated proliferation and migration as well as intracellular signal transduction pathways thought to be involved in the repair process. H292 cells were stimulated *in vitro* with FGF-1 or FGF-2 and proliferation was assessed. Both FGF-1 and FGF-2 stimulated proliferation and cell numbers after 24 and 48 h of treatment. A graphic representation of the time dependent thymidine uptake

following incubation with FGF-1 or FGF-2 is presented in Figure 3.4A. After 8 hours of stimulation, we observed a statistically significant increase in thymidine incorporation upon FGF-2 treatment (2.21±0.11 Vs1.26±0.07 counts per minute (CPM) X 10^4) and after 16 and 24 hours a statistically significant increase upon FGF-1 (3.4±0.19 and 6.8±0.4 CPM X 10^4) or FGF-2 treatment (4.3±0.28 and 7.6±0.48 CPM X 10^4) as compared to untreated cells (0.96±0.05 and 2.5±0.12 CPM X 10^4); Figure 4A. After 48h, FGF-1 and FGF-2 also induced significant increase in cell numbers 1.85±0.28 and 2.80±0.66 times, respectively; Figure 3.4B.

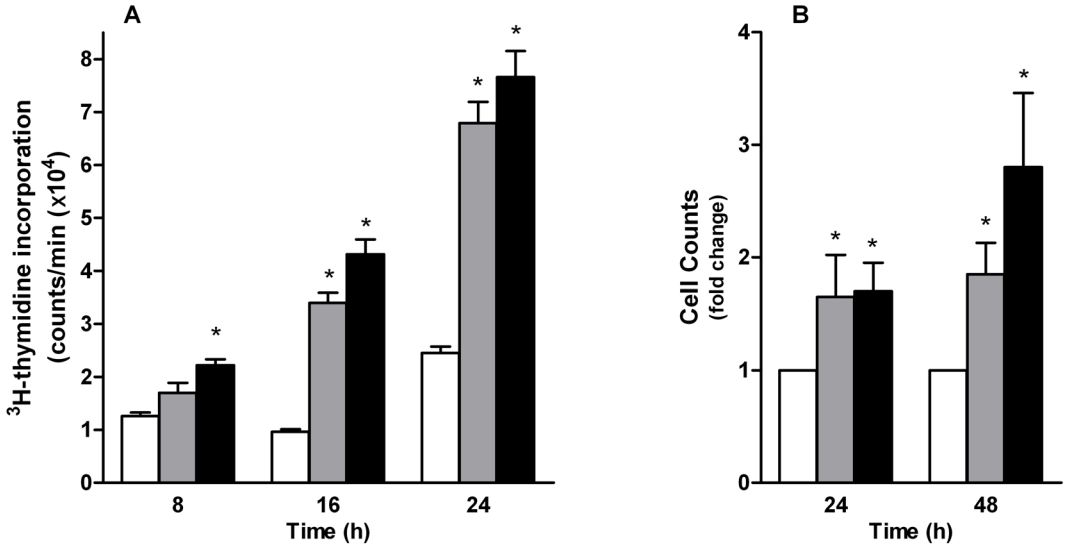

Figure 3.4: Assessment of NCI-H292 cell proliferation in relation to FGF-1 and FGF-2

*Time course of [^{3}H]thymidine uptake (8, 16, 24 h) in NCI-H292 cells after stimulation with 10 ng/ml of FGF-1(closed gray bar) and FGF-2 (closed black bar) (**A**). Fold increase in cell counts of NCI-H292 cells upon stimulation with 10 ng/ml of FGF-1 (closed gray bar) and FGF-2 (closed black bar) for 24 h and 48 h relative to control (open bar) culture conditions (**B**). Data are represented as mean values from three independent experiments performed in quadruplicate. Values are given as mean±SEM. Differences were statistically significant at P≤0.05 (*) as compared to the control group.*

3.3.5 In vitro wound repair potential of FGFs

To investigate the effects of the mitogenic FGF-1 or FGF-2 on epithelial wound closure, mechanically wounded NCI-H292 cell monolayers were incubated with medium alone (negative control, Figure 3.5A upper panels) or with FGF-1 (10 ng/ml, Figure 3.5A, middle panels) or FGF-2 (10 ng/ml, Figure 3.5A, lower panels) or 10% FCS. The wound area was measured at various time intervals (24, 48, and 72 h). Wounded epithelial monolayers showed enhanced wound closure in the presence of FGF-1 (67%, P <0.01) or FGF-2 (61%,

P<0.01) after 72 h as compared with monolayers incubated with medium alone (38%, Figure 3.5B). In FCS-treated monolayers, 58% of the wound area was closed after 72 h as compared to the control.

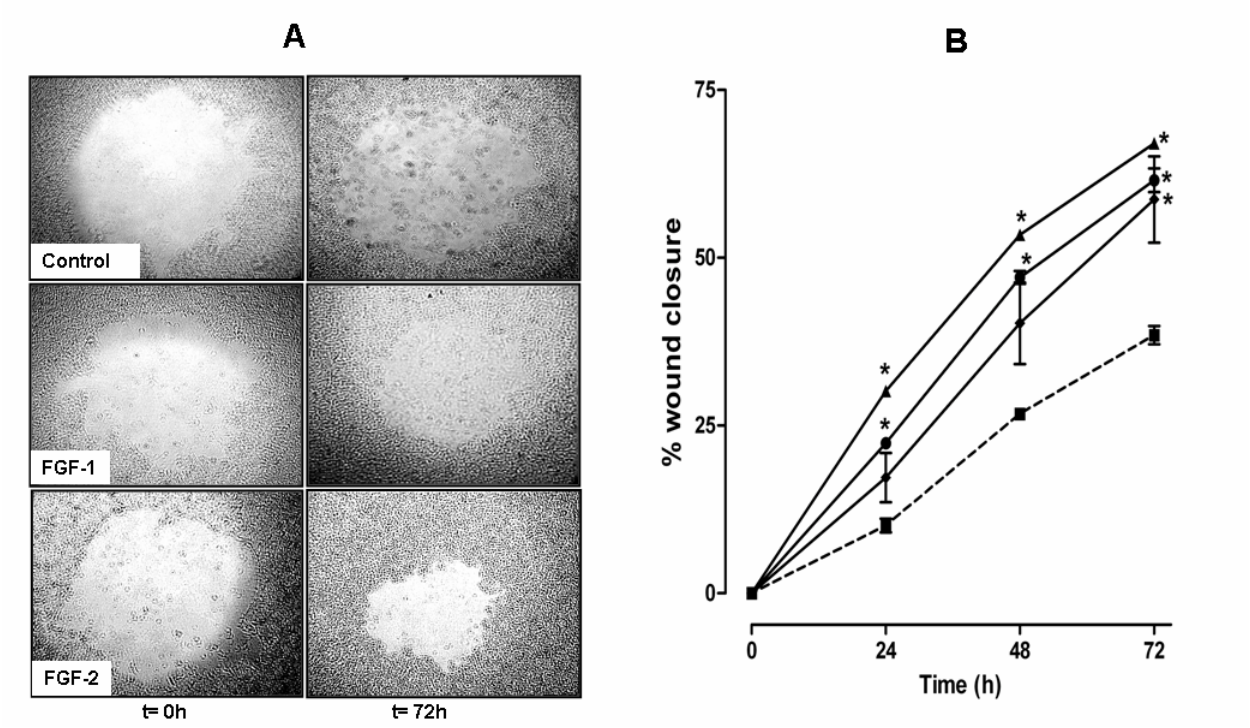

Figure 3.5: Time-dependent enhancement of airway epithelial wound closure by FGF-1 or FGF-2

*Representative images of wound closure treated with control medium alone (left panel), FGF-1 (middle panel) and FGF-2 (right panel) after time period 0 and 72 h (panel A). Mechanically wounded NCI-H292 monolayers were incubated with control medium alone (closed square) or supplemented with 10 ng/ml of FGF-1 (closed triangle) or FGF-2 (closed circle) or 10% FCS (closed diamond) and the percentage of closed wound area compared to t=0 h was measured after 24, 48 and 72 h (panel B). Data represent mean±SEM of three separate experiments, each performed in triplicate. *: P≤0.05 versus serum-free medium-treated cells.*

3.3.6 Involvement of FGFR-1 receptor and MAPK signalling in FGF-induced ERK 1/2 activation

FGF-1 and FGF-2 were demonstrated to stimulate both proliferation and migration of cells. Next we explored FGFR-1-dependent signal transduction pathways. We determined whether the intracellular signal transduction protein ERK1/2 was phosphorylated upon the induction of proliferation by FGF-1 and FGF–2. Incubation of NCI-H292 cells with 10 or 100 ng/ml FGF-1 and FGF-2 for 15 and 30 min resulted in an increase of ERK1/2 activation, as did the positive control TGFα (Figure 3.6A).

To further delineate the involvement of the FGFR-1 receptor signalling pathway, NCI-H292 cells were incubated with inhibitors of FGF receptor-1 (SU5402) or ERK 1/2 (U0126) 1 h before addition of FGF-1 or FGF-2. Subsequently, cells were treated with either FGF-1 or FGF-2 for 15 minutes. Western blot analysis of phosphorylated ERK1/2 demonstrated that both the inhibitors completely blocked FGF-1 and FGF-2 induced ERK1/2 activation (Figure 3.6B). This point to FGFR-1 being the major effector receptor as SU5402 specifically inhibits FGFR-1.

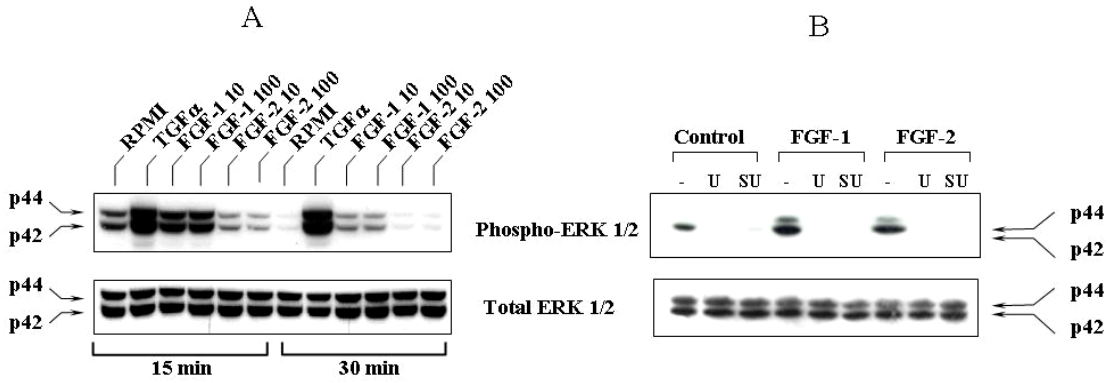

Figure 3.6: ERK1/2 activation in NCI-H292 cells treated with FGF-1 and FGF-2

(A) NCI-H292 cells were incubated with medium alone or supplemented with FGF-1 or FGF-2 (10 or 100 ng/ml) or TGFα (20 ng/ml) for 15 or 30 min. Activated and total ERK1/2 were determined in cell lysates by Western blot. (B) Effect of inhibitors of the FGFR-1 and MEK signalling pathway on FGF-induced ERK1/2 activation. NCI-H292 cells were preincubated with SU5402 (SU; 20μM) or U0126 (U; 20 μM), for 1 h before addition of 10 ng/ml FGF-1 or FGF-2 for 15 min. Data are of one experiment (similar results were obtained in two separate experiments).

3.3.7 Involvement of FGFR-1 and MAPK signalling in FGF-1, 2 enhanced wound closure

To investigate whether the observed induction of epithelial wound closure by FGF-1 and 2 is mediated via the FGFR-1 and ERK 1/2 pathway, the effect of the previously described inhibitors on FGFs enhanced wound closure was assessed. All individual inhibitors were shown to block the basal wound closure (Figure 3.7). Similar to the results on ERK1/2 activation, U0126 and SU5402 significantly blocked both FGF-1 (showed 6% and 23% wound closure respectively as compared to 67%) and FGF-2 (9% and 19 % wound closure respectively as compared to 61%) enhanced wound closure. PI-3K has been implicated in mediating cell migration and thereby may also contribute to wound repair (25). Inhibitors of these kinases, (LY294002) with or without SU5402, significantly blocked wound closure in FGF-1(28% Vs 67% wound closure) and FGF-2 (22% vs 61% wound closure) treated cells.

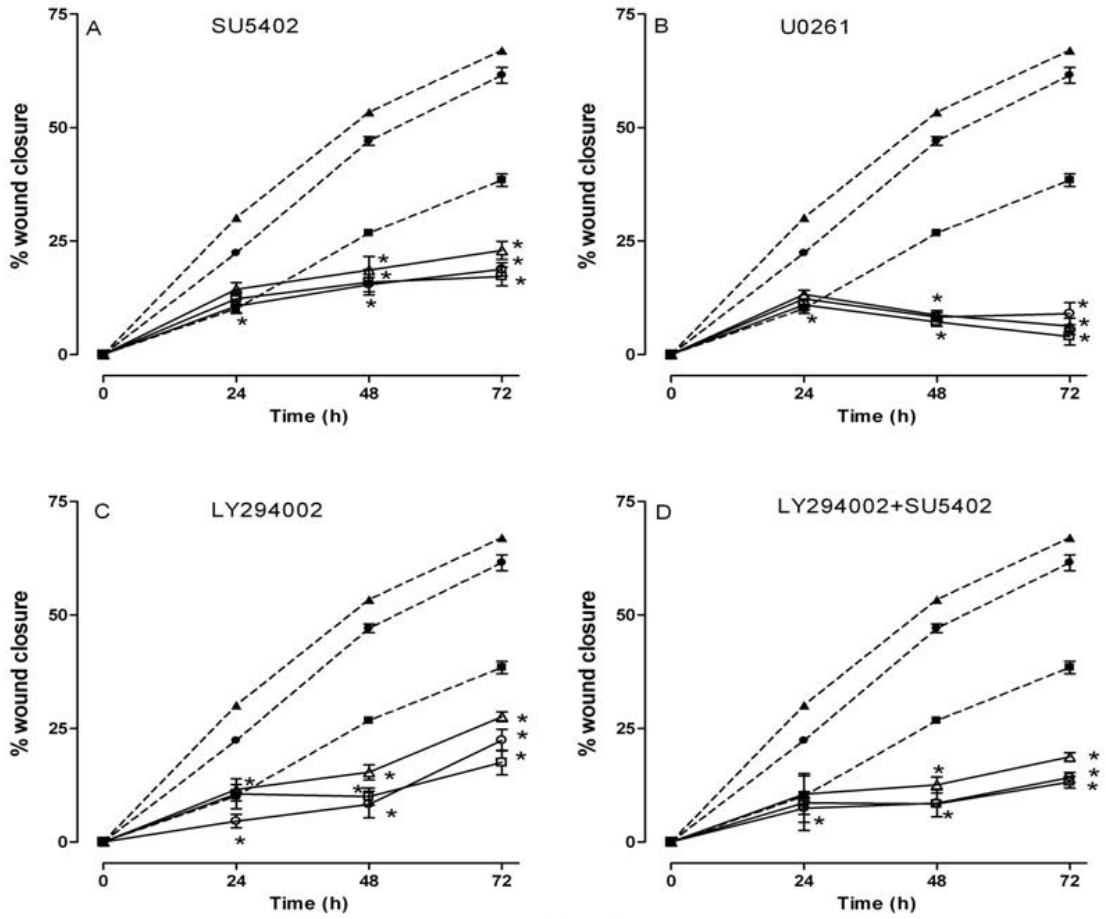

Figure 3.7: Effect of inhibitors of the FGFR-1, ERK 1/2 and PI3K signalling pathway on FGF–induced wound closure of NCI-H292 cells.

Mechanically wounded NCI-H292 cell monolayer's were preincubated with (A) SU5402 (20 μM), (B) U0126 (20 μM), (C) LY294002 (10 μM), or (D) LY294002 and SU5402 1 h before addition of 10 ng/ml FGF-1 or FGF-2. Data are mean±SEM of three experiments. Closed square, medium; closed triangle, FGF-1; closed circle, FGF-2; open square, inhibitor; open triangles, inhibitor + FGF-1; open circles, inhibitor + FGF-2.

3.4 Discussion

In this study we showed an association between the expression of Ki-67 in the bronchial epithelium and the presence of chronic airflow obstruction. Several studies have used this marker to assess proliferating airway epithelial cells in biopsies of healthy, asthma and chronic bronchitis patients (6, 26). Our *in vitro* results indicate that FGF-1 and FGF-2 are potent mitogens for NCI-H292 cells associated with activation of ERK1/2. Additionally, our *in vitro* wound repair experiments revealed that FGF-1 and FGF-2 significantly enhanced wound closure. Using specific inhibitors of signalling pathways revealed that ERK1/2 and PI3-K are involved in FGF enhanced wound closure via activation of FGFR-1.

Taken together, these findings support the idea that the FGF/FGFR-1 system contributes to epithelial remodelling as seen in chronic lung diseases like COPD.

In response to chronic injury there may be an abnormal persistent activation and chronic secretion of growth factors. Previously, we showed that there is an increased expression of growth factors such as vascular endothelial growth factors (VEGF) and FGFs in COPD patients (21, 27). This expression pattern also correlated with the severity of the disease or the lung function indicating that the repair machinery was activated. We observed that FGF-1, FGF-2 and FGFR-1 are constitutively expressed in non-COPD human lungs, particularly in bronchial epithelium, alveolar macrophages and monocytes, as well as in the intima and media of pulmonary blood vessels. Expression of these molecules was enhanced in COPD patients in the bronchial epithelium (21). FGF-1 and FGF-2 in the bronchial epithelium may be involved in proliferation and repair of epithelial layers after injury. Our findings of increased Ki-67 expression in bronchial epithelium and its correlation with the lung function data (FEV_1) indicate that such compensatory mechanisms are active in COPD (28).

Many experimental and clinical studies have shown the importance of exogenous growth factors in wound repair (29). Recently the functions of endogenous growth factors in the healing process are being addressed. Our *in vitro* data confirm the mitogenic potential of FGF-1 and FGF-2 on epithelial cells (30) and was similar to our previous results (21) on human airway smooth muscle cells. Our data suggest that FGF-1 and 2–enhanced wound closure requires activation of the FGFR-1 and involves the ERK 1/2 pathway, suggesting pivotal role of cell proliferation in the FGF-enhanced wound closure. It supports previous reports on ERK1/2 activation upon FGF-2 stimulation as early as 15 minutes in lens epithelial cells (30). However, in contrast to that report, we did not see correlation of the ERK 1/2 phosphorylation with the dose of FGFs.

The observation that the MEK inhibitor, U0126 blocked airway epithelial cell proliferation (31) and given the importance of ERK1/2 signalling pathway in the induction of epithelial cell, suggests a role for ERK1/2 signalling in wound repair. FGF induced epithelial cell proliferation has been shown to be ERK dependant. In addition, inhibition of this pathway has been reported to result in delayed epithelial wound healing (32). ERK1/2 and PI-3K pathways have also been implicated in cell migration during wound repair (25) and is

further supported by the current demonstration that inhibitors of ERK1/2, PI3K, and FGFR-1 block FGF-enhanced wound closure suggesting involvement of cell migration in FGF-enhanced wound repair. Previous reports on FGF-2 induced endothelial cell migration after injury support the role of PI3K pathway in this process (33).

In asthma and COPD injury of the epithelium is a common feature and this injury is followed by the activation of the remaining epithelial cells. Besides releasing pro-inflammatory cytokines, they also secrete factors that help in restoring the epithelial barrier through active repair (34). Among these secreted factors many growth factor/receptor systems are involved in tissue remodelling, including the EGF/EGFR, TGF-β/TGF-βR and IGF-1/IGFR-1. These growth factors have been suggested to regulate repair of airway epithelial injury by induction of extra cellular matrix deposition, cell migration, proliferation and differentiation (2, 4). Our results add up to the available evidences that FGF-1 and FGF-2 have the potential to induce airway epithelial repair similar to EGFs, IGFs and keratinocyte growth factor (15, 35-37).

Taken together, our results support the notion that increased bronchial expression of FGF-1, FGF-2 and FGFR-1 in patients with COPD could contribute to epithelial proliferation (as observed in the present study) and remodelling in COPD. Our observation that FGFs promote epithelial wound repair and that these growth factors are over expressed in the lungs of COPD patients, is in line with the hypothesis that excessive repair following injury may underlie epithelial remodelling in COPD. Modulation of the signalling pathways identified in the current study could provide a new therapeutic target for the treatment of COPD.

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Part 2

Vascular Remodelling

4

Bronchial Vascular Remodelling in Chronic Bronchitis/Emphysema

Based on: "Sharma HS, Kranenburg AR and Alagappan VKT: Bronchial Vascular Remodeling in Emphysema / Chronic Bronchitis. In Ed: Lazaar A, Bronchial Vascular Remodeling in Asthma and COPD, Monograph on Lung Biology in Health and Disease. Marcel Dekker, Inc. (Taylor & Francis CRC Press), New York, 2006 (216) 147-168.

Chapter 4: Bronchial Vascular Remodelling in Chronic Bronchitis/ Emphysema

Summary
At present, our knowledge of airway and vascular remodelling in COPD is far from complete. COPD is often called "emphysema and chronic bronchitis" since over time, alveolar destruction results in emphysema, and chronic bronchial inflammation leads to chronic bronchitis. Increased adventitial infiltration of inflammatory cells in pulmonary and bronchiolar arteries has been reported. Many growth factors, among them VEGF and FGF, play an essential role in maintaining pulmonary and vascular viability and in tissue repair. Based on the available evidence, we hypothesize that in chronic bronchitis, expression of VEGF and VEGFR-2 expression leads to increased vascular remodelling, which is inefficiently compensated by the low expression of VEGFR-1. In subjects with emphysema, however, VEGFR2 expression is lower. Preferential activation of VEGFR-1 results in higher MMP activity, alveolar destruction and endothelial apoptosis. Hence, the balance between VEGF, VEGFR-1 and VEGFR-2 are critical in the pathogenesis of COPD subtypes.

4.1 Introduction

Chronic obstructive pulmonary disease (COPD) is global health problem with increasing morbidity and mortality (1). COPD is characterized by airflow limitation that is not fully reversible, usually progressive and associated with an abnormal inflammatory response of the lungs following exposure to noxious particles and gases and inhaled cigarette smoke (2, 3). One important pathological feature of COPD is airway inflammation, characterized by an influx of neutrophils, macrophages and CD8+ T-lymphocytes in the lumen and wall of bronchial and bronchiolar airways and parenchyma (4-6). Over time, alveolar destruction results in emphysema, and chronic bronchial inflammation leads to chronic bronchitis, which is why COPD is often called "emphysema and chronic bronchitis" (7). Interestingly, only ten to twenty percent of all smokers develop symptomatic COPD; yet the causes of this variability in response of the airways and lung parenchyma to tobacco smoke exposure remain largely unclear.

4.1.1 Emphysema

The development of emphysema and its different pathological patterns is likely the result of interactions between external risk factors and intrinsic host susceptibility factors. The parenchymal destruction seen in emphysema may be caused by an inflammatory process due to imbalances in protease-antiprotease and oxidant-antioxidant levels (8) and also by activation of innate and adaptive immune responses (9). The protease-antiprotease theory has been further refined to an elastase-anti-elastase theory of pathogenesis of emphysema, as neutrophil elastase and macrophage-derived proteases are primarily implicated (10, 11). The role of cigarette smoke-induced free radicals in structural damage and inhibition of protease inhibitors also has been studied extensively (12-14). However, theories of antiprotease deficiency do not fully explain why only 10-20 % of all smokers develop emphysema (14). One additional possibility is the vascular atrophy model of Leibow (15), as has been suggested (16, 17). Leibow proposed that a reduction in the blood supply of the small precapillary blood vessels might induce the disappearance of alveolar septa and hence may result in the development of emphysema.

4.1.2 Chronic bronchitis

Mucous gland hypertrophy and goblet cell hyperplasia occur in chronic bronchitis and contribute to excess mucus production. Although the exact pathogenesis of chronic bronchitis remains unclear, bacterial colonization and the resulting inflammatory response are thought to be of central importance. The generation of pro-inflammatory cytokines and chemotactic stimuli by the airway epithelium are likely to play a central role in propagating the inflammatory response in patients with chronic bronchitis (18). Smokers with chronic sputum production have an increased infiltration of neutrophils and macrophages and an increased proportion of CD8+ T-lymphocytes in their bronchial glands, supporting the important role of bronchial-gland inflammation in the pathogenesis of chronic bronchitis (19), which results in epithelial disruption, smooth muscle hypertrophy and fibrosis (20). It has been reported that there are increased macrophage counts in chronic bronchitis patients with airflow limitation compared to patients without airflow limitation (21). In addition, neutrophils, compartmentalized in the mucosal surface of the airways and CD8+ T cells, distributed along the subepithelial zone of the airways and lung parenchyma are consistently associated with the chronic airflow limitation found in COPD (22). The number of

neutrophils further increases in the submucosa of patients with severe COPD, suggesting a role for these cells in the progression of disease (22).

Several studies have described changes consistent with airway remodelling in COPD, characterized by a thickened bronchiolar wall, an increase in airway smooth muscle (ASM) mass (5, 23, 24) and increased deposition of extracellular matrix (25), associated with peribronchiolar fibrosis. Airway remodelling and abnormal repair of small airways may explain the changes in small airways and parenchyma in these diseases (14).

4.2 Airway remodelling

4.2.1 Injury and repair

It is now well established that particles from cigarette smoke causes damage to the airways, particularly to the epithelial lining (26). Both non-symptomatic smokers and patients with COPD show signs of damage and repair to the epithelial surface in the form of denuded epithelial lining and squamous metaplasia (4). The processes of normal and abnormal wound healing as a response to injury have been studied extensively (27-31). 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. This process is characterized by an influx of platelets and inflammatory cells, predominantly neutrophils, followed by macrophages and T-lymphocytes. These platelets and inflammatory cells release many growth factors and cytokines, as well as fibrin and fibronectin, which act to repair wounded tissue. The environment of cytokines and growth factors, (myo)-fibroblast-derived extracellular matrix and adequate capillaries facilitate epithelial cell proliferation and migration, leading to wound closure (31). Within the airways, the bronchial epithelium, sub-epithelial myo-fibroblasts and ASM cells are the major cell types involved in tissue repair processes. Some of the important cellular and molecular events in the epithelial repair process and potential mechanisms leading to the remodelling of the airway are summarized in Figure 4.1.

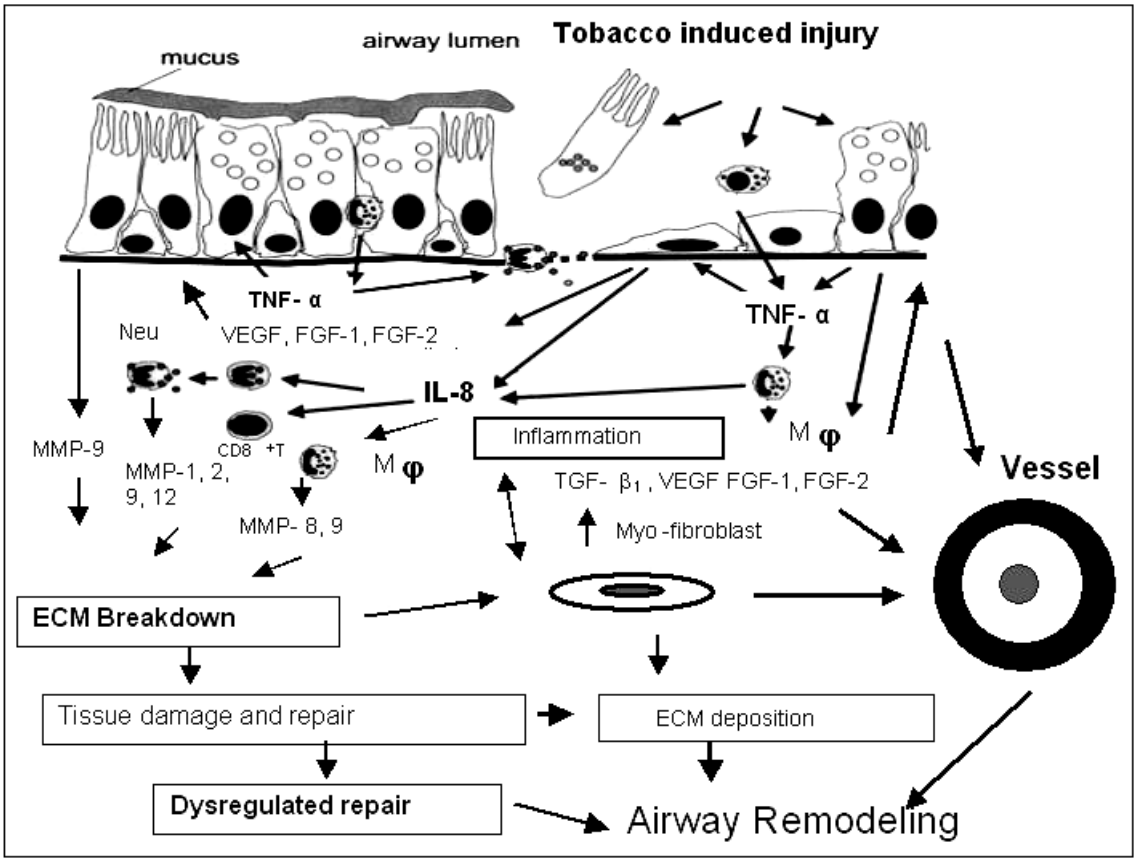

Figure 4.1: Cigarette smoke and airway injury leading to tissue remodelling

On exposure tobacco smoke, epithelial cells are damaged. Epithelial cells and resident macrophages produce inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8. In turn, inflammatory mediators stimulate migration of monocytes/macrophages, neutrophils, $CD8^+$ T-lymphocytes to the airway. Both TNF-α and IL-8 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 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-β_1 (TGF-β_1) inducing ECM production by myofibroblasts. Repetitive tissue damage and repair leads to excessive ECM deposition and subepithelial fibrosis, and ultimately to airway and vascular remodelling. Neu = neutrophil; Mφ = macrophage. Based on Refs (32, 33).

4.3 Vascular remodelling in COPD

Changes in the airway microvasculature have been described in many chronic respiratory diseases (34). Advances in staining methods and availability of vascular markers have improved the ability to visualize vessels in tissue specimens. The mechanisms and therapeutic implications of alterations in airway blood vessels are just beginning to be elucidated and changes in the microvasculature still represent an important gap in the understanding of the pathophysiology of asthma and other chronic inflammatory airway

disease (34). Patients with moderate to severe COPD display elevated pulmonary vascular pressures during exercise and pathological changes in the pulmonary circulation (4, 35). It has been postulated that emphysema actually may lead to loss of the pulmonary vascular bed and induce angiogenesis (36).

Vascular abnormalities are associated with the development of COPD; conversely, advanced COPD leads to pathological changes in the pulmonary circulation (35, 37). This is likely due, in part, to alveolar hypoxia, which is well known to cause pulmonary vasoconstriction and, if the hypoxic stimulus persists, pulmonary vascular remodelling (36). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (36). Intimal thickening due to fibrosis and emergence of smooth muscle cells within the intima of small pulmonary arterial branches has also been reported (38). Several studies have commented on the importance of structural and functional abnormalities in the pulmonary vasculature of COPD patients. Hypoxic vasoconstriction is considered to represent one of the major contributing factors of pulmonary hypertension and right-sided heart failure in COPD and other chronic pulmonary diseases (35, 39). In addition, emphysema, accompanied by loss of elastic recoil, increased pulmonary pressure and destruction of part of the pulmonary microvasculature, may contribute to the increased vascular resistance observed in COPD (36, 38). Thus, several phenomena acting in concert in COPD result in pulmonary vascular remodelling. Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

4.3.1 Angiogenesis

Mature endothelial cells are quiescent cells with an extremely low proliferative index. Smoke-induced injury with hypoxia, however, induces VEGF-A mRNA expression via hypoxia inducible transcription factors (HIF 1 to 3), (40, 41). 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 HSPGs (42). This, in turn, leads to ECM breakdown and the liberation of additional growth factors, predominantly VEGF-A, as well as FGF-2 and insulin-like growth factor-1 (IGF-1) sequestered within the surrounding matrix (40, 42). Proliferating endothelial cells migrate to distant sites in wounded or

inflamed tissue, guided by the actions of VEGF and FGF-2 in close contact with the collagen and heparan-sulfate proteoglycan matrix, thus resulting in endothelial tube formation (43).

4.3.2 Arteriogenesis and vascular remodelling

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 (ANGs) 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 (44). Binding ANG-1 to tie-2 induces endothelial cells to recruit fibroblasts or VSM, whereas ANG-2 binding to tie-2 inhibits this event (44). TGF-β_1 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 fibroblasts, thereby solidifying the endothelial-mural connections (42).

Pathological arteriogenesis involves hypoxia, tissue ischemia and increased sheer stress, which damage both endothelial and VSM cells (42). Inflammatory cells such as monocytes, macrophages and CD8+ T-lymphocytes infiltrate the vessel wall, causing additional damage to the vessel wall through release of mediators. Growth factors such as FGF-2, PDGF and TGF-β1 are released by endothelial and VSM cells in response to inflammatory mediators. Eventually dysregulated repair leads to fibrosis and vascular remodelling (42). Taken together, vascular remodelling and angiogenesis in peripheral, as well as in central airways could also be associated with the pathogenesis of COPD.

4.3.3 Bronchial vessels

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 by fibrosis, analogous to arteriosclerosis in cardiovascular disease (45, 46). In COPD, persistent alveolar hypoxia causes pulmonary vasoconstriction and increased muscularization of small arterial branches (36). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (36).

Recent observations indicate that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of $CD8^+$ T-lymphocytes and intimal thickening that correlates with collagen deposition (37, 47). We studied pulmonary vascular remodelling, assessed as the ratio of α-smooth muscle actin staining and vascular wall (VW) area to lumen diameter (48). Vascular medial thickness, assessed by video image analysis, was significantly increased in pulmonary vessels of various sizes in patients with COPD. Others used expression of the smooth muscle marker α-SMA to investigate whether the ratio of smooth muscle (α-SMA/VW area) in the vascular wall had changed during the progression of COPD (49). Surprisingly, the ratio of α-SMA stained area to VW area remained unchanged. Approximately 42% of cells in all vessels stained positive for α-SMA, indicating that the increase in wall thickness could be attributed to the deposition of extracellular matrix proteins and medial accumulation of inflammatory cells and fibroblasts. Recently, we demonstrated expression staining for extracellular matrix proteins, like fibronectin (Figure 4.2D) and collagen subtypes (Figure 4.2C) in the intimal vascular cells of these pulmonary vessels indicating for ongoing intimal fibrosis in COPD patients. Wall thickness of vessels 200 μm or more in diameter was increased in COPD (Figure 4.2A and B). Our results on pulmonary vascular remodelling particularly in terms of intimal and medial thickening (Figure 4.2) are in agreement with several earlier reports (35, 38, 47, 50, 51). Wright and coworkers (35, 38) also observed a correlation with the severity of disease with mild to moderate COPD with intimal thickening and in severe cases with medial thickening. Taken together, the data from this study indicate that vascular remodelling in COPD could be a contributing event in the pathogenesis of pulmonary hypertension in these patients. Furthermore, the observed changes in the intimal fibrosis as well as medial thickening could narrow the vessel calibre and may eventually lead to more severe vascular obstruction in COPD patients. Additionally, an inverse correlation of FEV_1 with medial thickening was found in pulmonary vessels of larger calibre, indicating that the degree of pulmonary vascular remodelling is related to the severity of obstructive lung function defect. Wright et al. (35, 38) also demonstrated increased wall thickness of small (<500 μm) pulmonary vessels in COPD subjects as compared to non-symptomatic smokers, which was correlated with the severity of the disease (as indicated by a decline in FEV_1).

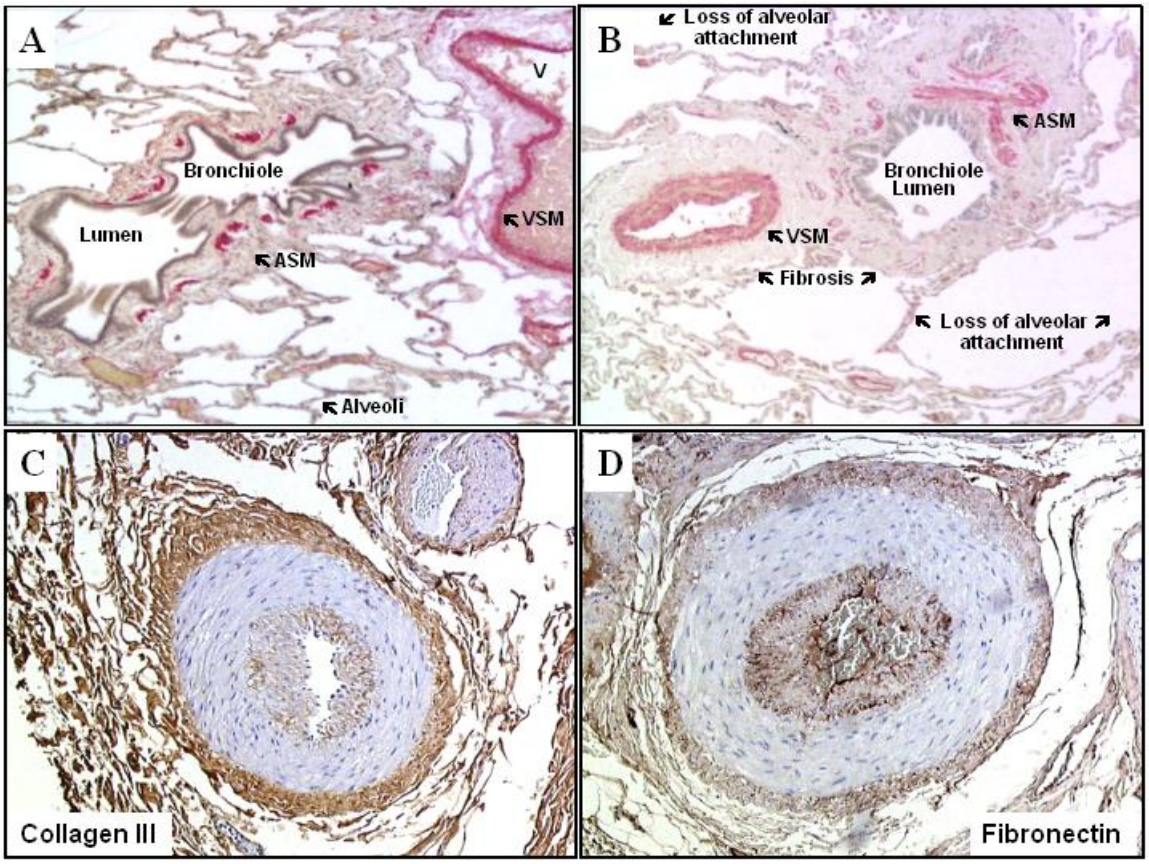

Figure 4.2: Histopathology of airway and vascular remodelling

*Photomicrographs of lung tissue sections from patients without COPD (**A**) and with COPD (**B**) showing α-smooth muscle actin staining (red new-fuchsine) in airway and vascular smooth muscle cells (ASM and VSM). Panel C and D represents the expression of Collagen III and Fibronectin respectively in a bronchial vessel of COPD patient depicting vascular intimal fibrosis and remodelling.*

Similar findings of vascular abnormalities in COPD were recently reported by Peinado and coworkers, who showed intimal but not medial thickening in the vasculature of mild COPD patients compared to non-smoking controls (47, 50). 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 correlated to the amount of total collagen deposition (37, 47).

Table 4.1: Major growth factors in vascular remodelling

Growth factor	Source	Target	Function
FGF-1	ECM	Fibroblast	Proliferation,
	Fibroblast	ASM	Collagen production
		VSM	Proliferation
		Epithelium	Collagen production
FGF-2	ECM	Endothelial cell	Proliferation
	Endothelial cell	Fibroblast	Proliferation
	ASM	ASM	Proliferation
	VSM	VSM	Proliferation
	Macrophages	Epithelium	Proliferation
VEGF	Epithelium	Endothelial cell	Proliferation,
	ASM		Migration
	Endothelial cells	Epithelial cells	Proliferation
	VSM	Fibroblast	Proliferation
	Macrophages		Recruitment
	ECM	Macrophages	Recruitment
TGF-ß	ECM	Fibroblast	ECM production
	Platelets,	ASM	Recruitment
	Macrophages	VSM	ECM production
	Fibroblast	Endothelial cell	ECM production
	ASM	Epithelium	Differentiation
		Neutrophil,	ECM production
		T-lymphocytes	Apoptosis
		Monocyt/macrophage	Differentiation
			ECM production
			Chemotaxis
PDGF	Platelets,	ASM	Proliferation
	Endothelial cell	Epithelium	Proliferation
	Macrophages	Fibroblast	Recruitment
	Fibroblast		Proliferation
	ASM		
	Epithelium		
IGF-1	ECM	Fibroblast	Proliferation and
	Fibroblast	ASM	Differentiation
		VSM	Collagen synthesis
IGF-2	ECM	Fibroblast	Proliferation
	Fibroblast		Differentiation
			Collagen synthesis

Abbreviations; Transforming growth factor beta (TGF-ß), 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 (27, 28, 52-54).

4.4 Growth factors involved in vascular remodelling

A variety of growth factors and cytokines released from various sites of airway and vascular walls have the potential to contribute to the pathogenesis of vascular remodelling in COPD.

The major sources, target cells and effects for several growth factors implicated in chronic lungs diseases are listed in Table 4.1. The main families of growth factors include the vascular endothelial growth factor (VEGF) family, fibroblast growth factors (FGFs), epidermal growth factor (EGF) family, transforming growth factor (TGF) β family, insulin-like growth factor (IGF) family, platelet-derived growth factor (PDGF) family and hepatocyte growth factor (HGF).

4.5 Vascular remodelling in Emphysema and Chronic Bronchitis

4.5.1 Expression of angiogenic growth factors in COPD

In patients with COPD, higher expression of VEGF is found in bronchial and alveolar epithelium and VSM as well as in alveolar macrophages, whereas higher VEGFR-1 and VEGFR-2 expression is found in the endothelium, compared to patients without COPD (55), (56). These data suggest a role for VEGF in tissue and vascular remodelling seen in COPD. One intriguing possibility is that functional differences exist for VEGFRs in the pathogenesis of COPD. For example, activation of VEGFR-2 is involved in angiogenesis by stimulating mitogenesis of endothelial cells during vascular damage-repair processes (57). In contrast, VEGFR1 is involved in stimulating vascular smooth muscle expression of MMP and endothelial expression of plasminogen activator and its inhibitor, activities needed for blood vessel maturation (58, 59). As VEGFR-1 has a higher affinity for VEGF than VEGFR-2, it is thought that VEGFR-1 is involved in resolving the angiogenic process (59, 60). Pulmonary vascular smooth muscle expression of VEGF and VEGFR-2 are increased in smokers with COPD or chronic bronchitis, whereas VEGFR-1 expression did not differ between smokers with or without COPD in these compartments (37, 55). In contrast to Kasahara *et al* (61) who showed that VEGF and its receptor VEGFR-2 were decreased in total lung extracts of emphysematous lungs as measured by ELISA or western blot analysis, we (55) found that the epithelial and endothelial cells in the alveolar spaces and in the most distal airways were intensely positive for VEGF and VEGFR-2 in patients with COPD. In our study the patients could be considered as having mild to moderate COPD whereas the lungs studied by Kasahara et al (61) were solely emphysematous. Likewise, in COPD, expression levels of FGF-1, FGF-2 and FGFR1 are increased in vascular and in epithelial compartments in patients with COPD compared to subjects without COPD (48, 62). Table 4.2 summarises the expression levels of angiogenic growth factors in COPD patients.

Table 4.2: Pulmonary expression of angiogenic growth factors in COPD, chronic bronchitis and emphysema

Growth Factor	Expression*	Disease
EGF	Increased	Chronic Bronchitis
EGFR	Increased	COPD
FGF-1	Increased	COPD
FGF-2	Increased	COPD
FGFR1	Increased	COPD
TGFβ-1	Increased	COPD
VEGF	Increased	COPD
VEGF	Decreased	Emphysema
VEGFR1	Increased	COPD
VEGFR2	Increased	COPD
VEGFR2	Decreased	Emphysema

**Pulmonary expression or circulating levels of various growth factors in comparison to age matched controls*

4.5.2 Role of VEGF and its receptors in pathogenesis of Emphysema and Chronic Bronchitis

The destruction of lung tissue in emphysema may involve the progressive loss of capillary endothelial and epithelial cells through the process of programmed cell death, apoptosis. Several lines of evidence suggest that VEGF induces the expression of anti-apoptotic proteins and thus acts as an endothelial survival factor (63-65). A recent study demonstrated that patients with emphysema had decreased levels of VEGF messenger RNA and protein, as well as decreased expression of VEGFR-2. Furthermore, decreased VEGF and VEGFR-2 expression were associated with endothelial and epithelial cell death in alveolar septa (61). Several recent animal studies have demonstrated that VEGFR-2 blockade, in combination with chronic hypobaric hypoxia, destroys lung capillaries by inducing endothelial cell apoptosis and at the same time causes occlusion of precapillary pulmonary arteries by proliferating endothelial cells (66-69). Chronic treatment of animals with SU5416, a VEGFR-2 inhibitor, results in increased pulmonary artery pressure in both normoxic and hypoxic animals, widespread endothelial cell apoptosis and emphysema, when compared with drug vehicle treated controls (17). Similar findings of emphysema have been reported in mice depleted of pulmonary VEGF by a Cre/LoxP-targeted deletion technique (70) and in animals treated with a soluble Flt-Fc chimaeric protein (71). Thus, the expression of VEGF may be protective against signals leading to apoptosis such as toxic agents from tobacco smoke. Cigarette smoke, possibly by inducing nitric oxide (72), may

also act to decrease the expression of VEGF and VEGFR-2, thus resulting in lung septal endothelial cell death (61).

In chronic bronchitis, increased VEGF levels may be due, in part, to ongoing repair processes (55, 73). Small airway changes are associated with thickening of the airway wall and disruption of mucociliary clearance, resulting in the accumulation of inflammatory exudates in the airway lumen. In addition, Hogg et al. postulated that colonization and infection of the lower airways was associated with an adaptive immune response that accounts for the increase in lymphocytes and their organization into lymphoid follicles in patients with severe COPD (74). Thus, both the innate mucociliary clearance system (75), the adaptive immune responses (74) appear to be important for the repair process.

Kanazawa et al. (73) examined VEGF levels in the induced sputum of patients with emphysema, chronic bronchitis and asthma and found correlations with both forced expiratory volume in 1 s [FEV_1] and diffusing capacity of lung for carbon monoxide (DLco). In emphysema patients, lower FEV_1 or DLco directly correlated with lower VEGF levels in sputum. In contrast, patients with chronic bronchitis or asthma demonstrated an inverse correlation between VEGF levels and FEV_1. These data suggest a positive association between the severity of airway inflammation and VEGF secretion in these two diseases. Given the scarcity of evidence, however, it is difficult to conclude that there is a differential role for VEGF in the pathogenesis of emphysema or chronic bronchitis. As Wagner rightly points out, it is unknown whether VEGF expression is below a critical threshold level and indeed the cause of emphysema, or whether reduced expression is merely a marker of the disease process, without functional importance (7). However, emerging evidences gives us a clear indication that there is modulation of angiogenic factors like VEGF in COPD patients that appears to correlate with the underlying pathophysiology.

Signalling through VEGFR-1/flt-1 may also be important to the pathogenesis of emphysema. A recent study of PlGF transgenic mice demonstrated enlarged airspaces without airway inflammation (76). Over expression of PlGF resulted in apoptosis of type II pneumocytes, leading to decreased VEGF secretion and subsequent endothelial cell death. These authors hypothesize that a positive feedback loop exists between type II pneumocytes

that secrete VEGF and integrity of the septal microcirculation that promotes pneumocyte survival (76).

4.5.3 Angiogenic growth factors as potential therapeutic targets

VEGF and its receptors are critical regulators of angiogenesis, tumorigenesis and metastasis. Several drugs have been developed to reduce tumour growth by impairing neovascularization. Among those in clinical trials are VEGF-Trap, bevacizumab (Avastin), CEP-7055, SU6668 and PTK787 (77). Avastin is a humanized monoclonal IgG1 antibody against VEGF that inhibits receptor binding. CEP-7055 is a small molecule, water-soluble indenopyrrolocarbazole derivate inhibitor of pan-VEGFR tyrosine kinase activity. It has been shown to inhibit pulmonary VEGFR-2 activation *in vivo*, capillary formation by human and rat endothelial cells *in vitro* and *in vivo*, granuloma formation and vascularisation in a chronic inflammation model, and tumorigenesis (78). VEGF Trap is a soluble VEGFR constructed from VEGFR1 and VEGFR2 binding domains linked to an IgG1 constant region. *In vivo* animal models have demonstrated that VEGF Trap reduce haemangiogenesis, lymphangiogenesis, and tumour growth (79, 80). SU6668 is an IgG2a fusion protein inhibiting the receptor tyrosine kinase activity of FGFR1, KDR and PDGF-Rβ. SU6668 has been shown to inhibit tumour growth, lung cancer metastasis and tumour vascularisation (81). Many other small molecule VEGFR inhibitors are being developed for clinical therapy (82).

Recent studies support a role for VEGF/VEGFR in pulmonary and vascular remodelling and inflammation. For example, VEGF transgenic mice exhibit alveolar vascular and airspace remodelling as well as a Th2-type airway inflammation in a murine model of experimental asthma (83, 84). In this study, the VEGF-R inhibitor SU1498, as well as VEGF-TRAP, decreased airway inflammation and airway hyperresponsiveness (83, 84). Therefore, VEGF inhibitors may be of potential use for the treatment of inflammation and neovascularization seen in asthma or specific subtypes of COPD, like chronic bronchitis. However, the redundancy of the VEGF-VEGFR system may limit the applicability and efficacy of VEGF/VEGFR inhibitors (85). In contrast, in patients with emphysema, therapy may need to be targeted more towards inhibition of apoptosis or stimulation of VEGF expression, in order to overcome endothelial and epithelial cell death and maintain alveolar

septal integrity (86). Hence, treatment with VEGF/VEGFR inhibitors should be further investigated and may be restricted to specific chronic lung diseases.

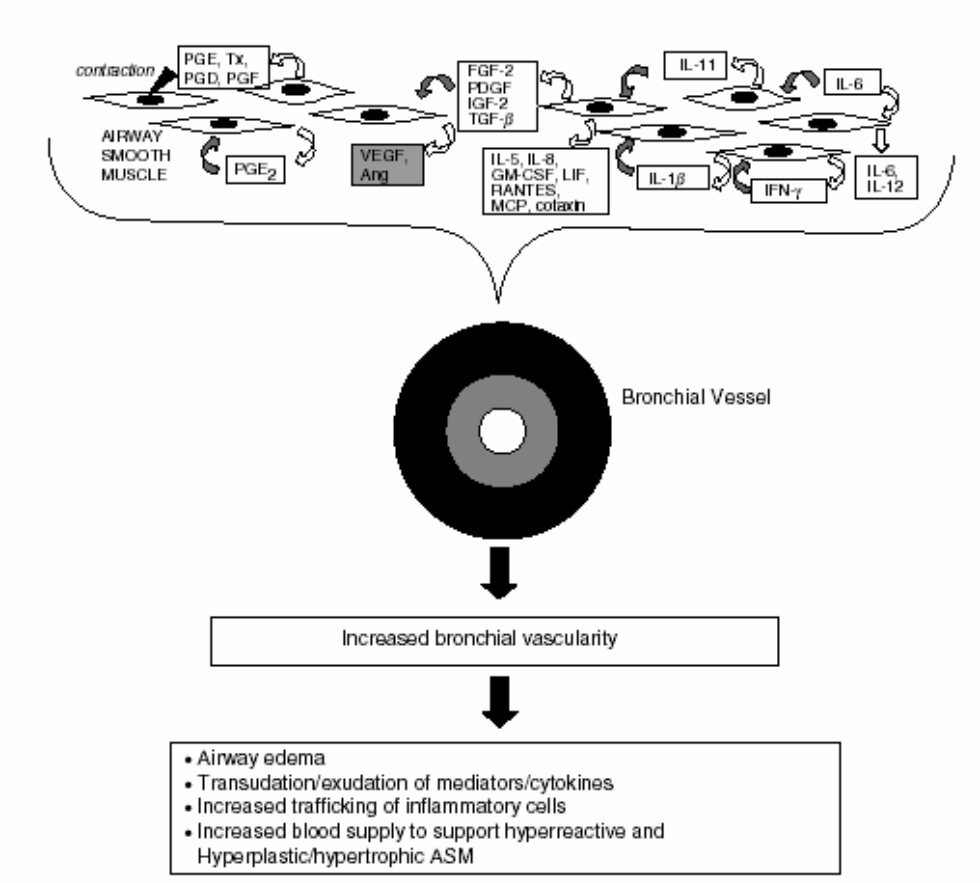

Figure 4.3: The potential role of airway smooth muscle cells in bronchial vascular remodelling

The expression and secretion of cytokines, chemokines and growth factors by ASM cells can alter the proliferative response of vascular endothelial and smooth muscle cells. These complex interactions may contribute to the overall airway and vascular remodelling process by promoting increased vascularity, endothelial permeability and airway wall oedema and trafficking of inflammatory cells during chronic lung diseases.

4.6 Conclusion

Current *in vivo* and *in vitro* data indicate that cross-talk between smooth muscle cells, endothelium, myofibroblasts and inflammatory cells via growth factors and cytokines, are major contributing factors to vascular remodelling during different pathophysiological conditions (49, 87-89). Figure 4.3 summarizes the possible interactions between the ASM-derived mediators and the adjacent endothelial and vascular smooth muscle cell compartment in the bronchial blood vessels and how they contribute to the remodelling process. At present, our knowledge of airway and vascular remodelling in COPD is far from complete. Increased adventitial infiltration of inflammatory cells, predominantly $CD8^+$

T-lymphocytes, in muscular pulmonary and bronchiolar arteries has been reported (47, 90). Many growth factors, among them VEGF and FGF, play an essential role in maintaining pulmonary and vascular viability and in tissue repair. Based on the available evidence, we hypothesize that in chronic bronchitis, expression of VEGF and VEGFR-2 expression leads to increased vascular remodelling, which is inefficiently compensated by the low expression of VEGFR-1. In subjects with emphysema, however, VEGFR2 expression is lower. Preferential activation of VEGFR-1 results in higher MMP activity, alveolar destruction and endothelial apoptosis. Hence, the balance between VEGF, VEGFR-1 and VEGFR-2 are critical in the pathogenesis of COPD subtypes.

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4.7 References

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5

Vascular Endothelial Growth Factor and its Receptors in COPD

Based on: "Kranenburg AR, de Boer WI, Alagappan VK, Sterk PJ, and Sharma HS. Enhanced Bronchial Expression of Vascular Endothelial Growth Factor and Receptors (Flk-1 and Flt-1) in Patients with Chronic Obstructive Pulmonary Disease. Thorax 2005; 60(2): 106-13".

Chapter 5: Vascular Endothelial Growth Factor and its Receptors in COPD

Summary
Ongoing inflammatory processes resulting in airway and vascular remodelling characterize chronic obstructive pulmonary disease (COPD). Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) could play a role in tissue remodelling and angiogenesis in COPD. We examined the cellular expression pattern of VEGF, Flt-1 and KDR/Flk-1 by immunohistochemistry in central and peripheral lung tissues obtained from (ex-) smokers with (FEV_1 <75% predicted; n=14) or without COPD (FEV_1 >85% predicted; n=14). The immunohistochemical staining of each molecule was quantified using a visual scoring method with grades ranging from 0 (no), 1 (weak), 2 (moderate) to 3 (intense). VEGF, Flt-1 and KDR/Flk-1 immunostaining was localized in vascular and airway smooth muscle (VSM and ASM) cells, bronchial, bronchiolar and alveolar epithelium and macrophages. Pulmonary endothelial cells abundantly expressed Flt-1 and KDR/Flk-1 but not VEGF. In COPD patients, bronchial VEGF expression was higher in microvascular VSM cells and ASM cells as compared to non-COPD patients (1.7 and 1.6 fold, $p \leq 0.01$, respectively). VEGF expression in intimal and medial VSM (1.7 and 1.3 fold, $p \leq 0.05$) of peripheral pulmonary arteries associated with the bronchiolar airways was more intense in COPD, as well as in small pulmonary vessels in the alveolar region (1.5 and 1.7 fold, $p \leq 0.02$). In COPD patients, KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM (1.3, 1.9 and 1.5 fold, $p \leq 0.02$), whereas endothelial Flt-1 expression was 1.7 times higher ($p \leq 0.03$). Furthermore, VEGF expression was significantly increased in bronchiolar and alveolar epithelium as well as bronchiolar macrophages (1.5 fold, $p \leq 0.001$). Additionally, expression of VEGF in bronchial VSM and mucosal microvessels as well as bronchiolar epithelium inversely correlated with FEV1 ($r < -0.45$; $p \leq 0.01$). Our results suggest that VEGF and its receptors Flt-1 and KDR/Flk-1 are involved in peripheral vascular and airway remodelling processes in an autocrine and/or paracrine manner. This system may also be associated with epithelial cell viability during airway wall remodelling in COPD.

5.1 Introduction

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

Vascular abnormalities have been associated with the development of COPD (7, 8). Wright et al. found an increase in wall area of small (<500 μm) pulmonary vessels, by intimal thickening in mild to moderate COPD patients and medial thickening in severe cases as well, which was correlated with a decline in FEV_1 (7, 9). Furthermore, recent observations indicated that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of $CD8^+$ T-lymphocytes and have intimal thickening that was correlated to the amount of total collagen deposition (8, 10). Finally, emphysema may lead to loss of the pulmonary vascular bed and induce angiogenesis (11). Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

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

Although the role of VEGF in the vascular biology is thoroughly studied, it has become clear that VEGF and its receptor system are involved in various other cellular events as well, including epithelial proliferation and survival, and the recruitment of mast cells, neutrophils and macrophages to sites of fibrosis (13, 15, 16). Recent studies indicate that VEGF is expressed in the lung by bronchiolar, submucosal glandular and alveolar type I and II epithelial cells, alveolar macrophages, airway and vascular smooth muscle (ASM and VSM) cells as well as myo-fibroblast in fibrotic lung lesions (14, 17, 18).

In order to assess the role of VEGF and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) in the pathophysiology of COPD, we first examined the expression of VEGF-A, Flt-1 and KDR/Flk-1 in central and peripheral lung tissue from (ex-) smokers with or without COPD. Furthermore, we investigated the relation of lung function with the expression data of VEGF and its receptors.

5.2 Materials and methods

5.2.1 Selection of patients

Central and peripheral lung tissues were obtained from current or ex-smokers who underwent lobectomy or pneumonectomy for lung cancer. Fourteen subjects with COPD (FEV_1 < 75% predicted) and fourteen subjects without COPD (FEV_1 > 84% predicted) were included as previously described (19-21). Total lung capacities (TLC) were not below normal levels (TLC >80% predicted). All patients lack upper respiratory tract infection and did not receive antibiotics perioperatively. None of the patients had received glucocorticosteroids during 3 months period before resection, but four patients received glucocorticosteroids perioperatively. Based on these criteria, subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone. Clinical data are given in Table 5.1. Subjects were excluded if the obstruction of the central bronchi was due to the tumour, or if diffuse pulmonary inflammation or fibrosis was present, or if no tissue free from tumour could be obtained. Lung tissue specimens used in this study were obtained from the archival collection at the Department of Pathology (LUMC, Leiden, NL). Medical Ethics Committee of LUMC approved the study. The patients in these two groups participated in a larger research project, part of which has been published previously (19-21). Lung tissue specimens were routinely fixed in 10% neutral buffered formalin by inflation-immersion fixation and embedded in paraffin for histopathological examination and immunohistochemistry.

5.2.2 Immunohistochemistry

Paraffin sections (4 μm thick) of the lung tissues were cut and mounted on silane-coated glass slides. Immunohistochemistry was performed using a method as described earlier (20, 22, 23). In brief, after deparaffinization in xylene and rehydration through graded alcohol, slides were rinsed with phosphate buffered saline (PBS). Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. For VEGF, VEGFR-1, VEGFR-2 and Ki-67

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

5.2.3 Quantitative scoring analysis of immunohistochemistry

Prior to screening, sections were coded so that the observers were unaware of the clinical details of the case under study. Expression of VEGF, Flt-1 and KDR/Flk-1 was analyzed semi-quantitatively, using a visual scoring method with grades ranging from 0 to 3 (0 = no staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining) as previously described (8, 19, 20, 24). The entire section of a tissue block was investigated and scored at

the same magnification. The staining intensity of VEGF, Flt-1 and KDR/Flk-1 was scored blindly by two independent observers, who were unaware of the clinical data of the case under study, in bronchial and bronchiolar airways as well as alveolar parenchyma in cells of epithelial, endothelial and smooth muscle origin as well as macrophages. We examined errors within and between observers by correlating the expression scores using Pearson's analysis and found a very high correlation ranging from 0.8 to 0.9. In the bronchial airways staining was assessed in the bronchial epithelium, mucosal microvasculature, submucosal bronchial wall vessels, airway smooth muscle (ASM) cells and macrophages in the bronchial airway wall. In peripheral lung tissues the staining of VEGF and receptors was analyzed in bronchiolar an alveolar epithelium, bronchiolar ASM cells, and bronchiolar and alveolar macrophages. The vasculature in the peripheral lung was further subdivided into the larger pulmonary vessels associated with the bronchiolar airways and smaller vessels situated within the alveolar parenchyma. In each the VEGF and receptor staining of endothelial, intimal and medial VSM cells were assessed. Since TGF-β_1 may also induce VEGF expression in epithelial cells (25, 26), we assessed the correlation between the epithelial VEGF expression from the current study and epithelial TGF-β_1 expression from one of our previous studies (20). In both studies the same patient groups were used and the staining was performed on adjacent or near sections.

5.2.4 Statistical analysis

Data were analyzed for statistical significance using the unpaired, two-tailed Students' t-test as well as the non-parametric Mann-Whitney test, where appropriate. The expression data for VEGF and its receptors were expressed as mean±SEM. Furthermore, VEGF and its receptors staining for different compartments were correlated with FEV_1 using Pearson's correlation analysis. Differences with $p \leq 0.05$ were considered to be statistically significant.

5.3 Results

5.3.1 Clinical parameters

The clinical and lung function characteristics of all subjects included in the study are listed in Table 5.1. As defined, the COPD group demonstrated decreased FEV_1 and FEV_1/FVC values, ($p \leq 0.001$) as has been described previously (19-21). The subjects in the two groups did not differ significantly in age and smoking status (pack-years) or steroid use (Table 5.1).

Table 5.1: A summary of the clinical characteristics of subjects with and without chronic obstructive pulmonary disease

Group	Sex (M/F)	Age	PY	FEV1 (% Pred.)	FEV1/FVC (%)	Steroid treatment
Non-COPD	10/4	64 (3.7)	42 (7.7)	101 (3.3)	0.72 (0.02)	None
COPD	14/0	64 (2.3)	44 (0.8)	63 (2)	0.54 (0.02)	4
p-value		0.84	0.82	≤0.001	≤0.001	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; Forced expiratory volume in 1 s (FEV1) and Forced vital capacity (FVC) are given as percentages of the predicted values (% Pred.) before bronchodilatation. M = Male; F = Female. PY = number of pack years. Data shown represent means with standard deviation in brackets. The patients in these two groups participated in a larger project, part of which has been published previously (19-21).

5.3.2 Immunolocalisation of VEGF, Flt-1 and KDR/Flk-1

Bronchial airways

Examples of VEGF expression in central airways of non-COPD and COPD subjects are given in Figure 5.1A and 5.1B, whereas 5.1C and 5.1D (both taken from COPD subjects) show the VEGF receptors Flt-1 and KDR/Flk-1, respectively. In all subjects, within the airways VEGF, Flt-1 and KDR/Flk-1 were localized in the bronchial epithelium and airway smooth muscle (ASM) cells, bronchial microvasculature of mucosa and submucosa and on inflammatory cells, predominantly macrophages, (Figure 5.1A-D). In the vessel wall, vascular smooth muscle (VSM) cells were positive for VEGF, Flt-1 and KDR/Flk-1, whereas endothelial cells did not stain for VEGF protein but were positive for the Flt-1 and KDR/Flk-1 (Figure 5.1). To assess the intensities of VEGF, Flt-1 and KDR/Flk-1 expression in various bronchial airway compartments, we opted for a visual scoring method as previously described (8, 19, 20, 24). VEGF expression was increased in bronchial airway smooth muscle cells of COPD patients as compared to non-COPD subjects (1.6 fold, $p \leq 0.01$) but not in bronchial epithelial cells and macrophages (Figure 5.2A). In the central airways of patients with COPD as compared to non-COPD subjects, VEGF staining was more intense in VSM of microvasculature the bronchial mucosal (lamina propria) (1.7 fold, $p \leq 0.001$) and bronchial VSM in the submucosa (1.4 fold, $p \leq 0.01$, Figure 5.2A). No significant differences were observed when considering the expression levels of KDR/Flk-1 and Flt-1 between COPD subjects and non-COPD patients (Figure 5.2B and 5.2C, respectively). In all subjects VEGFR-2 (KDR/Flk-1) expression was more intense than

VEGFR-1 (Flt-1) expression, except for the expression in endothelial cells of bronchial microvessels and on bronchial macrophages, which were comparable (Figure 5.2B and 5.2C).

Bronchiolar airways

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

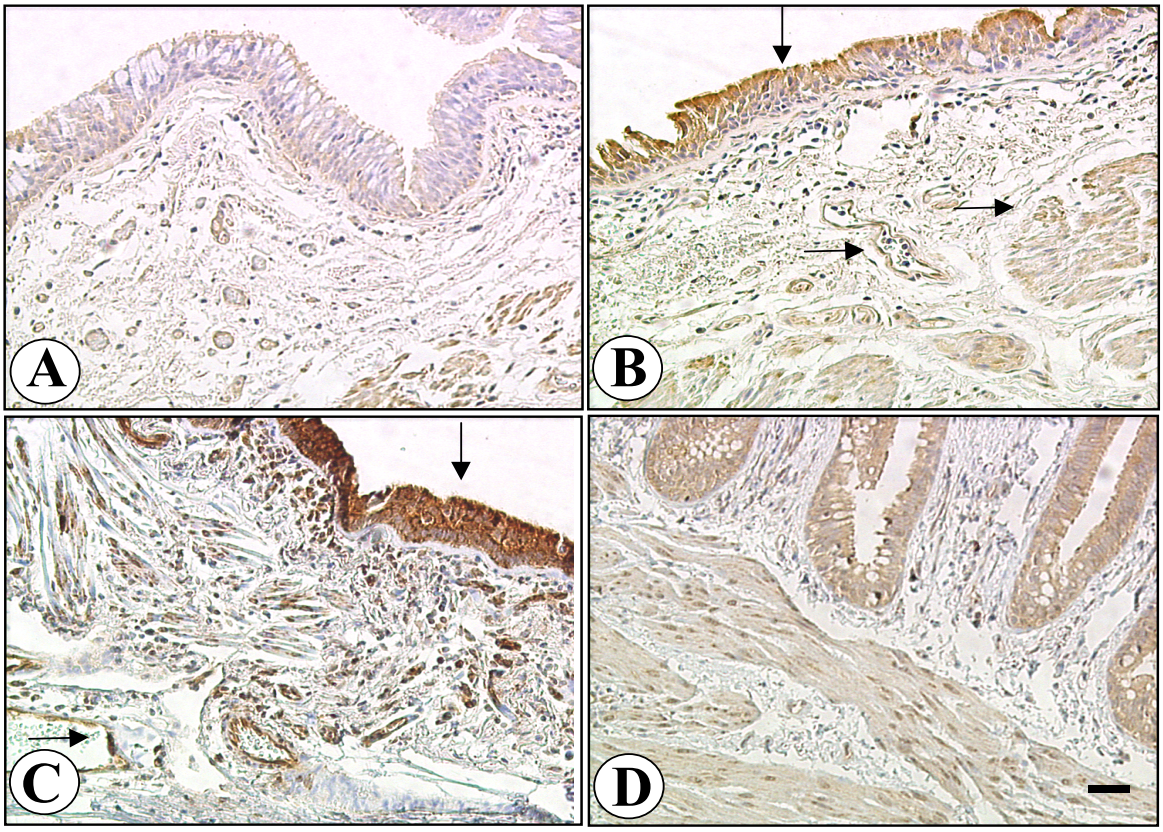

Figure 5.1: Expression of VEGF and its receptors in bronchial tissues

Immunohistochemical localization of VEGF (A-B), KDR/flk-1 (C) and flt-1 (D) in bronchial tissues from non-COPD (ex-) smoking subjects (A) and patients with COPD (B, C, D). Immunoreactive VEGF, KDR/flk-1 and flt-1 were localized in bronchial epithelial cells, airway smooth muscle (ASM) cells and in macrophages, endothelial and vascular smooth muscle (VSM) cells. Colour is developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown colour) and counterstained with Mayer's haematoxylin. Arrows indicate sites of positivity for VEGF, flt-1 or KDR/flk-1. Original magnification: x100; Scale bar = 50 µm.

Airway smooth muscle cells showed slightly increased VEGF expression in bronchiolar region (1.3 fold, $p \leq 0.05$), whereas the expression of both the receptors remained unchanged in two patient groups. However, the expression of KDR/Flk-1 was more intense than Flt-1 in all patients (Figure 5.4B and 5.4C).

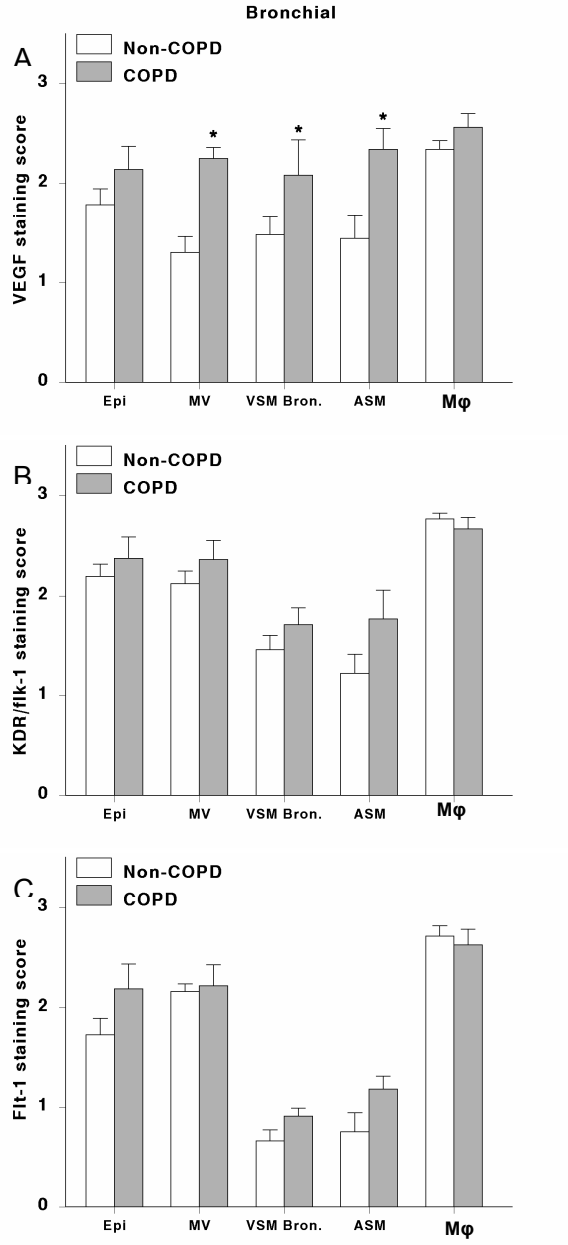

Figure 5.2: Quantification of expression pattern of VEGF, KDR/flk-1 and flt-1 in COPD

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

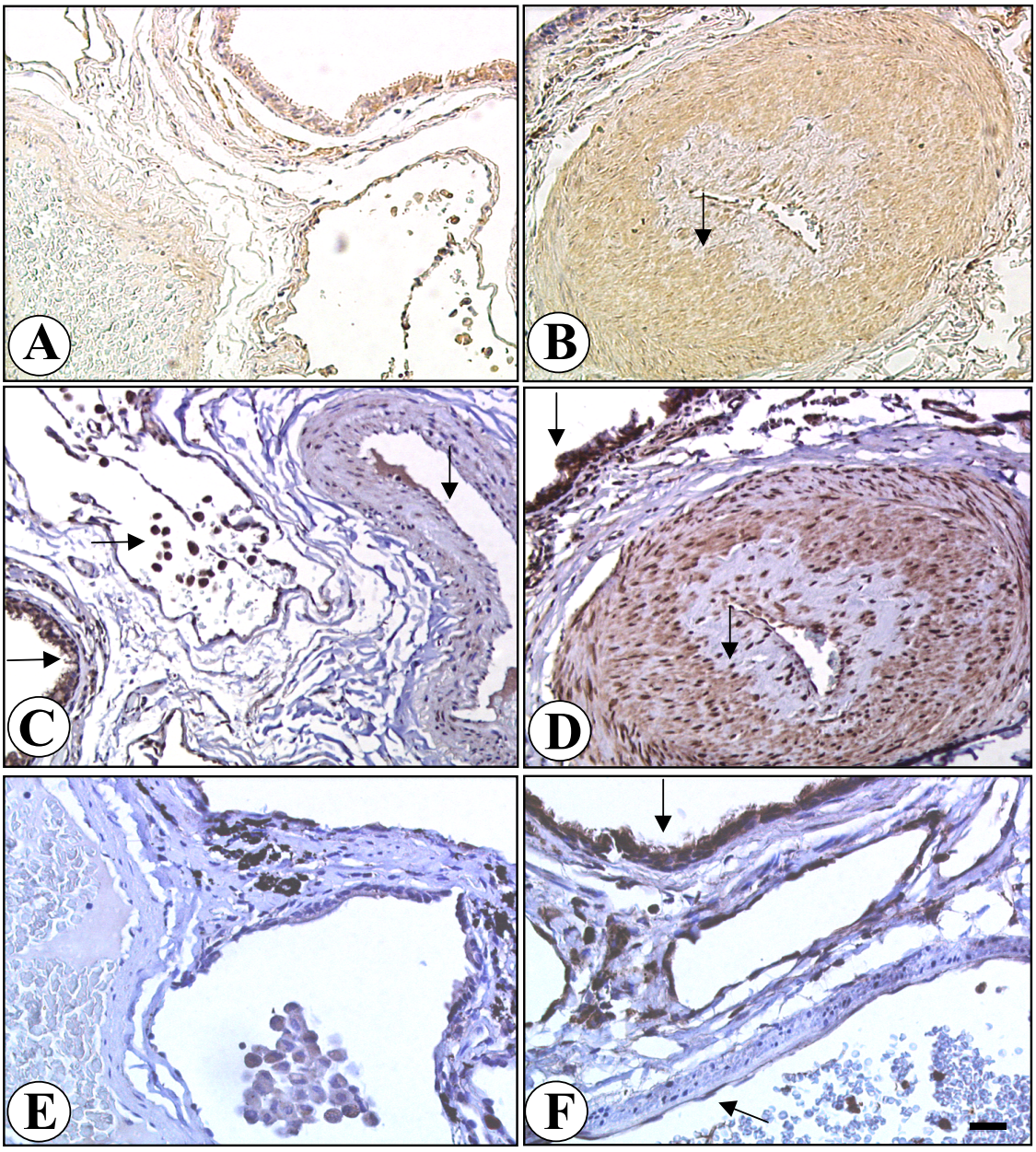

Figure 5.3: Expression of VEGF and its receptors in peripheral lung tissues

Immunohistochemical localization of VEGF (A-B), KDR/flk-1 (C-D) and flt-1 (E-F) in peripheral tissues from non-COPD (ex-) smoking subjects (A, C, E) and patients with COPD (B, D, F). Immunoreactive VEGF, flt-1 and KDR/flk-1 were localized in bronchiolar and alveolar epithelial cells, airway smooth muscle (ASM) cells, macrophages and in endothelial and intimal/medial vascular smooth muscle (VSM) cells. Colour is developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown colour) and counterstained with Mayer's haematoxylin. Arrows indicate sites of positivity for VEGF, flt-1 or KDR/flk-1. Original magnification: x100; Scale bar = 50 µm.

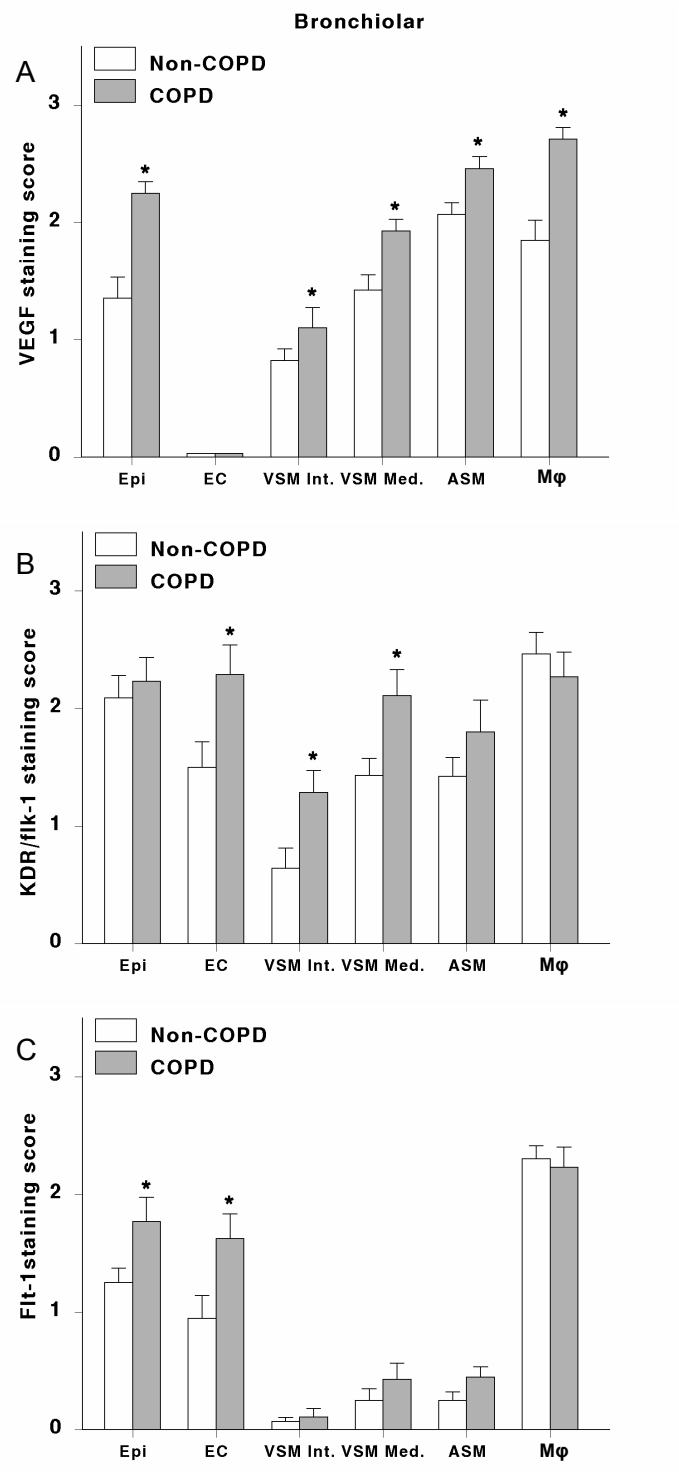

Figure 5.4: Quantification of expression pattern of VEGF, KDR/flk-1 and flt-1 in bronchiolar airways and pulmonary arteries

*Graphic representations of VEGF (**panel A**), KDR/flk-1 (**panel B**) and flt-1 (**panel C**) protein expression in different cell types in bronchiolar airways and associated pulmonary arteries using visual scoring. Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean±SEM. An asterisk indicates a significant difference ($p \leq 0.05$, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchiolar epithelium (Epi), endothelial cells (EC), intimal and medial vascular smooth muscle cells (VSM int. and med), airway smooth muscle (ASM) and bronchiolar macrophages (Mφ).*

When considering the expression of VEGF in the larger pulmonary arteries associated with the bronchiolar airways, the fold in intimal and medial VSM staining was 1.7 and 1.3 ($p \leq 0.05$, Figure 5.4A) between COPD and control subjects respectively, whereas endothelial cells did not express VEGF. KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM (1.3, 1.9 and 1.5 fold, $p \leq 0.02$, Figure 5.4B), whereas the corresponding value in endothelial cells for Flt-1 expression is 1.7 ($p \leq 0.03$, Figure 5.4C).In both patient groups, the intimal VSM stained 2-3 times less intense than medial VSM for VEGF, Flt-1 and KDR/Flk-1. Moreover, the vascular Flt-1 expression was lower than KDR/Flk-1 and VEGF in each of the investigated vessel wall areas ($p \leq 0.002$, Figure 5.4). Staining of VEGF in bronchiolar macrophages (1.5 fold, $p \leq 0.001$, Figure 5.4A) was increased in COPD as compared to non-COPD subjects, whereas the staining on macrophages of Flt-1 or KDR/Flk-1 expression in bronchiolar airways as well as VEGF, Flt-1 or KDR/Flk-1 in the alveolar region remained unchanged (Figure 5.5).

Alveolar parenchyma

Staining of alveolar epithelial cells (type I and II) for COPD was more intense than for non-COPD controls (1.5 fold, $p \leq 0.0001$, Figure 5.5A). KDR/Flk-1 and Flt-1 expression were not changed in alveolar epithelial cells (Figure 5.5B and 5.5C). VEGF expression was increased in intimal and medial VSM (1.5 and 1.7 fold, $p \leq 0.01$, Figure 5.5A) of small pulmonary vessels in the alveolar region whereas the corresponding values for KDR/Flk-1 were 2.0 and 1.8 ($p \leq 0.02$), respectively (Figure 5.5B). Furthermore, the expression of both KDR/Flk-1 and Flt-1 were increased in endothelial cells of small pulmonary vessels in lung parenchyma (1.7 and 2.1 fold, $p \leq 0.001$, Figure 5.5B and 5.5C).

5.3.3 Correlation between staining and clinical data

We examined the relation between FEV1 values of patients in both groups and the staining scores of VEGF, Flt-1 and KDR/Flk-1 in the investigated areas. Within the bronchial airways, FEV1 values were inversely correlated with VEGF staining sores in bronchial mucosal microvasculature ($r = -0.65$; $p \leq 0.001$, Figure 5.6A), bronchial ASM cells ($r = -0.45$; $p \leq 0.01$, Figure 5.6B) if all subjects were analyzed together.

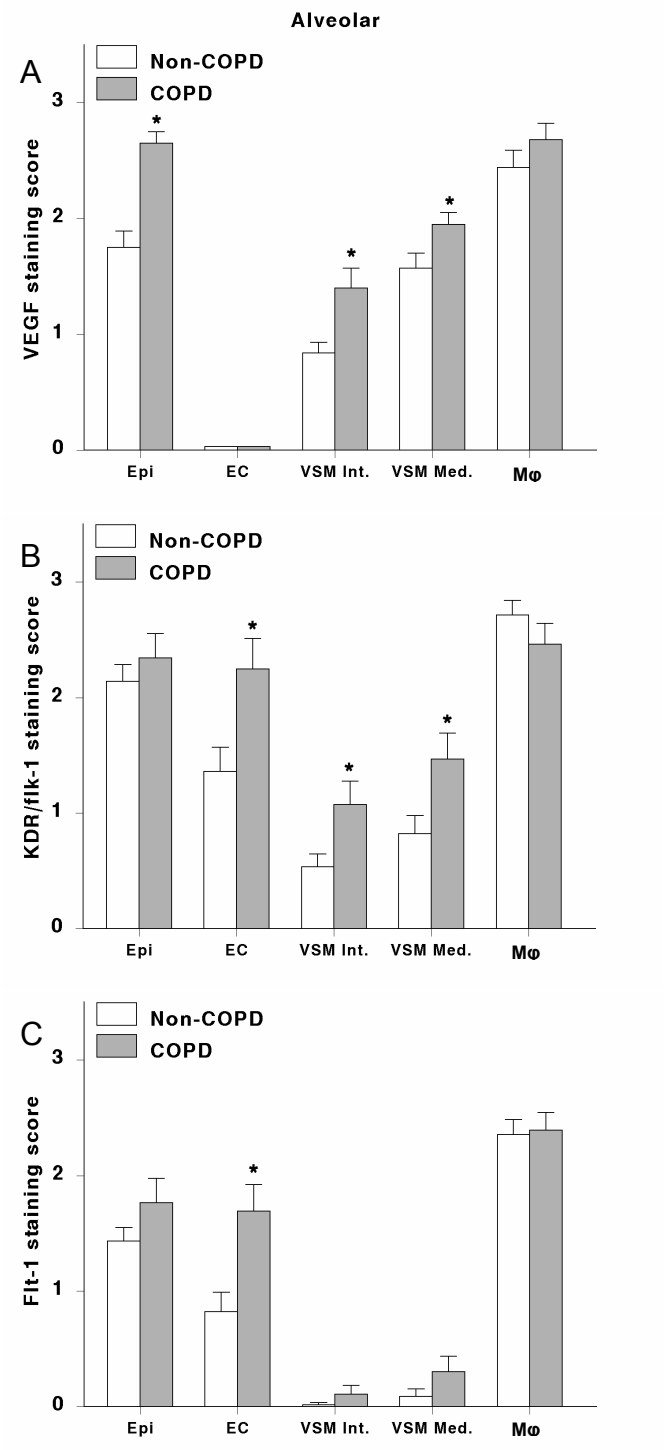

Figure 5.5: Quantification of expression pattern of VEGF, KDR/flk-1 and flt-1 in alveolar parenchyma

Graphic representations of VEGF (panel A), KDR/flk-1 (panel B) and flt-1 (panel C) protein expression in different cell types in alveolar parenchyma and pulmonary vasculature using visual scoring. Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean±SEM. An asterisk indicates a significant difference ($p \leq 0.05$, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchiolar epithelium (Epi), endothelial cells (EC), intimal and medial vascular smooth muscle cells (VSM int. and med.), and alveolar macrophages (Mφ).

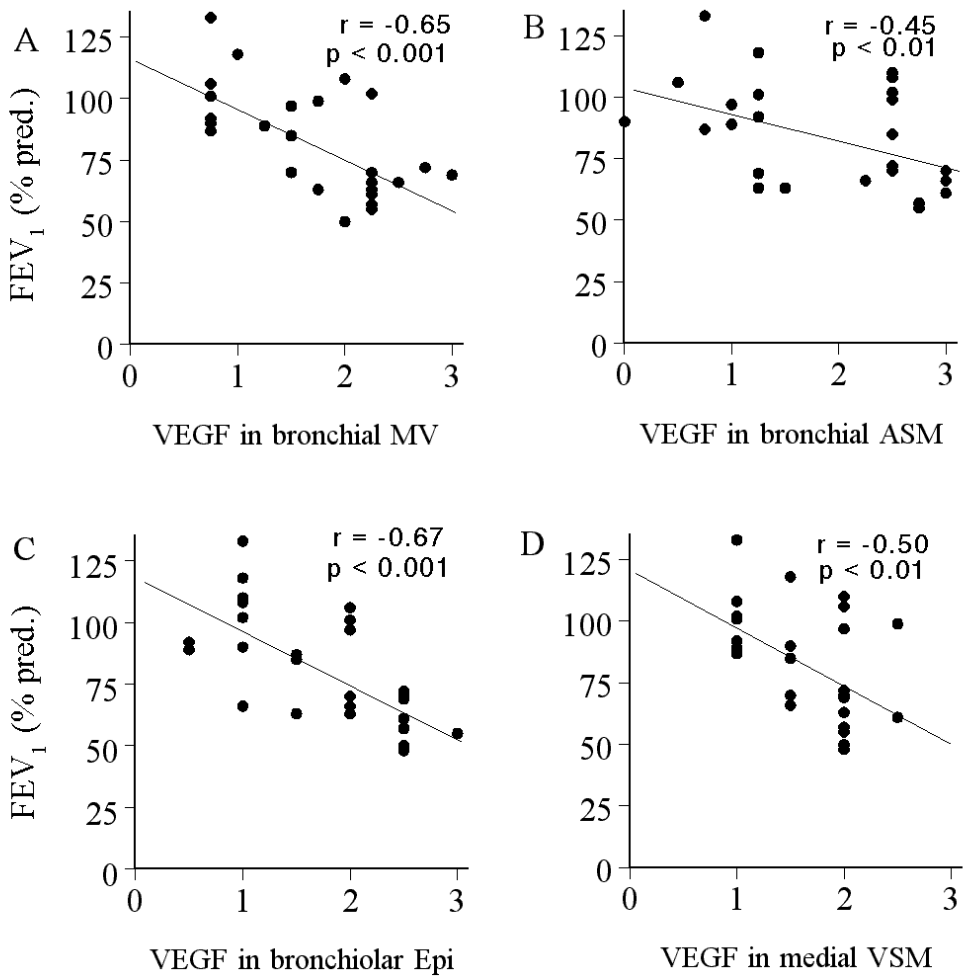

Figure 5.6: Correlation of lung function with VEGF expression in airway

*Correlation with FEV_1 (% predicted) of VEGF protein expression in microvessels (MV) in the bronchial mucosa (**A**), bronchial airway smooth muscle (ASM) cells (**B**), bronchiolar epithelial (Epi) cells (**C**) and medial vascular smooth muscle (VSM) cells of pulmonary arteries associated with the bronchiolar airways (**D**). Correlation was assessed for the combined patient groups (non-COPD and COPD). Correlation coefficient (r) was obtained using linear regression (Pearson's) analysis.*

The bronchiolar epithelium (r = -0.67; p≤0.001, Figure 5.6C) and medial VSM of larger pulmonary arteries associated with bronchiolar airways (r = -0.50; p≤0.01, Figure 5.6D) also showed an inverse correlation with FEV_1 values from the total group. Additionally, VEGF expression in medial VSM was correlated with KDR/Flk-1 expression in endothelium of pulmonary arteries (r = 0.41; p≤0.01) as well as smaller alveolar vessels (r = 0.48; p≤0.01). Furthermore, we found correlation for the expression pattern of KDR/Flk-1 and Flt-1 in the endothelium of pulmonary arteries (r = 0.67; p≤0.001) as well as in alveolar vessels (r = 0.80; p≤0.0005).

Additionally, we examined correlation between the epithelial VEGF expression from the current study and epithelial TGF-β_1 expression from one of our previous studies (20). In both studies the same patient groups were used and the staining was performed on adjacent or near sections. With regard to the bronchiolar epithelium, Pearson's analysis revealed a significant positive correlation between the VEGF protein and TGF-β_1 protein levels ($r = 0.55$; $p \leq 0.004$) and VEGF protein and TGF-β_1 mRNA expression ($r = 0.45$; $p \leq 0.02$). With regard to the alveolar epithelium, the VEGF protein levels correlated significantly with the TGF-β_1 mRNA expression only ($r = 0.58$; $p \leq 0.002$), but not with the TGF-β_1 protein levels ($r = 0.31$; $p \leq 0.12$).

5.4 Discussion

In this study we show that COPD is associated with an increased expression of VEGF in the bronchial, bronchiolar and alveolar epithelium and in bronchiolar macrophages as well as ASM and VSM cells in both bronchiolar and alveolar region. KDR/Flk-1 and Flt-1 were increased in COPD as compared to non-COPD in endothelial, intimal and medial VSM cells of larger pulmonary arteries and of smaller calibre alveolar vessels. Interestingly, we observed a significant inverse correlation of VEGF with FEV_1 in bronchial mucosal microvessels and ASM cells, bronchiolar epithelium and medial VSM of larger pulmonary arteries associated with bronchiolar airways. TGF-β_1 staining in the bronchiolar epithelium also correlated with VEGF in the same patients as described in our previous study (20).

Our results indicate that VEGF and its receptors Flt-1 and KDR/Flk-1 are localized within the airways and vasculature in endothelial and epithelial cells as well as smooth muscle cell origin and furthermore on various inflammatory cells, predominantly macrophages. The localization of VEGF and its receptors in the lungs of our patient groups is in agreement with earlier reports, which described a similar staining pattern in human developing and normal adult as well as in emphysematous lungs (17, 27, 28). In contrast to Kasahara et al. (28), where authors showed in emphysematous lungs that VEGF and its receptor VEGF-R2 were decreased in total lung extracts, as measured with ELISA or western blot analysis, we found that the epithelial and endothelial cells in the alveolar spaces and in the most distal airways were intensely positive for VEGF and KDR/Flk-1 in COPD patients. Furthermore, our patient groups could be considered as mild to moderate COPD, whereas in the study of Kasahara (28) the selected patients were solely emphysematous in origin. Our findings of

increased VEGF expression in viable cell populations represent in part a successful attempt to repair sustained damage and perhaps contribution to vascular remodelling and their participation in the establishment and maintenance of the functional blood-gas interface, maturation, survival and proliferation of capillary endothelial cells (29). In adult lungs, VEGF and its receptor system could contribute in the maintenance of endothelial and epithelial cell viability in response to injury (30).

Interestingly, immunoreactivity for VEGF in intimal and medial VSM cells and for Flt-1 as well as KDR/Flk-1 in endothelial cells of pulmonary arteries and alveolar vessels was elevated in patients with COPD. The highest levels of VEGF expression in the pulmonary vasculature were observed in the medial VSM cells and of KDR/Flk-1 in endothelial cells of arteries with a diameter of approximately 200 μm which are known to play an important role in pulmonary blood pressure regulation and vascular resistance (14, 31). Pulmonary hypoxia and hypertension with increased sheer stress are pathophysiological conditions that have been shown to increase the expression of VEGF in VSM cells (13, 14). Blockade of KDR/Flk-1 is associated with obliterative endothelial cell proliferation in pre-capillary arterioles with abnormal vessel development and at the same time with induction of capillary endothelial and cell death by apoptosis, together leading to death in rat embryos, similar to that seen in human primary pulmonary hypertension subjects (13, 18, 30, 32). In a follow-up study they found that after VEGFR-2 blockade apoptosis predominated in areas of oxidative stress and that apoptosis blockade by a broad spectrum caspase inhibitor markedly reduced the expression of markers of oxidative stress (33). Hypoxia, oxidative stress and pulmonary hypertension are pathological features often seen in advanced COPD patients and increased VEGF expression may lead to increased or even abnormal proliferation of endothelial and VSM cells in pulmonary vessels. This suggests a potential role of this endothelial mitogen in peripheral angiogenesis and vascular remodelling, possibly in orchestration with other smooth muscle specific growth factors like FGF-2, PDGF and TGF-$ß_1$ (12, 34-36).

We observed increased expression for VEGF and unchanged expression levels for Flt-1 and KDR/Flk-1 in bronchiolar and alveolar epithelial cells as well as in airway smooth muscle cells in COPD. It has been previously documented that the expression of VEGF and receptor KDR/Flk-1 can also be induced by stimuli like hypoxia and oxidative stress in other than endothelial cells, such as epithelial and smooth muscle cells (33, 37, 38). In a

recent report, Kanazawa and colleagues (39) have demonstrated that VEGF levels in induced sputum were higher in patients with bronchitis and lower in emphysema as compared to normal controls. Moreover, VEGF levels in bronchitis patients were inversely correlated with FEV_1 values. Our data on inverse correlation of VEGF levels in various airway and vascular cells is in agreement with this report. In our study subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone. Furthermore, the nature of the human material examined (sputum) in the study of Kanazawa and colleagues is different than the lung tissue where we immunohistochemically localize and quantify the VEGF and its receptor levels.

Recent studies indicated that the expression of VEGF was increased in bronchial and alveolar epithelial cells and also was induced in α-SMA positive (myo-)fibroblasts in bleomycin induced fibrosis in the rat and in human patients with pulmonary fibrosis and that these fibrotic regions were densely populated by mast cells and macrophages with elevated KDR/Flk-1 expression (15, 38). We have shown earlier that mast cells and macrophages were increased in bronchiolar airway epithelium and reported an increased expression of TGF-$ß_1$ in bronchiolar and alveolar epithelial cells in patients with COPD (20, 21). We found a significant correlation between VEGF expression in epithelial cells with the expression of TGF-$ß_1$ published on same patient groups earlier (20) suggesting that the VEGF/Flk-1 system, possibly together with TGF-$ß_1$, represents a molecular link between inflammatory cell accumulation and proliferation of myo-fibroblasts. Summarizing, the elevated VEGF and TGF-$ß_1$ expression on bronchiolar epithelial cells and macrophages and the presence of KDR/Flk-1 and Flt-1 suggests a mechanism of initiating and perpetuating fibrosis at sites of tobacco induced injury contributing to airway remodelling in COPD. As inhaled corticosteroids could decrease the VEGF expression levels (40), but this was not the case in our study as none of the patient received inhaled corticosteroid therapy except 4 patients received corticosteroids perioperatively. However, caution must be exercised in extrapolating the expression data based on fourteen patients in each group as the increased trend of VEGF expression in bronchial airways and KDR/Flk-1 in bronchial and bronchiolar airway smooth muscle could reach significance if more patients would have been examined.

Taken together, these findings strongly suggest a role for VEGF and its receptors in airway and vascular remodelling, and thereby in the development of airway obstruction in COPD.

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

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6

Pro-inflammatory Cytokines and Vascular Remodelling

Based on: "Alagappan VK, McKay S, Widyastuti A, Garrelds IM, Bogers AJJC, Hoogsteden HC, Hirst SJ and Sharma HS. Pro-inflammatory Cytokines Up-regulate mRNA Expression and Secretion of Vascular Endothelial Growth Factor in Cultured Human Airway Smooth Muscle Cells. Cell Biochem Biophys 2005; 43(1): 119-30"

Chapter 6: Pro-inflammatory Cytokines and Vascular Remodelling

Summary
Airflow obstruction in chronic airway disease is associated with airway and pulmonary vascular remodelling of which the molecular mechanisms are poorly understood. Paracrine actions of angiogenic factors released by resident or infiltrating inflammatory cells following activation by pro-inflammatory cytokines in diseased airways could play a major role in the airway vascular remodelling process. Here, the pro-inflammatory cytokines, IL-1β and TNF-α were investigated on cell cultures of human airway smooth muscle (ASM) for their effects on mRNA induction and protein release of the angiogenic peptide, vascular endothelial growth factor (VEGF). IL-1β (0.5 ng/ml) and TNF-α (10 ng/ml) each increased VEGF mRNAs (3.9 and 1.7 kb) expression in human ASM cells, reaching maximal levels between 16-24 h and 4-8 h, respectively. Both cytokines also induced time dependent release of VEGF, which was not associated with increased ASM growth. Pre-incubation of cells with 1 μM dexamethasone abolished enhanced release of VEGF by TNF-α. Data suggest that human ASM cells express and secrete VEGF in response to pro-inflammatory cytokines and may participate in paracrine inflammatory mechanisms of vascular remodelling in chronic airways disease.

6.1 Introduction

Airway remodelling is a common feature of both asthma and chronic obstructive pulmonary disease (COPD) and is characterized by structural changes including increased airway smooth muscle (ASM) mass, subepithelial fibrosis, glandular hypertrophy and peribronchial fibrosis, widely believed to culminate in poorly reversible airway narrowing. Recently, it has been demonstrated that vascular changes also occur during airway remodelling (1-6). For instance, in asthma there is hyperaemia of the bronchial vasculature and increased number and size of blood vessels. These changes correlate with the severity of disease in asthma (7, 8) and the release of angiogenic growth factors such as vascular endothelial growth factor (VEGF) from infiltrating inflammatory or resident structural cells has been implicated in their induction (9).

It is well established that ASM cells exhibit functions in addition to their structural and contractile properties, rather they proliferate and can express a wide range of adhesion molecules and pro-inflammatory or mitogenic factors, including, but not limited to tumour necrosis factor-α (TNF-α), interleukin (IL)-1β (10-12). TNF-α is an important cytokine in chronic inflammation, its presence may further perpetuate airway inflammation by inducing the secretion of other pro-inflammatory cytokines from multiple cells types in the airways during chronic lung diseases (13, 14). Elevated levels of TNF-α are found in bronchial

tissues during chronic airway diseases. Similarly, IL-1β is found in high levels in bronchoalveolar fluid and in the airway epithelium of asthmatic patients (13). Many of the actions of IL-1β are similar to those of TNF-α and the signal transduction pathways of these cytokines may interact closely often resulting in synergism (15).

In many tissues, angiogenesis and increased vascular permeability are characteristic features of the wound healing process and of inflammation (16). VEGF is a potent endothelial cell mitogen, which is reported to regulate vasculogenesis and postnatal vascular remodelling (17). Its expression in the lung is increased in COPD and asthma (6), as well as during hypoxia (18) and levels of VEGF are reported to correlate with the increased size and number of blood vessels found in these conditions (8). Moreover, the degree of VEGF expression correlates with levels of airway hyperresponsiveness in subjects with asthma (2). VEGF is highly mitogenic for endothelial cells and induces their survival leading to nearby angiogenesis or bronchial vascular remodelling. It is also associated with increased endothelial permeability and induction of endothelial cell expression of chemokines (IL-8), adhesion molecules (ICAM-1) and proteolytic enzymes (matrix metalloproteinases) that promote changes in tissue ECM composition required for endothelial and inflammatory cell migration. Thus, VEGF may play an important role in chronic airway diseases like asthma by contributing to airway and vascular remodelling (19). Consistent with this possibility, in an animal model, VEGF receptor inhibition prevents both airway inflammation and hyperresponsiveness induced by toluene diisocyanate (20).

The molecular mechanisms underlying the expression and release of VEGF during airway inflammation are largely unknown. The aim of the present study was to investigate whether ASM cells release VEGF when stimulated with the proinflammatory cytokines TNF-α or IL-1β and might therefore contribute to paracrine mechanisms of bronchial vascular remodelling during inflammation in chronic airways disease.

6.2 Materials and methods

6.2.1 Materials

All cell culture reagents were obtained from Invitrogen (Life Technologies BV, Breda, The Netherlands). Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Recombinant human (rh) TNF-α was purchased from Knoll AG (Ludwigshaven, Germany). Foetal bovine

serum (FBS) was obtained from Bio-Whitaker BV (Verviers, Belgium). [*Methyl*-^{3}H]thymidine and [*Methyl*-^{3}H]leucine were from Amersham Nederland BV ('s-Hertogenbosch, The Netherlands). Human specific antibodies and the enzyme-linked immunosorbent assay (ELISA) kits were from R & D Systems Europe Ltd (Abingdon, UK). All other reagents including rhIL-1β were from Sigma-Aldrich BV (Zwijndrecht, The Netherlands).

6.2.2 Human ASM cell culture

Human ASM cells were obtained in accordance with procedures approved by the Erasmus University Hospital Research Ethics Committee from the lobar or main bronchus of non-asthmatic patients undergoing lung resection for carcinoma of the bronchus using cell isolation and culture methods described previously (21). Fluorescent immunocytochemical labelling confirmed that near confluent, FBS-deprived human ASM cells stained >95% for smooth muscle-α-actin. Under these conditions approximately 87% of propidium-iodide labelled cells remained in the G_0/G_1 phase of the cell cycle. Confluent cells in the 4th - 6th passage were used for all experiments.

6.2.3 Cytokine stimulation of human ASM cells in vitro

The ASM cell growth was synchronized prior to treatment by washing the cell monolayers twice in phosphate buffered saline (PBS, 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM $Na_2HPO_4.2H_2O$, pH 7.4) and then replacing the medium with serum free DMEM supplemented with 1 μM insulin, 5 μg/ml transferrin and 100 μM ascorbate for 60 h. Growth-arrested cell monolayers were treated with TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml) in fresh FBS-free DMEM for 1, 2, 4, 8, 16, 24 h. In additional sets of experiments, ASM cells were pretreated with 1 μM dexamethasone for 1 hr and then TNF-α was added and the incubation continued for 24 h. Cells were harvested for total RNA isolation and the conditioned media were collected after each time point. Cell-conditioned media were stored at -80°C until assayed for VEGF levels by ELISA.

6.2.4 [^{3}H]Thymidine and [^{3}H]leucine incorporation assay

Effects of pro-inflammatory cytokines on DNA biosynthesis and total protein biosynthesis were evaluated by incorporation of [*methyl*-^{3}H]thymidine and [*methyl*-^{3}H]leucine,

respectively. Sub-confluent cell monolayers were growth arrested as described above. Cells were incubated with [*methyl*-^{3}H]thymidine or [*methyl*-^{3}H]leucine (1 μCi/well) in either fresh FBS-free DMEM or similar containing TNF-α, IL-1β or 10% FBS for 8, 24 or 48 h. Following stimulation cells were washed in PBS, fixed with ice-cold methanol and exposed to ice-cold trichloroacetic acid (5% w/v). The acid-insoluble fraction was lysed in 0.3 M NaOH and the incorporated radioactivity determined in a Packard 1500 Tri-carb liquid-scintillation counter (Packard-Becker BV, Delft, The Netherlands). Data are expressed as counts per min of [^{3}H]thymidine or [^{3}H]leucine incorporation.

6.2.5 Isolation of total cellular RNA and Northern blot analysis

Treated and untreated human ASM cells were washed in PBS and directly lysed in guanidinium thiocyanate buffer. Genomic DNA was sheared by passing lysates repeatedly through 23-gauge needles. Total cellular RNA was then isolated using as described previously (21). Total RNA (10 μg) was denatured at 65°C in formaldehyde containing loading buffer and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide stained gels were photographed and RNA was transferred onto hybond-N membrane (Amersham Nederland BV, 's-Hertogenbosch, The Netherlands) by the alkaline downward capillary transfer method (21). Filters were air-dried and UV cross-linked (Biorad Laboratories B.V., Veenendaal, The Netherlands) and blots hybridized at 42°C. cDNA insert (950 bp DNA fragment encoding human VEGF) was labelled using 32P-dCTP with a multiprime labelling system. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (American Type Culture Collection, Rockville, USA) was used to rehybridize membranes for reference purposes. Filters were washed under stringent conditions and subsequently exposed to Kodak X-OMAT AR films (Amersham Nederland BV, 's-Hertogenbosch, The Netherlands) at -80°C. Hybridisation signals were quantified by scanning laser densitometry using the Ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden) and normalised against GAPDH mRNA values and expressed as relative optical density (OD) in stimulated cells versus controls.

6.2.6 Measurement of VEGF protein by ELISA

Conditioned media were collected from TNF-α- or IL-1β-treated human ASM cells after 1, 2, 4, 8, or 24 h and VEGF levels assessed using a human VEGF specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit. Cell-conditioned medium

samples were diluted until VEGF levels were within the linearity limits of the assay standard curve. The concentration of VEGF was expressed in pg/ml. The detection limit of the ELISA assay was 20 pg of VEGF/ml.

6.2.7 Statistical analysis

All data in the figures are given as mean±SEM. Statistical analysis was performed by using two-tailed, independent sample "t"-test. Significance was accepted at $p \leq 0.05$.

6.3 Results

6.3.1 Effect of pro-inflammatory cytokines on human ASM cell growth

There are reports that TNF-α and IL-1β can act as mitogens for ASM cells in culture (14, 22). Moreover, VEGF is a potent mitogen, particularly for endothelial cells but also indirectly for ASM cells (23, 24). Thus, we examined whether TNF-α or IL-1β, acting either directly or possibly indirectly via VEGF production, induced significant changes in DNA synthesis ([^{3}H]thymidine incorporation) or total protein synthesis ([^{3}H]leucine) in human ASM cells. However, treatment of the cells with TNFα (10 ng/ml) or IL-1β (0.5 ng/ml) for 8, 24 or 48 h did not induce significant changes in [^{3}H]thymidine or [^{3}H]leucine uptake when compared with unstimulated cells (Figure 6.1).

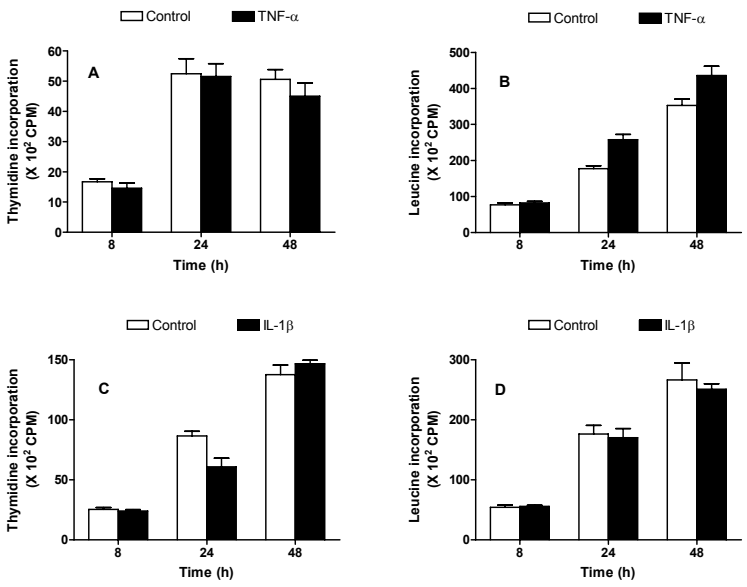

Figure 6.1: Growth of ASM cells treated with TNF-α or IL-1β

Thymidine (Panels A and C) and Leucine (Panels B and D) incorporation measured after 8, 24 and 48 h in semi-confluent, growth arrested, human ASM cells stimulated with 10 ng/ml of TNF-α

(upper panels) or 0.5 ng/ml IL-1β (lower panels). Values were calculated from quadruplicate experiments and expressed as mean counts per min (CPM)±SEM. No significant differences were found between stimulated and unstimulated control cells.

6.3.2 VEGF mRNA expression in relation to TNF-α or IL-1β

To examine VEGF mRNA expression human ASM cells were treated with TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml) for 1, 2, 4, 8, 16 or 24 h. Using Northern blot hybridization, two mRNA species of 3.9 and 1.7 kb encoding VEGF were detected in cultured human ASM cells treated with TNF-α (Figure 6.2, Panel A) or IL-1β (Figure 6.2, Panel B), which were absent in unstimulated cells. TNF-α-induced VEGF mRNA levels were maximal during 4-8 h; whereas IL-1β-dependent VEGF mRNA content peaked at 16 h and remained elevated after 24 h, when compared with unstimulated cells. 10% FBS also induced the mRNA expression of VEGF in human ASM cells *in vitro* (not shown).

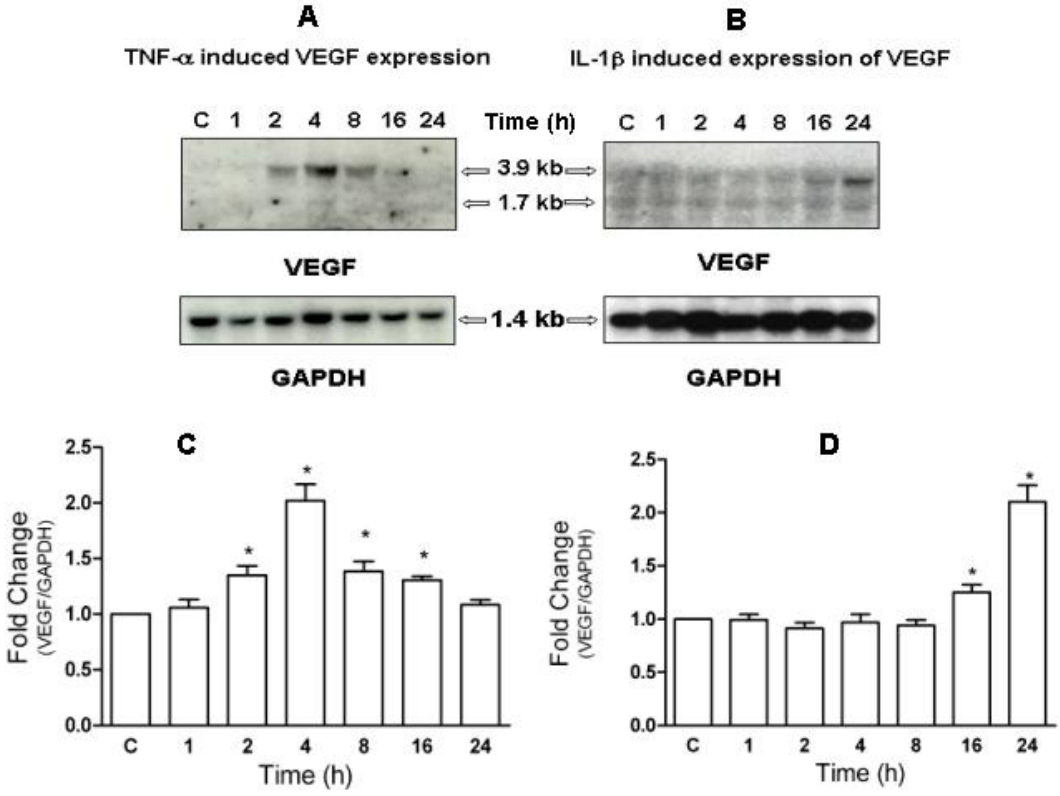

Figure 6.2: Pro-inflammatory cytokines induce expression of VEGF mRNAs in human airway smooth muscle cells

*Representative Northern blots showing a major 3.9 and a minor 1.7 kb mRNA band for VEGF. Total RNA samples from control (C) and TNF-α (Panel A) or IL-1β (Panel B) treated human ASM cells were hybridized with a cDNA insert encoding human VEGF. Human ASM cells were incubated with TNF-α or with IL-1β or left unstimulated for the time points as indicated on top of the blot. Panel C and D show the densitometric analysis for the blots represented as fold change in VEGF expression relative to GAPDH (as compared to control). Data represent the mean± SEM of triplicate values from independent blots. * Significant values (P ≤ 0.05) compared with controls.*

6.3.3 Release of VEGF protein from human ASM cells in response to TNF-α or IL-1β

Conditioned medium from human ASM cells treated with varying concentrations of TNF-α (0.2, 1, 2, 5, 10, 20 and 100 ng/ml) for 24 h revealed a concentration-dependent release of VEGF (Figure 6.3). TNF-α induced VEGF secretion, as measured by ELISA, was 20±3 pg/ml, 110±0.5 pg/ml and 78.5±12.5 pg/ml after 0, 20 and 100 ng/ml stimulation, respectively. VEGF release reached maximal levels (166 ±4 pg/ml) after stimulation with 10 ng/ml of TNF-α.

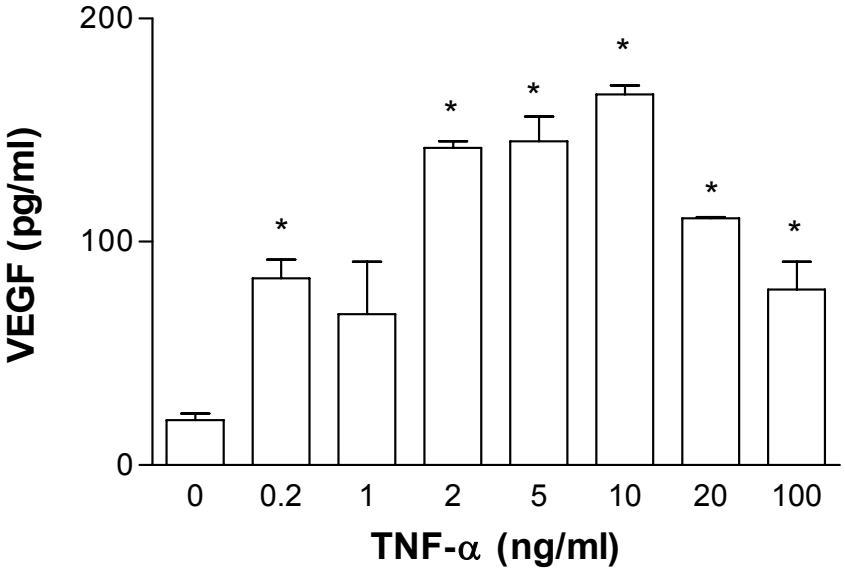

Figure 6.3: Concentration-dependent production of VEGF protein by TNF-α

*Growth-arrested human ASM cells were stimulated with 0.2, 1, 2, 5, 10, and 20 ng/ml of TNF-α for 24 h. Data represent the mean±SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from three patients. *Significant values ($p \leq 0.05$) compared with unstimulated cells.*

Likewise, the release of VEGF protein from human ASM cells treated either with TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml) was time-dependent. TNF-α-induced VEGF secretion (Figure 6.4, panel A) was 40.67±6.8 pg/ml, 88.67±11.7 pg/ml and 162.3±59 pg/ml at 1, 4 or 8 h, respectively. TNF-α-dependent VEGF release was maximal at 24 h (173.3±33.9 pg/ml), compared with unstimulated cells (37.67±7.2 pg/ml). Incubation with IL-1β resulted in 66 ±18 pg/ml, 267.3±15.5 pg/ml and 356.7±31.9 pg/ml at 1, 4 and 8 h and was maximal after 24 h where levels reached 668±116.4 pg/ml (Figure 6.4, panel B). Additionally, FBS induced a modest release of VEGF at 24 h (290.67±53.9 pg/ml).

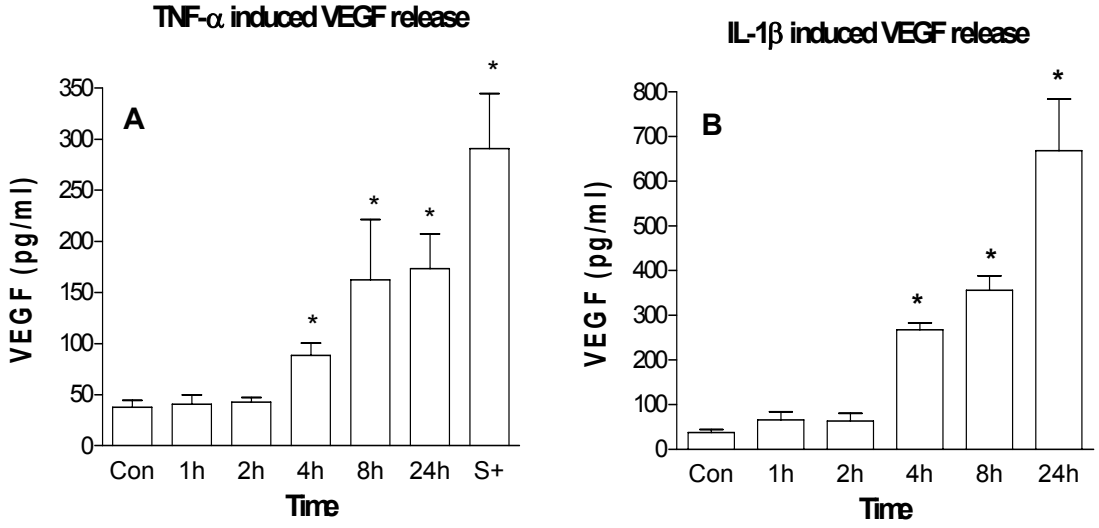

Figure 6.4: Time-dependent production of VEGF protein by pro- inflammatory cytokines

*Growth arrested human ASM cells were stimulated with TNF-α or with IL-1β for varying times (1, 2, 4, 8, 24 h). Control cells (Con) received only serum free medium. Data represent the mean±SEM of triplicate values from independent experiments using conditioned media from ASM cells cultured from three patients. *P ≤ 0.05 as compared with controls.*

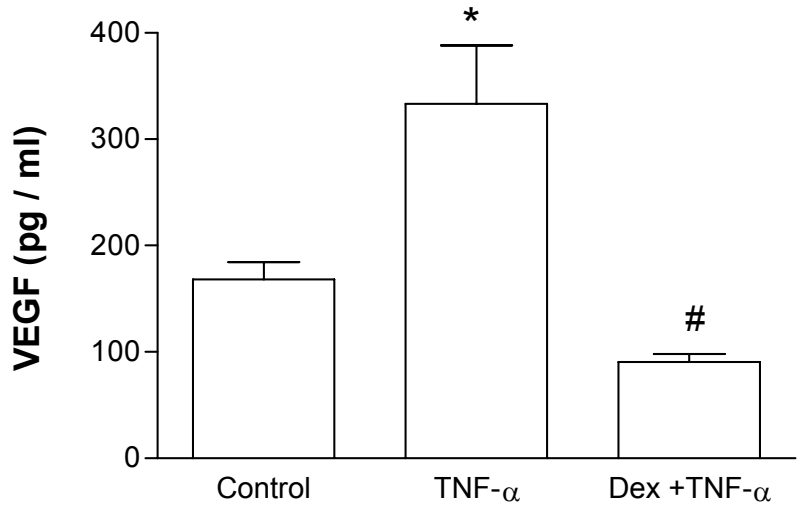

Figure 6.5: Effect of Dexamethasone on TNF-α induced VEGF release

*Growth arrested human ASM cells were stimulated with 10 ng/ml of TNF-α for 24 h. Human ASM cells were pretreated with 1 μM dexamethasone for 1 h prior to stimulation with TNF-α for 24 h. Control cells received only serum free medium. Data represent the mean±SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from three patients. *P ≤ 0.05 as compared with controls; # compared with TNF-α stimulated cells.*

6.3.4 Effect of dexamethasone on TNF-α induced expression of VEGF

To assess whether anti-inflammatory glucocorticoids could inhibit release of VEGF by TNF-α (10 ng/ml), ASM cells were pretreated with dexamethasone (1 μM). Under these conditions, VEGF release was abolished by dexamethasone (Figure 6.5).

6.4 Discussion

Changes in airway microvasculature and the presence of angiogenesis in inflammatory respiratory diseases are now being documented (3). Our study provides insight into possible mechanisms through which the angiogenic events may take place in chronic airway disease. We confirmed that human ASM cells express mRNA encoding VEGF and secrete VEGF protein and found that VEGF expression was increased by the proinflammatory cytokines, TNF-α and IL-1β, important modulators of airway function in asthma, and suppressed by anti-inflammatory glucocorticoids. We speculate that paracrine actions of VEGF derived from ASM cells could perpetuate the chronic inflammatory process in asthma or COPD in several ways. For example, VEGF is highly mitogenic for endothelial cells and induces their survival leading to nearby angiogenesis or bronchial vascular remodelling. It is also associated with increased endothelial permeability and induction of endothelial cell expression of chemokines (IL-8), adhesion molecules (ICAM-1) and proteolytic enzymes (matrix metalloproteinases) that promote changes in tissue ECM composition required for endothelial and inflammatory cell migration (20, 25, 26).

Recent reports describe the secretion of VEGF by human ASM cells in response to bradykinin and prostanoids (9) as well as by Th2 cytokines, such as IL-4, IL-5 and IL-13 (21). Other reports reveal only a modest effect of these cytokines on VEGF secretion by bronchial fibroblasts (27). Cytokines including IL-1 and IL-6 have also been shown to upregulate VEGF protein expression, but their effects appear variable and species or tissue specific. In the present study, up regulation of VEGF by human ASM cells occurred both at the mRNA and protein level, corroborating recent similar findings with IL-1β (24, 28). We also report that TNF-α, another major proinflammatory cytokine, increased VEGF mRNA and protein secretion in human ASM cells. Induction of VEGF secretion by these cytokines was time dependent, peaking at 24 h and was concentration-dependent in case of TNF-α with optimum concentration being 10 ng/ml. Our findings with IL-1β showing a relatively slow induction of mRNA and release of protein are in broad agreement with those of others (24) where IL-1β-dependent VEGF secretion was increased at 16 h after stimulation. However, accumulation of VEGF mRNA after stimulation by TNFα appeared more rapid occurring within 2 to 4 h and contrasts with Kazi and colleagues (24), who reported no increase in VEGF by TNFα. Reasons for this discrepancy are unclear, but our observation that neither TNFα nor IL-1β induced proliferation of ASM cells under these conditions

suggests the increased VEGF levels we observed were not artificially increased due to increased cell numbers.

Increasing experimental evidence suggests inhibition of VEGF activity via blockade of its receptors has potential therapeutic value as an intervention method for decreasing angiogenesis and vascular remodelling (29). This approach reverses pathophysiological symptoms including airway hyper-responsiveness and inflammation in a mouse model of asthma (30). One likely mechanism for the effectiveness of this approach could be prevention of VEGF-induced vascular permeability, thereby suppressing inflammation by reducing vascular leakage and migration of cells and mediators into the airways. However, the VEGF inhibition could be both beneficial and detrimental in different forms of chronic airway diseases. For example, some reports suggest increased levels of VEGF are associated with airflow limitation in bronchitis, whilst decreased levels are also associated with airflow limitation and alveolar destruction in patients with emphysema (31, 32). Likewise, recent evidence demonstrates that long-term glucocorticoids treatment in patients with asthma significantly affects airway remodelling by reducing basement membrane thickness, and reducing indices of submucosal vascularity including blood vessel number and total vascular area (33, 34). Our finding that dexamethasone completely inhibits the release of the angiogenic factor VEGF would support this notion. The inhibitory action of dexamethasone on TNF-α induced VEGF release may be mediated by several mechanisms, including the reduction of cAMP levels or inhibition of p38 MAPK phosphorylation (35). Dexamethasone suppresses TNF-α-induced AP-1 DNA binding suggesting that glucocorticoids may exert their inhibitory effect on TNF-α by *trans*-repression of AP-1 (36). The effect of glucocorticoids on airway remodelling, however, remains controversial. Inhibition of VEGF production by ASM cells suggests one mechanism by which glucocorticoids might affect tissue remodelling.

Localization studies in the airways of asthmatics suggest VEGF is expressed by both infiltrating inflammatory and resident cell types including submucosal glandular and alveolar type I and II epithelial cells, myofibroblasts, eosinophils, macrophages and CD34+ cells (2). The relative importance of each cell type for release of VEGF under pathophysiological conditions is unknown. However, consistent with ASM also participating in these processes we and others have demonstrated that human ASM cells express VEGF both *in vitro* and in

vivo in the intact myobundles of patients with COPD (6, 37, 38). These latter findings suggest that VEGF release by cultured human ASM cells is not a simple culture artifact rather ASM represents a possible pathophysiological important pool of VEGF available during airways inflammation that may be amenable to therapeutic intervention.

In summary, VEGF is secreted by ASM cells in response to the proinflammatory mediators, IL-1β and TNFα acting via pathways that are sensitive to inhibition by anti-inflammatory glucocorticoids. The current study supports the hypothesis that ASM cells are active sources of mediators relevant to the pathogenesis of airway disease. Proinflammatory cytokines such as IL-1β and TNF-α that associated with chronic inflammation of the airways may drive production of VEGF by ASM cells, which via paracrine mechanisms in the vicinity of bronchial endothelial cells may perpetuate the bronchial vascular remodelling that characterizes asthma and COPD.

Acknowledgements

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Vasoactive Peptides and Bronchial Vascular Remodelling

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Chapter 7: Vasoactive Peptides and Bronchial Vascular Remodelling

Summary
Airway remodelling and associated angiogenesis are documented features of asthma, of which the molecular mechanisms are not fully understood. Angiotensin II (ANG II) and endothelin-1 (ET-1) are potent vasoconstricting circulatory hormones implicated in asthma. We investigated the effects of ANG II and ET-1 on human airway smooth muscle (ASM) cells proliferation and growth and examined the mRNA expression and release of the angiogenic peptide, vascular endothelial growth factor (VEGF). Serum deprived (48 h) human ASM cells were incubated with ANG II (100 nM) or ET-1 (10 nM) for 30 min, 1, 2, 4, 8, 16 and 24 h and the endogenous synthesis of VEGF was examined in relation to control cells receiving serum free culture medium. ET-1 induced time dependent DNA biosynthesis as determined by [^{3}H]thymidine incorporation assay. Using northern blot hybridisation, we detected two mRNA species of 3.9 and 1.7 kb encoding VEGF in the cultured smooth muscle cells. Both ANG II and ET-1 induced the mRNA expression (2-3 fold) and secretion (1.8-2.8 fold) of VEGF reaching maximal levels between 4-8 h of incubation. Induced expression and release of VEGF declined after 8 h of ANG II incubation while levels remained elevated in the case of ET-1. The conditioned medium derived from ET-1 treated ASM cells induced [^{3}H]thymidine incorporation and cell number in porcine pulmonary artery endothelial as well as human umbilical vein endothelial cells. Moreover, the VEGF tyrosine kinase receptor inhibitor blocked the conditioned medium induced mitogenesis in endothelial cells. Our results suggest a potential role for ANG II and ET-1 in ASM cell growth and up-regulation of VEGF that may participate in endothelial cell proliferation via paracrine mechanisms and thus causing pathological angiogenesis and vascular remodelling seen during asthma.

7.1 Introduction

Increased airway smooth muscle (ASM) mass and airway remodelling is a common feature in chronic airway diseases such as asthma (1). Airway remodelling is accompanied with angiogenesis and microvascular changes wherein the ongoing inflammation seems to be the main contributing factor (2-5). Evidence suggests that the number and size of bronchial vessels is moderately increased in patients with asthma as compared with normal individuals (6-9). Moreover, Salvato and colleagues (10) found that patients with severe asthma had significantly more vessels than those with a mild or moderate disease. Previously, we reported that fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) play a pivotal role in the pathogenesis of chronic obstructive pulmonary disease (COPD) (2, 3). Similarly, enhanced bronchial localization of various angiogenic growth factors has been observed in patients with asthma and COPD (2, 3, 11, 12). However, the molecular mechanisms underlying bronchial angiogenesis and vascular remodelling remain unclear.

VEGF is a potent endothelial cell mitogen that regulates vasculogenesis and postnatal vascular remodelling (13). Expression of VEGF is upregulated under a variety of pathophysiological conditions, including hypoxia in the lung (14) and during angiogenesis in other biological systems (15, 16). The most important role of VEGF in inflammation may be in mediating the angiogenic response (17). Since angiogenesis and increased vascular permeability are characteristic features of wound healing, VEGF may play an important role in airway remodelling in asthma where wound healing after injury is a common phenomenon (12). VEGF is expressed by human airway smooth muscle cells in culture and in lungs under pathophysiological conditions (2, 9, 18, 19) and we have shown that COPD is associated with an increased expression of VEGF in both ASM and vascular smooth muscle (VSM) cells in bronchiolar as well as alveolar regions; this increased expression is inversely co-related with the lung function data (2).

Angiotensin II (ANG II) and endothelin-1 (ET-1) are potent vasoconstricting circulatory hormones implicated in asthma (20-24). Angiotensin II has been reported to induce VEGF mRNA expression in rat heart endothelial cells (25) and mitogenesis in human ASM cells (26). Evidence suggests that ET-1 acts as a co-mitogen with EGF and that its action is mediated through activation of ET_A receptors in lungs (27). However, the underlying molecular mechanisms of mitogenic and subsequent signalling elicited by ET-1 in human ASM cells are not yet characterised. We therefore investigated the responses to ANG II and ET-1 in human ASM cells *in vitro* and examined the expression and release of VEGF that may contribute to pulmonary vascular remodelling in chronic airway diseases. Furthermore, we assessed the conditioned medium obtained from human ASM cells treated with ANG II and ET-1 for *in vitro* angiogenic potential by examining endothelial cell proliferation and growth.

7.2 Materials and methods

7.2.1 Human ASM cell isolation and culture

Human ASM cells were isolated and cultured as described previously (28). Briefly, bronchial smooth muscle was dissected from fresh macroscopically normal lobar or main bronchi. After removal of the epithelium, parts of smooth muscle was dissected, free of adherent tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (HBSS; Invitrogen, Breda, The Netherlands) containing bovine serum

albumin (BSA, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (3.3 U/ml; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) at 37 °C in a humidified incubator containing 5% CO_2/95% air for 30 min. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Breda, The Netherlands) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; Cambrex, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml) (Invitrogen, Breda, The Netherlands). Cells were subsequently seeded at $2x10^5$ cells per 35 mm dish and maintained in culture by replacing the medium every 48 h. After 10-14 days in culture, ASM cells grew to confluence and were then detached by trypsinization (0.5% trypsin; 0.02% EDTA; Invitrogen, Breda, The Netherlands) and subcultured into 25 cm^2 and 75 cm^2 tissue culture flasks.

7.2.2 Immunocytochemical characterization of cultured human ASM cells

Immunocytochemical staining of confluent serum-deprived primary cultures of human ASM cells, using monoclonal antibodies to smooth muscle α-actin and smooth muscle-myosin heavy chain (SM1 and SM2; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) demonstrated that the cultures were essentially free (<5%) of other contaminating cell types (26, 28). In order to perform immunocytochemistry, monoclonal antibodies against α-smooth muscle actin were used as markers. In brief, cells were allowed to attach to multiwell slides for 24 h in FBS-containing medium and were subsequently growth-arrested by incubating for 60-72 h in FBS-free DMEM supplemented with apo-transferrin (5 μg/ml), ascorbate (100 μM), and insulin (1 μM) prior to fixation and staining. Following two washes in ice-cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2\ H_2O$, pH 7.4), the cells were fixed in ice-cold methanol and permeabilized in PBS containing 0.1% Tween-20. Non-specific bindings were blocked by incubating the cells in 1% BSA in PBS, and the cells were then washed and subsequently incubated with anti-α smooth muscle actin antibodies. The cells were then washed twice in PBS and further incubated with affinity purified FITC-conjugated antimouse antibody. Unbound antibody was washed away using distilled water and the sections were dehydrated and mounted in glycerol. Specimens were visualized under a fluorescence microscope (Carl Zeiss BV, Weesp, The Netherlands) and photographed.

Human ASM cells stained positive for anti-α smooth muscle actin (Figure 7.1, upper right panel). Under the light microscope, the human ASM cells appeared elongated and spindle-shaped with central oval nuclei containing prominent nucleoli. Confluent human ASM cells in culture showed a specific pattern, aligned in parallel so that the broad nuclear region of one cell lies adjacent to the thin cytoplasmic area of another, giving rise to a typical "hill and valley" appearance (Figure 7.1, upper left panel).

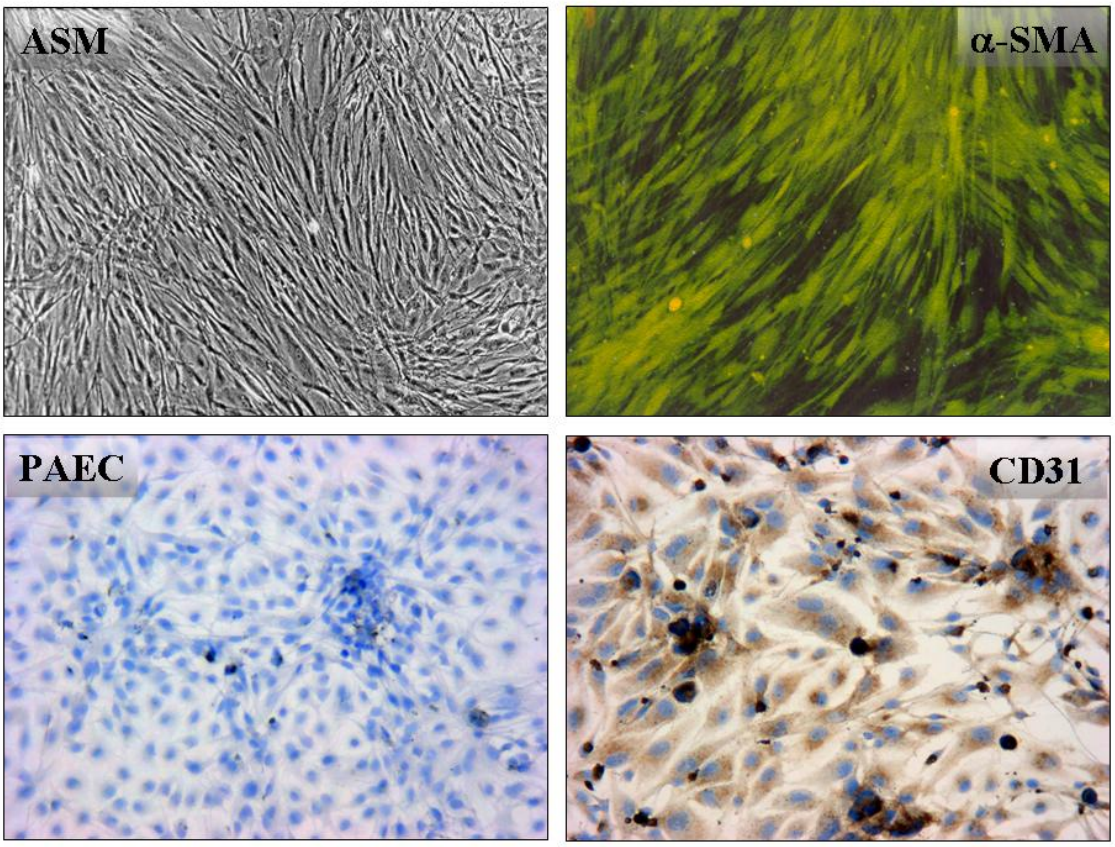

Figure 7.1: Representative examples of human ASM and pulmonary artery endothelial cells in culture

Photomicrograph depicts the monolayer (upper left panel) of human ASM cells with the characteristic hill and valley appearance. Smooth muscle α–actin (fluorescence) antibody (upper right panel) characterizes the staining in longitudinal actin filaments and assures the purity of the human ASM cells. Photomicrograph depicts the monolayer (lower left panel) of porcine pulmonary artery endothelial cells (PAEC) with the characteristic cobble stone appearance. CD31 (brown) antibody staining (lower right panel) characterizes the purity of the endothelial cells.

7.2.3 Porcine pulmonary artery endothelial cell isolation and culture

The lungs were obtained from Yorkshire x Landrace female pigs after sacrifice and the major pulmonary artery was identified and dissected free of all connective tissue and cleaned with HBSS. After incubation with collagen I for 30 min endothelial cells were isolated by scraping gently the inner surface of the artery with a surgical blade and cultured

up to fourth passage in M-199 medium containing 10% foetal calf serum (FCS; Cambrex, Verviers, Belgium) at 37 °C in a 5% CO_2-humidified atmosphere. Cells were maintained in culture by replacing the medium every 48 h and when confluent was further passaged using trypsin/EDTA solution into 75 cm^2 tissue culture flasks. Porcine pulmonary artery endothelial cells had the characteristic cobblestone appearance when cultured in monolayer. Cells were characterized immunocytochemically, using monoclonal antibodies against CD31 and visualised under the light microscope. Porcine pulmonary artery endothelial cells in culture revealed cobblestone appearance (Figure 7.1, lower left panel). Immunocytochemical staining of confluent serum-deprived primary cell cultures of using monoclonal antibodies against CD31 (Figure 7.1, lower right panel) demonstrated that the cultures were essentially free (<5%) of other contaminating cell types.

7.2.4 Stimulation of human ASM cells in vitro with ANG II or ET-1

Human ASM cells were added in 500µl medium to 24 wells multiplate (Corning BV, Schiphol, The Netherlands), at a final concentration of $3x10^4$ cells/well. After 72 h, cells were serum deprived for 60 h and subsequently incubated with ANG II (100 nM), optimal concentration based on our previous report (26) or ET-1 (10 nM), optimal concentration based on pilot experiments, for 30 min, 1, 2, 4, 8, 16 and 24 h. Control cells were cultured in serum free medium (24 h).

7.2.5 Isolation of total cellular RNA and Northern blot analysis

Total RNA was extracted from the cells incubated with ANG II (100 nM) or ET-1 (10 nM) for 30 min, 1, 2, 4, 8, 16 and 24 h by the guanidinium thiocyanate-phenol-chloroform method. For Northern hybridization, 10 µg of total RNA was electrophoresed on 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to hybond-N membranes, which were eventually UV cross-linked and hybridized at 42 °C. cDNA insert (950 bp DNA fragment encoding human VEGF) was labelled using (^{32}P)dCTP with a multiprime labelling system. Filters were washed under stringent conditions and subsequently exposed to Kodak X-OMAT AR films at -80 °C. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to rehybridize membranes for reference purposes. After autoradiography and densitometric measurement of signals, the optical density (OD) of the VEGF signal was divided by OD of the corresponding GAPDH signal and relative mRNA levels were calculated. Values were

expressed as fold induction (mean±SEM) from four different autoradiograms. Statistical significance was accepted at $p \leq 0.05$.

7.2.6 Measurement of VEGF protein by ELISA

Conditioned media were collected from human ASM cells treated with ANG II or ET-1 and VEGF levels were assessed using a commercial ELISA kit according to the manufacturer's instruction (R&D Systems, Abington, UK). As a control, conditioned medium from human ASM cells incubated with only serum-free medium were was used. A standard curve using recombinant VEGF protein was first established and subsequently (100 µl) of conditioned medium was used to assay VEGF. Subsequently, the samples were pre-incubated with VEGF capture antibody followed by biotinylated VEGF detecting antibody. After addition of the streptavidine-peroxidase conjugate tetramethylbenzidine (TMB) was added and the absorbance of the resulting coloured product was measured using an automated spectrophotometer (Biorad Laboratories BV, Veenendaal, The Netherlands). The concentration of VEGF was expressed in ng/ml. The detection limit of the VEGF ELISA method was 20 pg of VEGF/ml.

7.2.7 Cell proliferation assays

7.2.8 [^{3}H]Thymidine incorporation assay in human ASM cells treated with ET-1

Effects of ET-1 on human ASM DNA biosynthesis were evaluated by incorporation of [*methyl*-^{3}H]-thymidine. Confluent cells were washed in PBS, loosened by trypsinisation and transferred into 96-well plates at a seeding density of $1x10^4$ cells/well. After 24 h in culture the sub-confluent cell monolayers were growth arrested by 48 h serum deprivation and incubated with different concentrations of ET-1 (1, 10 and 100 nM) for 48 h. Additionally, cells were incubated with ET-1 (10 nM) for varying time periods from 24 to 96 h. Five hours prior to the end of the treatment periods, 10 µl (1 µCi/10 µl in HBSS) of [^{3}H]thymidine was added to the wells, at a final concentration of 1 µCi/110 µl per well. The plates were frozen overnight at -20 °C after which the cells were harvested on glass fibre filters using a Filtermate 196 cell harvester (Packard, Meridan, USA) and the activity was counted using a Microplate Scintillation ß-counter (Topcount, Packard, Meridan, USA). Measured radioactivity was expressed as counts/min (CPM). Data represented is the mean CPM of experiments performed with three different cell isolations.

Endothelial cell proliferation assay

Porcine pulmonary artery endothelial cells were added in 100 µl medium to 96 wells multiplate (Corning BV, Schiphol, the Netherlands), at a final concentration of 1 x 10^4 cells per ml. After 48 hours, the cells were serum deprived for 24 hours and subsequently incubated with the conditioned medium from human ASM cells treated for 8 h with ANG II (100 nM) or ET-1 (10 nM) and positive controls with VEGF (5 ng/ml) or PlGF (5 ng/ml) in 100 µl serum-free M199 medium for 24 h. Control incubations consisted of cells that were incubated with serum-free M199 medium alone. DNA biosynthesis was measured employing [^{3}H]thymidine incorporation assay as described above.

Endothelial cell count

In a parallel series of experiments, serum-deprived porcine pulmonary artery endothelial cells were stimulated with the same factors as above for 48 h and processed for cell counting in the Casy®1 system (Schärfe system GmbH, Reutelingen, Germany). After stimulation, cells were harvested with mild trypsinisation. Cells in suspension were added to 10 ml of Casy®1 isotonic solution (6.38 g/l NaCl, 0.2 g/l Na-tetraborate, 1.0 g/l Boric acid and 0.2 g/l EDTA), counted and further analyzed using Casy®1 system software.

7.2.9 VEGF receptor blockade and endothelial cell growth

Human umbilical vein endothelial cells (HUVEC) were isolated from normal human umbilical cords as described earlier (29). Briefly, cells were cultured in fibronectin-coated tissue culture flasks in culture medium (human endothelial-SFM, Invitrogen), with 20% newborn calf serum, 10% human serum (Cambrex, Verviers, Belgium), 20 ng/ml basic fibroblast growth factor, and 100 ng/ml epidermal growth factor (Peprotech, London, UK). Passages 5 to 7 were used for the experiments. Cells were plated in fibronectin-coated 96-well plates and allowed to grow for 24 h and then serum deprived and cultured in DMEM for 24 h. Serum deprived cells were incubated for 48 h with control medium (DMEM alone) or the conditioned medium from ET-1 (10 nM)-treated (8 h) human ASM cells in 1:1 dilution. Control HUVEC was exposed to medium from untreated ASM cells. Activity of VEGF present in the conditioned medium on the growth of HUVEC cells was assessed by pre-treating the cells with 10 µM VEGF tyrosine kinase receptor blocker 4-[(4′-Chloro-2′-fluoro)phenylamino]-6,7-dimethoxyquinazoline (Calbiochem, Darmstadt,

Germany). The growth of HUVEC cells was measured using the sulforhodamine-B (SRB) assay as described earlier. In short, cells were washed twice with PBS, incubated with 10% trichloric acetic acid (1 h, 4 °C), and washed in distilled water. Cells were stained with 0.4% SRB (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) for 15 to 30 min, washed with 1% acetic acid, and were allowed to dry. Protein-bound SRB was dissolved in TRIS (10 mmol/l, pH 9.4). The absorbance was read at 540 nm. Growth was calculated using the formula: percentage growth = (absorbance test well / absorbance control well) x 100%.

7.2.10 Statistical analysis

All data in the figures are given as mean±SEM. Statistical analysis was performed by using two-tailed, independent sample "t"-test. Significance was accepted at $p \leq 0.05$.

7.3 Results

7.3.1 Effect of ET-1 on human ASM cell proliferation

In order to investigate the role of ET-1 on airway smooth muscle cell proliferation and remodelling, isolated human airway smooth muscle cells were stimulated *in vitro* with increasing concentrations of ET-1 (1, 10 and 100 nM). Graphic representations of dose-dependent increase in [^{3}H]thymidine incorporation are shown in Figure 7.2 (Panel A). ET-1 significantly increased cell numbers at all concentrations after 48 h of incubation. There was no significant difference in the increase in cell proliferation in relation to increase in dose of ET-1. Figure 7.2 (Panel B) is a graphic representation of time-dependent [^{3}H]thymidine incorporation at a concentration of 10 nM of ET-1. After 48 h of stimulation, we found significantly increased cell proliferation as measured by thymidine incorporation as compared to the control cells (13.6±1.9 x10^3 CPM vs. 8.9±0.8 x10^3 CPM). Thereafter, cell proliferation induced by ET-1 was significantly higher than the control even up to 96 h of treatment.

7.3.2 VEGF mRNA expression in relation to ANG II and ET-1

Human ASM cells were treated for 30 min, 1, 2, 4, 8, 16 and 24 h either with ANG II (100 nM) or ET-1 (10 nM) in order to examine the VEGF mRNA expression. Using Northern blot hybridisation, we detected two mRNA species of 3.9 and 1.7 kb encoding VEGF in human ASM cells treated with ANG II (Figure 7.3, panel A) and ET-1 (Figure 7.3, panel B). In order to compare the expression pattern and to verify the integrity of total

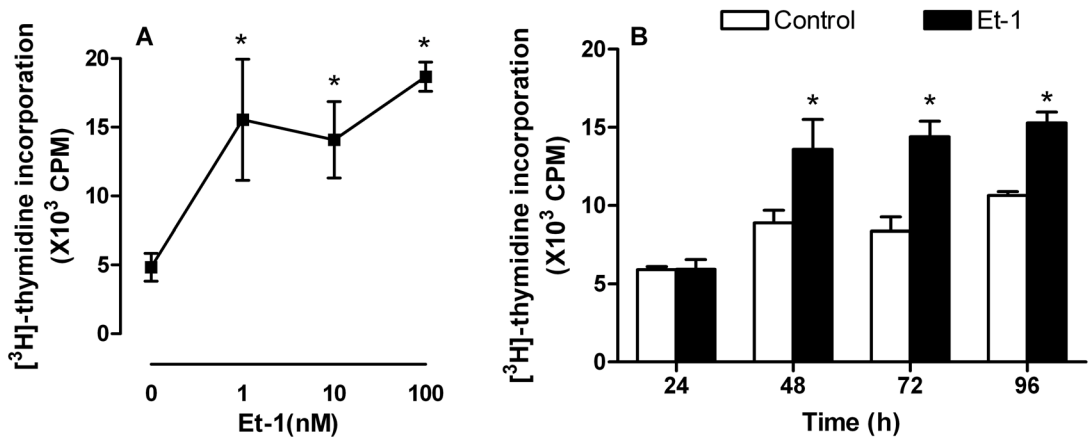

Figure 7.2: Graphic representation of human ASM cell proliferation in relation to ET-1

*Panel A: Dose-dependent increases in [^{3}H]thymidine uptake in ASM cells after 48 h stimulation with increasing concentrations (0, 1, 10, 100 nM) of ET-1. Panel B: Time course of [^{3}H]thymidine uptake in ASM cells after stimulation with 10 nM of ET-1. Data represent mean fold increase in relation to control from three independent experiments performed in quadruplicate. Values are shown as mean±SEM. *P≤0.05 versus the control serum deprived cells.*

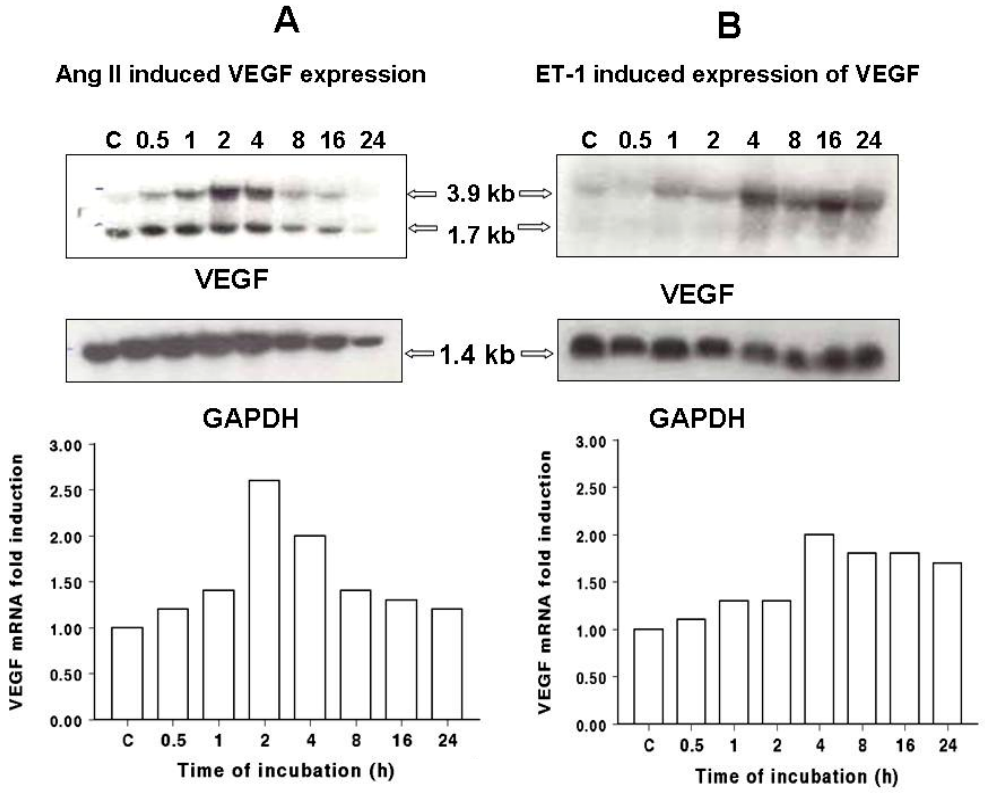

Figure 7.3: ANG II and ET-1 induced expression of VEGF in human airway smooth muscle cells

A representative Northern blot showing a major 3.9 and a minor 1.7 kb mRNA band of VEGF. Total RNA from control and treated human ASM cells was hybridised with a cDNA insert encoding human VEGF. Growth arrested cells were incubated with ANG-II or ET-1 in serum free growth medium for the times indicated on the top. Filters were rehybridised with GAPDH cDNA probe for reference purposes.

RNA samples, GAPDH, a housekeeping gene was used to re-hybridise the membranes. A strong dark band at 1.4 kb, hybridising to a cDNA insert encoding GAPDH in each RNA preparation is visible (Figure 7.3 lower panels). As shown in the blot and in the graphs expressing the relative mRNA levels, stimulation with ANG II induced the expression of VEGF mRNA reaching maximal levels at 2 h, whereas ET-1 induced VEGF mRNA expression peaked at 4 h and remained elevated as compared to serum free controls. Serum also induced mRNA expression of VEGF in human ASM cells *in vitro* (data not shown).

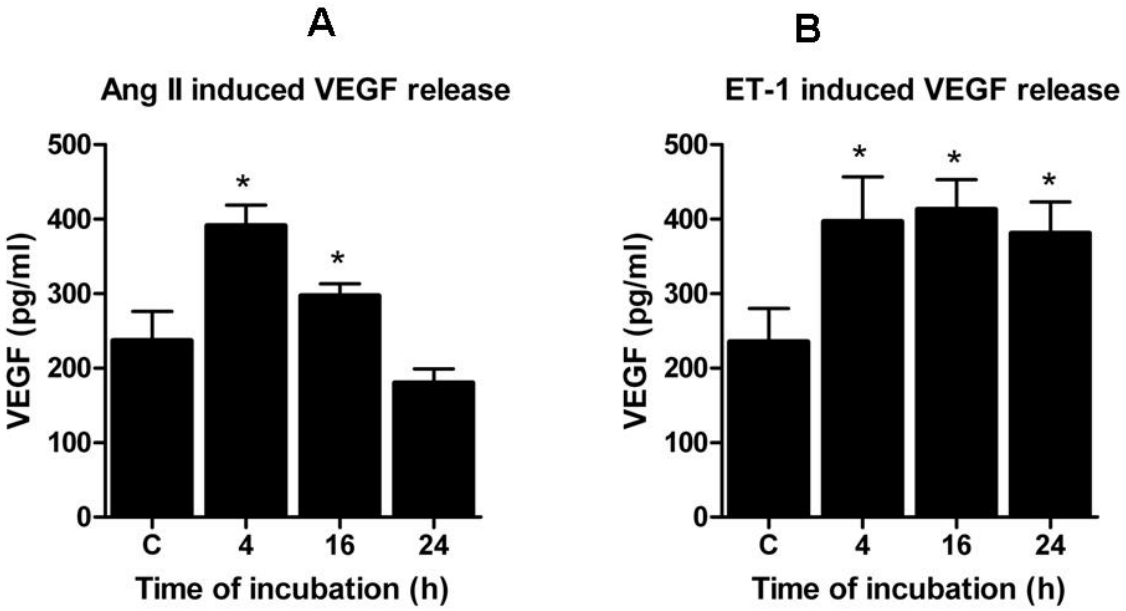

Figure 7.4: Time-dependent production of VEGF protein in relation to ANG II or ET-1

*Growth arrested human ASM cells were stimulated with 100 nM ANG II or 10 nM of ET-1 for various time points. Control cells (C) received serum free medium. Data represent the mean±SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from four patients. *P ≤ 0.05 as compared to respective controls.*

7.3.3 ANG II and ET-1 induced release of VEGF from human airway smooth muscle cells

The effects of ANG II and ET-1 on VEGF release in human ASM cells are shown in Figure 7.4. Conditioned media obtained from ANG II (100 nM)- and ET-1(10 nM)-treated human ASM cells were assessed for the VEGF release using ELISA method. In our study, we observed a time-dependent release of VEGF from human ASM cells treated with ANG II and ET-1. We found a significant increase in VEGF concentration in conditioned medium from ANG II treated ASM cells at 4h of incubation (391±28 pg/ml) as compared to the control (237±39 pg/ml) (Figure 7.4, panel A). In the conditioned medium from ET-1 treated

cells, we found significant increase at 4h (397±60 pg/ml) and 16h (413±40 pg/ml) and then the levels of secreted VEGF remained elevated at later time points (Figure 7.4, panel B). However, ANG II induced the secretion of VEGF protein in a transient manner where the levels declined after 8 h of incubation.

7.3.4 Effects of ASM conditioned medium on DNA biosynthesis and cell number in porcine pulmonary artery endothelial cells

To decipher the angiogenic potential of the conditioned medium obtained from vasoactive peptides treated human ASM cells, proliferation assays were conducted on porcine pulmonary artery endothelial cells. Figure 7.5 (Panel A) shows significant increase in [^{3}H]thymidine incorporation after 24 h treatment with conditioned medium from ET-1-treated ASM cells (2.4±0.5 x10^3 CPM), 5 ng/ml VEGF (1.07±0.3 x10^3 CPM) and 5 ng/ml PlGF (1.07±0.2 x10^3 CPM) as compared to serum free control cells (0.35±0.06 x10^3 CPM). However, the conditioned medium obtained from ANG II-treated human ASM cells did not induce significant proliferation (0.48±0.6 x10^3 CPM) as compared to the control. Similarly, the cell count experiments revealed enhanced cell numbers (2-4 fold) after treatment with the conditioned medium from cells treated with ET-1, VEGF and PlGF at 48 h (Figure 7.5, Panel B).

7.3.5 Inhibition of conditioned medium induced endothelial cell proliferation by VEGF receptor blocker

To verify the potential mitogenic activity of VEGF secreted from ET-1 treated human ASM cells, a HUVEC based cell proliferation model was exploited. Serum deprived cultured HUVECs were pre-treated with the VEGF receptor tyrosine kinase inhibitor (VRI) prior to treatment with the conditioned medium of ET-1 treated human ASM cells and the data is shown in Figure 7.6. We observed a significant increase (1.45 ±0.23 fold) in HUVEC proliferation when cells were incubated with conditioned medium as compared to respective control cells ($P<0.05$). However, pre-treating the HUVEC with VRI showed a drastic inhibition in HUVEC proliferation as compared to cells without the VRI pre-treatment ($P<0.001$) as well as in comparison to control cells ($P<0.05$).

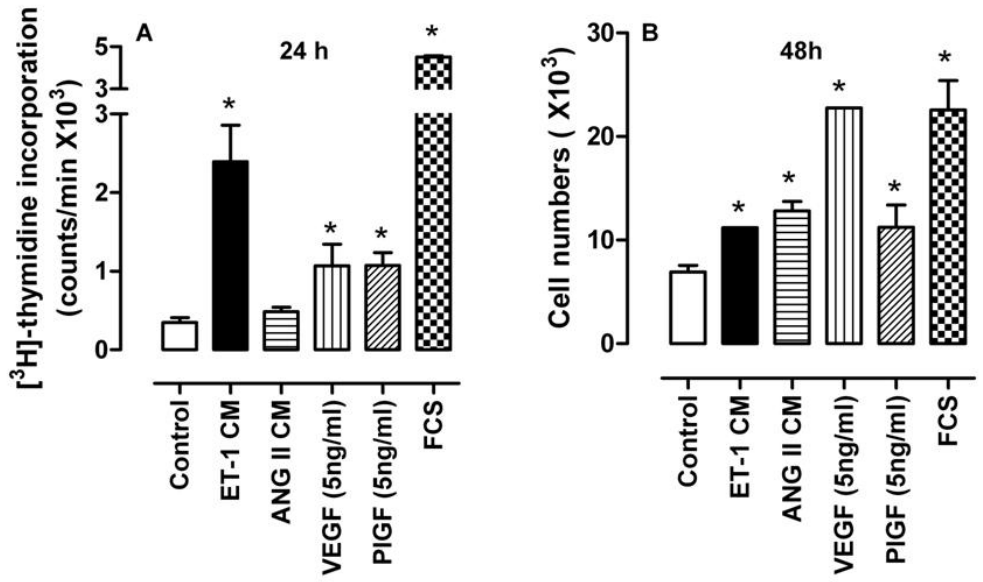

Figure 7.5: Effects of angiogenic factors and conditioned medium (CM) obtained from ANG II or ET-1 treated ASM cells on the biosynthesis of DNA in porcine pulmonary artery endothelial cells

Conditioned medium (CM, 100 µl) obtained from human ASM cells treated with ANG II (100 nM-) or ET-1 (10 nM)-were added to serum deprived endothelial cells and [3H]thymidine incorporation assay was performed. Values are mean ± SEM from three experimental determinations. Note that VEGF (5 ng/ml), PlGF (5 ng/ml), and fetal calf serum (FCS) are potent mitogens for the endothelial cells (P≤0.05). CM from ET-1 (10 nM- treated ASM cells is mitogenic for the cells at 24 h and 48 h. However, CM from ANG II-treated ASM cells did not elicit the mitogenic effect (P≤0.05) at 24 h.

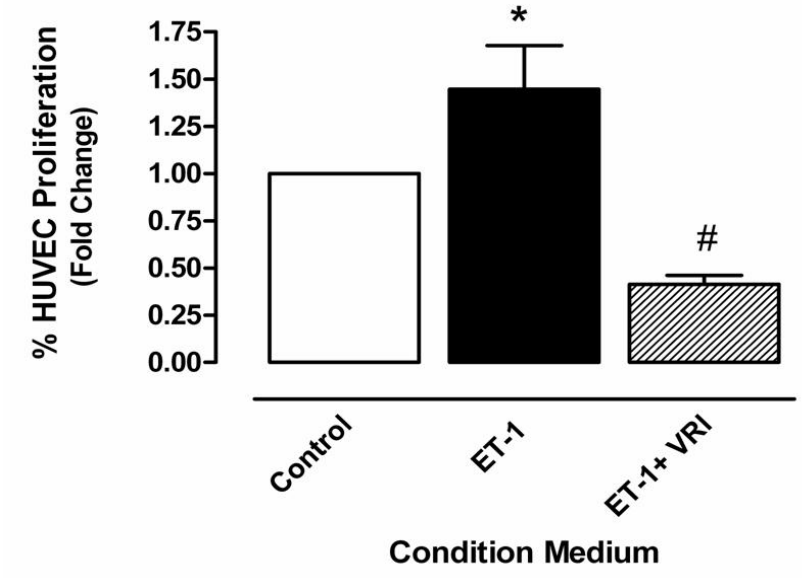

Figure 7.6: Pretreatment with VEGF receptor inhibitor and HUVEC proliferation in response to condition medium

*Graphic representation of fold change in percentage HUVEC proliferation in response to DMEM (Control), and condition medium from human ASM cells treated with ET-1 for 8 h . Proliferation of HUVEC with the ET-1 conditioned medium increased significantly compared to the control. This stimulating effect was strongly diminished when the HUVECs were pre-treated with 10 µM VEGF receptor blocker (VRI) before addition of the ET-1 conditioned medium. Data represents values ± SEM from two experimental determinations. * compared to medium alone (P<0.05), # in relation to ET-1 treated human ASM conditioned medium (P<0.05) at 48h.*

7.4 Discussion

In this study, we have shown that ET-1 induced a time dependent DNA biosynthesis in cultured human ASM cells. We have previously demonstrated the mitogenic and hypertrophic potential of ANG II in human ASM cells (26). Furthermore, both ANG II and ET-1 induced the mRNA expression and secretion of VEGF from human ASM cells. The conditioned medium derived from ET-1 treated ASM cells induced cell proliferation in porcine pulmonary artery endothelial as well as human umbilical vein endothelial cells and VEGF tyrosine kinase receptor inhibitor blocked the conditioned medium induced mitogenesis in endothelial cells. These results suggest that ANG II and ET-1 contribute differentially to human airway smooth muscle cell growth and up-regulation of VEGF for paracrine action on endothelial cells, which could potentially lead to angiogenesis and vascular remodelling during chronic airway diseases. Besides regulating the bronchial tone and contributing to the hyper responsiveness (30), studies have clearly shown the important role of airway smooth muscle in the perpetuation of inflammation by its enhanced secretory nature and thus contributing to the remodelling process in chronic airway diseases. Remodelling is a complex process (31) involving cell migration, proliferation, angiogenesis (2) and extra cellular matrix alteration (32) ultimately leading to a modified airway vascular structure.

In our study, we used confluent human ASM cells as a model to study the role of vasoactive peptides in expression and secretion of VEGF, thereby mimicking vascular remodelling pathways in airways. Our data on the increased expression of VEGF in human ASM cells support the notion that this angiogenic molecule is transcribed and translated in these cells for paracrine action on neighbouring endothelial cells. This is in accordance with previous studies where its expression is regulated by growth factors and cytokines, including TNF-α, TGF-β, and interleukin-1β (18, 33). Several lines of evidence suggest that VEGF levels are up-regulated in asthma (34, 35) and COPD patient (2, 11). Moreover, increased expression of VEGF in asthmatic airways with positive correlation with increase in blood vessel number and size suggests that VEGF significantly contribute to angiogenesis (36) and vascular remodelling and hence to the airway wall remodelling processes. Moreover, the mitogenic effects of the conditioned medium derived from ET-1 treated ASM cells on the pulmonary artery endothelial cells supports the hypothesis that the VEGF synthesised by ASM under influence of these molecules is biologically active. However, the conditioned medium derived from ANG II-treated human ASM cells could not result in endothelial cell

proliferation and warrants further investigation. This could also be attributed to the sub-threshold levels of secreted VEGF from ANG II treated cells as compared to ET-1-treated ASM cells. Both mRNA and protein data on VEGF production in ANG II treated cells showed transient and rapid increase which declined within 8 h indicating for degradation as compared to stable levels in ET-1 treated cells. Also itcan not be ruled out that either the released VEGF is an inactive variant or that VEGF, a known survival factor, is being utilised by the cells rapidly. Additionally, the fact that only ET-1 induces significant proliferation of ASM cells, while ANG II induces cellular hypotrophy but does not increase cell numbers, as we reported previously (26), may suggest that predominantly proliferating ASM cells secrete VEGF. Inhibition of the conditioned medium-induced HUVEC cell proliferation with the VEGF receptor blocker clearly demonstrates and confirms the importance of the angiogenic molecule, VEGF in whole milieu.

In conclusion, our study demonstrated that potent vasoactive peptides (ANG II and ET-1), via increased production of VEGF in proliferating human ASM cells, could indirectly contribute to vascular remodelling and angiogenic processes during bronchial pathophysiology. Our previous results (18, 2) and the current data clearly demonstrate that ASM cells are an important source of angiogenic mediators in these airways. The mechanism underlying the signalling events leading to the expression of VEGF mRNA in response to vasoactive peptides are not entirely clear. It is now well established that the angiogenic factors such as, VEGF produced by ASM cells in the vicinity of the endothelial cells appear to be a key paracrine event in the airways that contributes to the pulmonary vascular remodelling. Undoubtedly, more research focus has been recently directed towards the molecular events underlying the airway vascular remodelling. Though the precise nature of these events is far from clear, our study sheds a possible mechanism of vascular remodelling that could take place in chronic airway diseases.

Acknowledgements

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8

Regulation of VEGF Expression by Nitric Oxide in ASM Cells

Based on: "Alagappan VK, Arulmani U, Garrelds IM, Saxena PR, de Boer WI and Sharma HS: Nitric Oxide Donors Augment Interleukin-1β Induced Vascular Endothelial Growth Factor in Airway Smooth Muscle Cells" – submitted.

Chapter 8: Regulation of VEGF Expression by Nitric Oxide in ASM Cells

Summary
Angiogenesis and microvascular changes are features of chronic inflammatory diseases, including asthma where vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) have been implicated. We investigated the effects of nitric oxide (NO) pathway on interleukin-1β (IL-1β)-induced expression and secretion of VEGF and PlGF from porcine airway smooth muscle (ASM) cells. Serum deprived (for 48 h) porcine ASM cells were stimulated with IL-1β (5 ng/ml) alone or with nitric oxide donor L-arginine (10 mM) and/or NO synthase inhibitor L-NAME (2 mM) for 4 and 24 h. IL-1β did not affect PlGF release, but augmented VEGF release at 24 h from control levels (398.6±11 pg/ml) to 968.4±123 pg/ml. This VEGF release was inhibited by L-NAME (531.8±52 pg/ml), restored and further elevated by L-arginine (1529±287 pg/ml). IL-1β induced expression of VEGF mRNA (1.8 fold vs. control) and this response was attenuated by L-NAME (1.1 fold vs. control cells) and augmented by L-arginine (3.8 fold vs. control) at 4 h. Restoration of nitric oxide pathway by L-arginine in L-NAME-treated cells increased VEGF mRNA expression (2.2 fold). [^{3}H]Thymidine incorporation assay revealed enhanced pulmonary artery endothelial cell proliferation in response to IL-1β, VEGF and PlGF (5 ng/ml each). Interestingly, the conditioned medium obtained from porcine ASM cells treated with IL-1β did not induce endothelial cell proliferation. Moreover, the IL-1β-induced endothelial cell proliferation was not affected by interference with nitric oxide pathway. Taken together, our findings suggest that nitric oxide pathway modulates the synthesis of VEGF during airway inflammation and this may subsequently result in vascular remodelling in chronic airway diseases.

8.1 Introduction

Chronic airway diseases are characterized by reversible/irreversible airway obstruction, hyperresponsiveness and wall inflammation (1, 2) and are associated with increased airway smooth muscle (ASM) mass (3, 4). Airflow obstruction has been linked with airway and pulmonary vascular remodelling (5, 6), of which the molecular mechanisms are poorly understood. Angiogenesis and microvascular changes are documented features of chronic inflammatory diseases, including asthma and other airway diseases (7-10). Evidence suggests that the number and size of bronchial vessels is moderately increased in patients with asthma compared with normal individuals (8, 9, 11-15). Bronchial vascular changes and the associated hyperaemia are contributing factors in airway wall remodelling in patients with chronic asthma, proposing for the ongoing mechanisms like angiogenesis and vascular dilatation (5, 6).

Specific angiogenic and hyperpermeability factors, like vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) could directly contribute to the pathological

events including wound healing in chronic lung diseases such as asthma (16). Several studies have shown that VEGF and PlGF play an important role in airway and vascular remodelling (6, 17, 18). The potent mitogen VEGF, which is produced by different cell types, including endothelial, vascular smooth muscle and tumour cells, induces proliferation and growth of these cells (19, 20). Among various cytokines and growth factors that mediate inflammation in chronic airway diseases, interleukin (IL)-1β has been shown to augment VEGF (21) and nitric oxide (22, 23) production by smooth muscle cells.

Nitric oxide is known to mediate many physiological and pathological functions, including angiogenesis and vascular permeability (24). VEGF induces nitric oxide synthase (NOS) expression and promotes nitric oxide production in vascular endothelial cells *in vitro* (25). Inhibition of *in vivo* nitric oxide production results in reduced angiogenesis and vascular permeability induced by VEGF (24). Recent evidence shows that nitric oxide induces VEGF synthesis in numerous cell types, including vascular smooth muscle cells, macrophages, keratinocytes, and tumor cells (23). Moreover, recently the role of nitric oxide as been discussed as ‘actor’ as well as 'director’ of angiogenesis, both functions being equally expressed during physiological and pathological processes (26).

In this study, we investigated the role of nitric oxide pathway on IL-1β-induced expression and secretion of VEGF and PlGF from porcine ASM cells in culture. Furthermore, we elucidated the of nitric oxide in the mitogenic action of IL-1β on porcine pulmonary artery endothelial cells by using the the NOS-inhibitor N(omega)-nitro-L-arginine methyl ester (L-NAME) and nitric oxide donor L-arginine.

8.2 Materials and methods

8.2.1 Materials

All cell culture reagents were obtained from Invitrogen (Life Technologies BV, Breda, The Netherlands) and recombinant (rh) IL-1β was from Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Foetal calf serum (FCS) was obtained from Bio-Whitaker BV (Verviers, Belgium). Antibodies and the enzyme-linked immunosorbent assay (ELISA) kits were from R & D Systems Europe Ltd (Abingdon, UK).

8.2.2 Porcine ASM cell isolation and culture

The lungs were obtained from Yorkshire x Landrace female pigs after sacrifice and ASM cells were isolated and cultured as described previously (21). Briefly, after removal of the epithelium, parts of smooth muscle was dissected, free of adherent tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (HBSS; Invitrogen, Breda, The Netherlands) containing bovine serum albumin (BSA, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (3.3 U/ml; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) at 37 °C in a humidified incubator containing 5% CO_2/95% air for 30 min. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Breda, The Netherlands) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; Cambrex, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml) (Invitrogen, Breda, The Netherlands). Cells were subsequently seeded at $2x10^5$ cells per 35 mm dish and maintained in culture by replacing the medium every 48 h. After 10-14 days in culture, ASM cells grew to confluence and were then detached by trypsinization (0.5% trypsin; 0.02% EDTA; Invitrogen, Breda, The Netherlands) and subcultured into 25 cm^2 and 75 cm^2 tissue culture flasks. Confluent serum-deprived primary cultures of porcine ASM cells were characterized by immunocytochemistry using monoclonal antibodies to smooth muscle α-actin (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) demonstrating that the cultures were essentially free (<5%) of other contaminating cell types. Cells in 4^{th} -5^{th} passage were used for experiments.

8.2.3 Porcine pulmonary artery endothelial cell isolation and culture

Major porcine pulmonary artery was dissected free of all connective tissue and cleaned with HBSS. After incubation with collagen I for 30 min endothelial cells were isolated by scraping gently the inner surface of the artery with a surgical blade and cultured up to fourth passage in M-199 medium containing 10% foetal calf serum (FCS; Cambrex, Verviers, Belgium) at 37 °C in a 5% CO_2-humidified atmosphere. Cells were maintained in culture by replacing the medium every 48 h and when confluent was further passaged using trypsin/EDTA solution into 75 cm^2 tissue culture flasks. Porcine pulmonary artery endothelial cells had the characteristic cobblestone appearance when cultured in monolayer.

Cells were characterized immunocytochemically, using monoclonal antibodies against CD31 and visualised under the light microscope. Porcine pulmonary artery endothelial cells in culture revealed cobblestone appearanc. Immunocytochemical staining of confluent serum-deprived primary cell cultures of using monoclonal antibodies against CD31 demonstrated that the cultures were essentially free (<5%) of other contaminating cell types. Cells were maintained in culture by replacing the medium every 48 h and, when confluent, were further passaged using trypsin/EDTA solution into 75 cm^2 tissue culture flasks up to 4^{th} passage.

8.2.4 Measurement of VEGF and PlGF in conditioned media

To obtain information on the release of VEGF and PlGF and the role of nitric oxide pathway from stimulated pulmonary ASM cells in culture, conditioned medium was collected at various time points from control (untreated) cells and cells treated with IL-1β with or without L-NAME (NOS-inhibitor) and L-arginine (nitric oxide donor). Human specific solid-phase ELISA was performed for measuring the secreted VEGF and PlGF in the media. A standard curve using recombinant VEGF and PlGF protein was first established and then 0.5 ml of conditioned medium was used for the assay. Samples of conditioned medium were diluted until the levels of angiogenic proteins were within the linearity limits of the standard curve of the assay. Subsequently, the samples were pre-incubated with respective capture antibodies followed by biotinylated detecting antibody. After addition of the streptavidine-peroxidase conjugate tetramethylbenzidine (TMB), the absorbance of the resulting coloured product was measured using an automated spectrophotometer (Biorad Laboratories BV, Veenendaal, The Netherlands). The detection limit of the ELISA method was 20 pg/ml for both VEGF and PlGF.

8.2.5 Isolation of total cellular RNA and RT-PCR

Porcine ASM cells were washed in phosphate buffer solution (PBS, 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM $Na_2HPO_4.2H_2O$, pH 7.4) and directly lysed in guanidinium thiocyanate buffer (4M guanidium thiocyanate, 25mM sodium citrate, 0.5% N-lauroylsarcosine, 0.1% anti foam A, 1% beta-mercaptoethanol). Genomic DNA was sheared by passing lysates repeatedly through 23-gauge needles. Total cellular RNA was isolated, as described previously (21), treated with RNase-free DNase to eliminate contaminating genomic DNA and processed for the synthesis of cDNA and PCR. Porcine

specific forward and reverse primers spanning over a 319 bp fragment encoding VEGF-A (27) and a 625 bp fragment of β-actin (28) were employed. The human primers used were checked with the Basic Local Alignment Search Tool (BLAST) from the national centre for biotechnology information (NCBI) to be compatible with the porcine sequence. The PCR products were separated on 1.5% agarose gel, digitally photographed and the intensity of the bands was quantified in relation to β-actin band using Molecular Analyst (V 1.5) image analysis program (Biorad Laboratories, Hercules, USA). The arbitrary optical densitometric (OD) values were expressed as a ratio to the controls.

8.2.6 [^{3}H]Thymidine incorporation assay

Porcine growth-arrested pulmonary artery endothelial cells were incubated for 24 h with IL-1β, with or without L-NAME and L-arginine, in 100 µl FCS-free M199 medium. Control incubations consisted of cells that were incubated with FCS-free M199. Five hours prior to the end of the incubation period, 10 µl of [^{3}H]thymidine was added at a final concentration of 1 µCi/110 µl per well. The plates were frozen overnight at -20 °C after which the cells were harvested on glass fibre filters using a Filtermate 196-cell harvester (Packard, Meridan, USA) and the activity was counted using a Microplate Scintillation β-counter (Topcount, Packard, Meridan, USA). Measured radioactivity was expressed as counts per min (CPM) and the mean ± SEM CPM of quadruple wells and subsequently three different cell isolations were expressed as fold induction (as ratio to the control cells).

8.2.7 Cell counting

In a parallel series of experiments, cells were incubated with same compounds as above for 24 and 48 h. Thereafter, the cells were harvested in 50 µl of trypsin/EDTA solution for 10 min and the suspension were added to 10 ml of Casy®1 isotonic solution (6.38 g/l NaCl, 0.2 g/l Na-tetraborate, 1.0 g/l Boric acid and 0.2 g/l EDTA). An aliquot of 200 ul cell suspension was inserted in to Casy®1 coulter cell counter and cell numbers in one ml were assessed using system software (Sch¨arfe System GmbH, Reutingen, Germany).

8.2.8 Statistical analysis

All data in the figures are given as mean±SEM. Statistical analysis was performed by using two-tailed, independent sample "t"-test. Significance was accepted at $p \leq 0.05$.

8.3 Results

8.3.1 Regulation of IL-1β induced VEGF and PlGF secretion by nitric oxide pathway

Figure 8.1 depicts the release of VEGF (upper panel) and PlGF (lower panel) following incubation of ASM cells with IL-1β in the absence or presence of L-NAME and L-arginine. IL-1β augmented VEGF release at 24 h from 398.6±11 pg/ml (control) to 968.4±123 pg/ml. This VEGF release was inhibited by L-NAME (531.8±52 pg/ml) and restored by L-arginine (1529±287 pg/ml). In contrast to VEGF levels, PlGF could not be detected at 4 h of IL-1β treatment and then levels rose in all experimental conditions from non detecting level to 28-50 pg/ml and did not differ significantly.

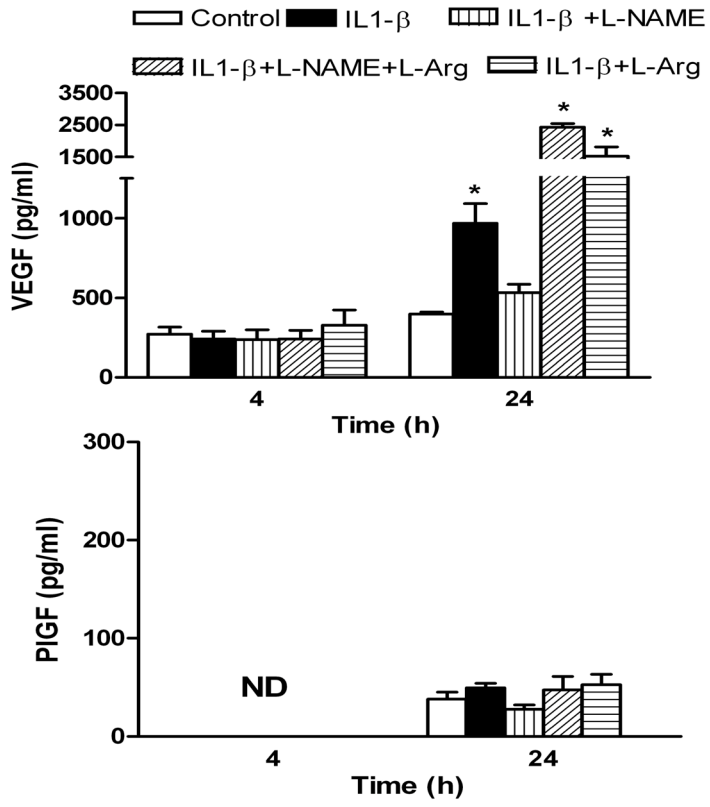

Figure 8.1: Role of nitric oxide in VEGF and PlGF secretion by IL-1β treated porcine ASM cells
*Graphic representation of VEGF (pg/ml) and PlGF (pg/ml) release at 4 and 24 h time in culture medium of ASM cells stimulated with vehicle (control) or IL-1β (5 ng/ml), in the absence or presence of L-NAME (2 µmol/ml) and/or L-arginine (L-Arg, 10 µmol/ml). Values represent mean ± SEM of three different cell batches. * p≤0.05 as compared to control (untreated ASM cells).*

8.3.2 Effects of NO pathway on IL-1β induced VEGF mRNA expression

IL-1β induced the expression of VEGF mRNA (1.8 fold vs. control) at 4 h, but apparently this effect had dissipated by 24 h as no increase was detected at this latter time point (Figure 8.2). The IL-1β-induced VEGF mRNA expression was attenuated by L-NAME (1.1 fold vs. control cells) whereas, L-arginine restored the response to IL-1β by increasing the levels by 3.8 folds vs. control (Figure 8.2).

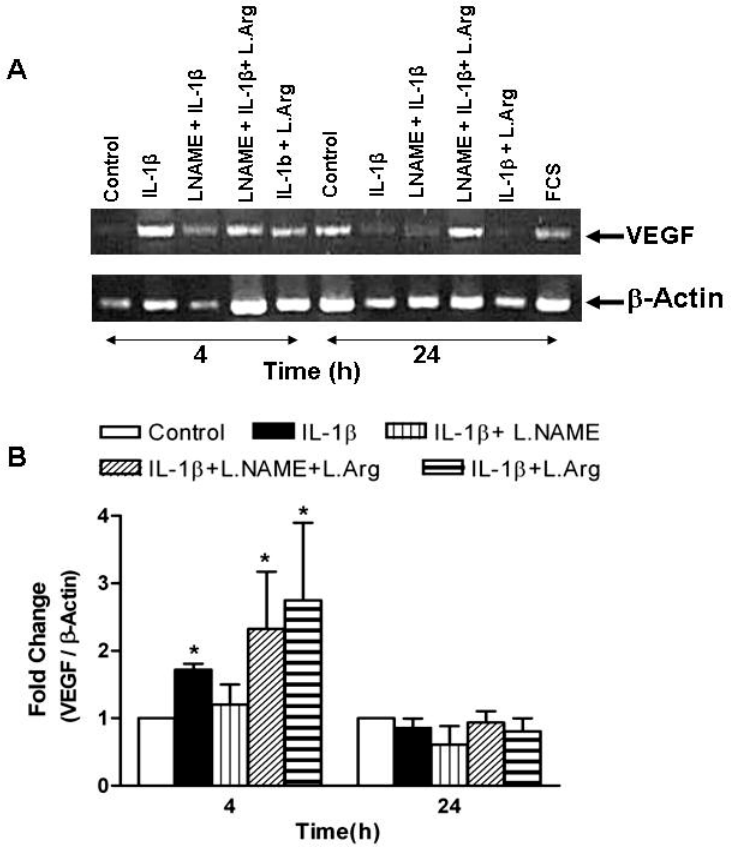

Figure 8.2: RT-PCR analysis of VEGF mRNA expression in porcine ASM cells

*Panel **A**: Agarose gel electrophoresis of RT-PCR products of VEGF (319 bp) and β-actin (625 bp) synthesised from porcine ASM cells treated with vehicle (control) or IL-1β (5 ng/ml), in the absence or presence of L-NAME (2 µmol/ml) and/or L-arginine (L-Arg, 10 µmol/ml).*

*Panel **B**: The intensity of the bands was analysed using digital image analysis software and VEGF/β-actin ratio was calculated as described in the text. Values are mean ± SEM from 3 independent measurements.*

8.3.3 Endothelial cell proliferation

Endothelial cell proliferation was assessed in response to various mitogens and IL-β treated ASM conditioned media. [^{3}H]thymidine incorporation assay revealed an increased cell proliferation in cells treated with IL-1β (5 ng/ml), VEGF (5 ng/ml) or PlGF (5 ng/ml), but not in the case of pulmonary artery endothelial cells incubated with conditioned medium obtained from IL-1β (5 ng/ml)-treated porcine ASM cells (Figure 8.3). Similarly, cell count experiments revealed increased cell number in pulmonary artery endothelial cells treated with IL-1β (5 ng/ml), VEGF (5 ng/ml), PlGF (5 ng/ml) or FCS as compared to control cells. However, conditioned medium collected at 24 h from porcine ASM cells treated with IL-1β (5 ng/ml) did not increase cell number significantly (Figure 8.4).

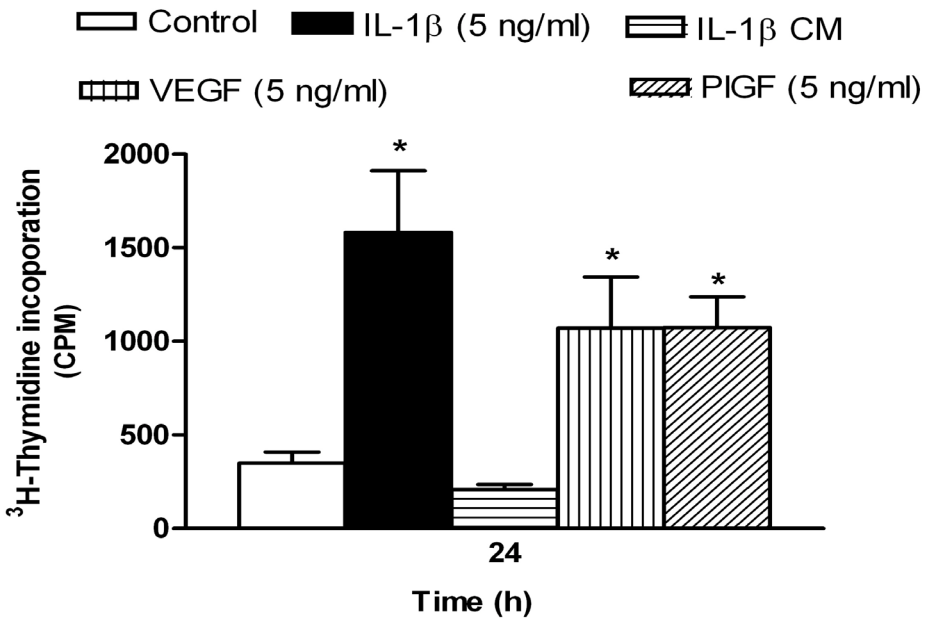

Figure 8.3: [^{3}H]Thymidine incorporation showing DNA biosynthesis in pulmonary artery endothelial cells

*Effects of vehicle (control), IL-1β, VEGF, PlGF and 100 µl of conditioned medium obtained at 24 h from IL-1β-treated porcine ASM cells (IL-1β CM) on [^{3}H]thymidine incorporation. Values are mean±SEM from three experimental determinations. Note that IL-1β, VEGF and PlGF, but not the conditioned medium, showed a potent mitogenic activity. *P≤0.05 vs. control.*

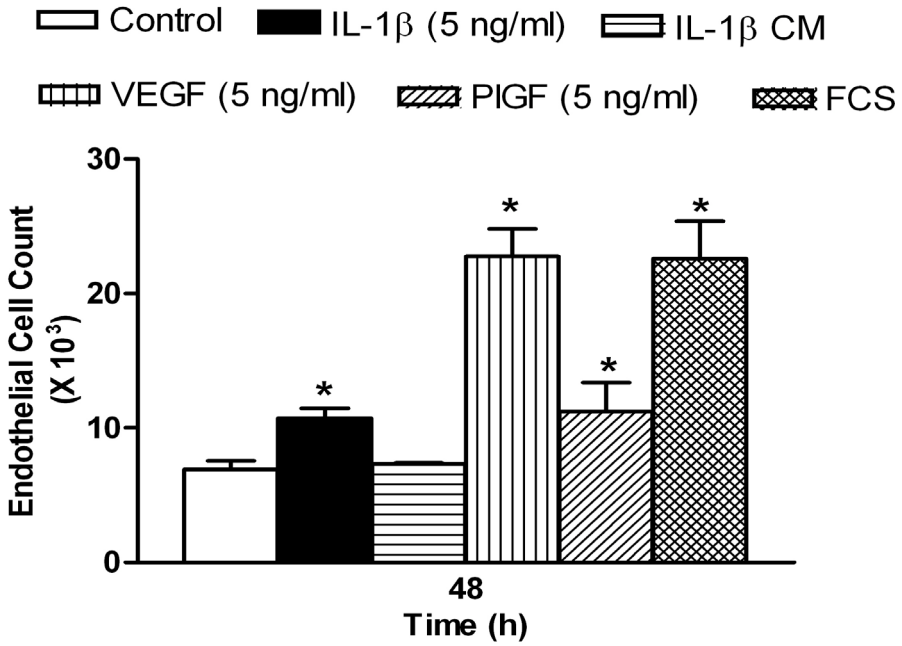

Figure 8.4: Cell count in pulmonary artery endothelial cells

*Effects of vehicle (control), IL-1β, VEGF, PlGF, 100 µl of conditioned medium obtained at 24 h from IL-1β-treated porcine ASM cells (IL-1β CM) and FCS (positive control) on pulmonary endothelial cell count. Values are mean±SEM from three experimental determinations. Note that IL-1β, VEGF, PlGF and FCS, but not the conditioned medium, showed a potent mitogenic activity. *P≤0.05 vs. control.*

8.3.4 Role of nitric oxide in IL-1β-induced cell proliferation in pulmonary artery endothelial cells

In order to decipher the role of nitric oxide pathway in the increase in cell number due to IL-1β, cells were pretreated with the NOS inhibitor L-NAME and the nitric oxide donor L-arginine and their number was counted after 24 and 48 h. Figure 8.5 shows that compared to serum deprived (control) cells, IL-1β (as well as FCS) induced clear cell proliferation at 48 h and this effect of IL-1β was not inhibited by the pretreatment of the cells with L-NAME. Moreover, treatment with L-arginine did not significantly affect the response to IL-1β.

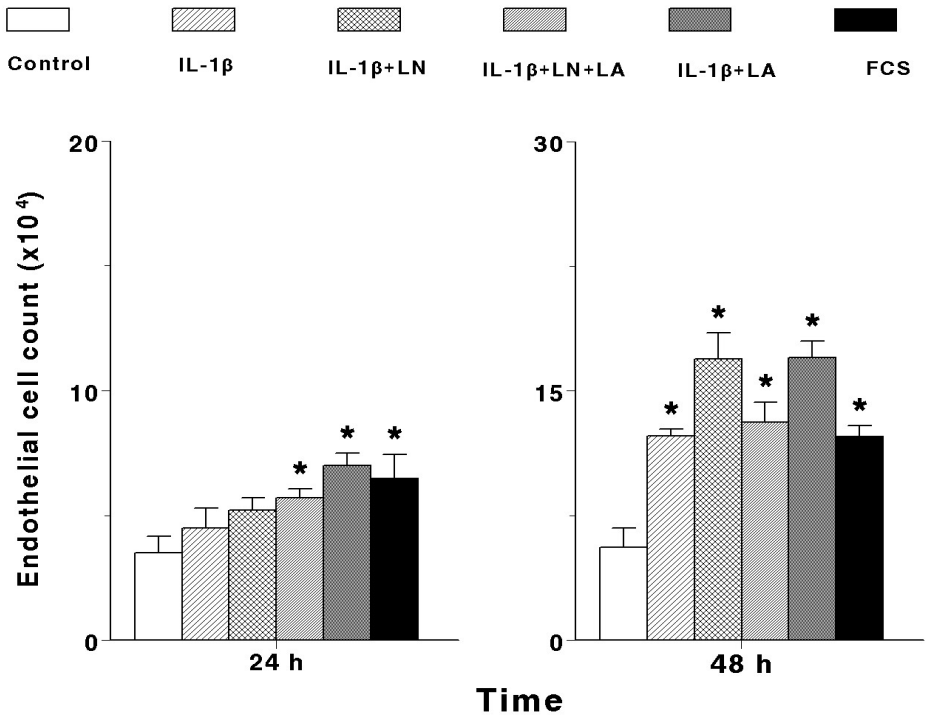

Figure 8.5: Role of nitric oxide in IL-1β-induced proliferation of pulmonary artery endothelial cells

*Serum deprived pulmonary artery endothelial cells were treated with medium alone (control), IL-1β (5 ng/ml) alone or in combination with 2 µmol/ml L-NAME (IL-1β+LN), 10 µmol/ml L-arginine (IL-1β+LA) or (IL-1β+LN+LA). FCS was used as positive control. Values are mean±SEM from three experimental determinations. *P≤0.05 vs. control.*

8.4 Discussion

Our data clearly demonstrate that IL-1β stimulates porcine airway smooth muscle cells by upregulating VEGF for paracrine action on endothelial cells. This clearly supports our

previous results that IL-1β induces mRNA expression and the secretion of VEGF from human ASM cells (21). Furthermore, in this study we established that nitric oxide pathway modulates the synthesis of VEGF in ASM cells by using inhibitors of NOS and nitric oxide augmenter L-arginine. This suggests that nitric oxide pathway may play a potential role in vascular remodelling in chronic airway diseases.

The inhibition of IL-1β-induced VEGF expression and secretion by NOS inhibitor L-NAME has been reported (22), though in a different cell type and species. Dulak et al. (22) described in rat vascular smooth muscle cells the role of nitric oxide in the synthesis of VEGF. This effect of NO on VEGF is also reported in other cell types, such as tumour cells (29) and keratinocytes (30). Our results are consistent with previous observations that IL-1β induces the NO generation via inducible nitric oxide synthase (31, 32) and/or VEGF (21, 33) as reported for vascular smooth muscle cells. However, we show for the first time that nitric oxide synthesis augments VEGF expression in porcine ASM cells. L-NAME inhibited VEGF generation in IL-β-stimulated porcine ASM cells suggesting the pivotal role played by nitric oxide in the whole milieu of pulmonary angiogenesis and remodelling since VEGF is one of the key angiogenic factor known (34). We have earlier reported that bronchial expression of VEGF is enhanced during COPD and that this molecule plays a role in bronchial remodelling associated lung function decline (6). Inhibition of IL-β-induced VEGF by L-NAME was reversed by addition of nitric oxide donor L-arginine reinforcing the critical role of nitric oxide. Recently, Kimura et al. (30) demonstrated that nitric oxide donors up-regulated the activity of the human VEGF promoter in human glioblastoma and hepatoma cells, independently of a cGMP-mediated pathway and supporting our findings in porcine ASM cells.

Angiogenesis is a complex multistep process that involves endothelial cell migration, proliferation and differentiation into vascular tubes. Nitric oxide has been reported to be a downstream mediator in the angiogenic response to a variety of growth factors, but the mechanisms by which nitric oxide promotes neovessel formation is not clear. Our study has focused on the regulation of VEGF by nitric oxide in ASM cells and it is clear that nitric oxide indeed plays a pivotal role. Babaei et al. (35) demonstrated that nitric oxide directly contributes to endothelial cell migration and capillary tube formation. These data support an important paracrine role for endogenously produced nitric oxide in endothelial cell

migration and differentiation *in vitro*, and suggest that the cell-based eNOS gene transfer may be a useful approach to increase new blood vessel formation *in vivo*. Figure 8.6 summarizes the possible role of nitric oxide in autocrine/paracrine manner in the regulation of VEGF and, in turn, angiogenesis in relation to smooth muscle and endothelial cells.

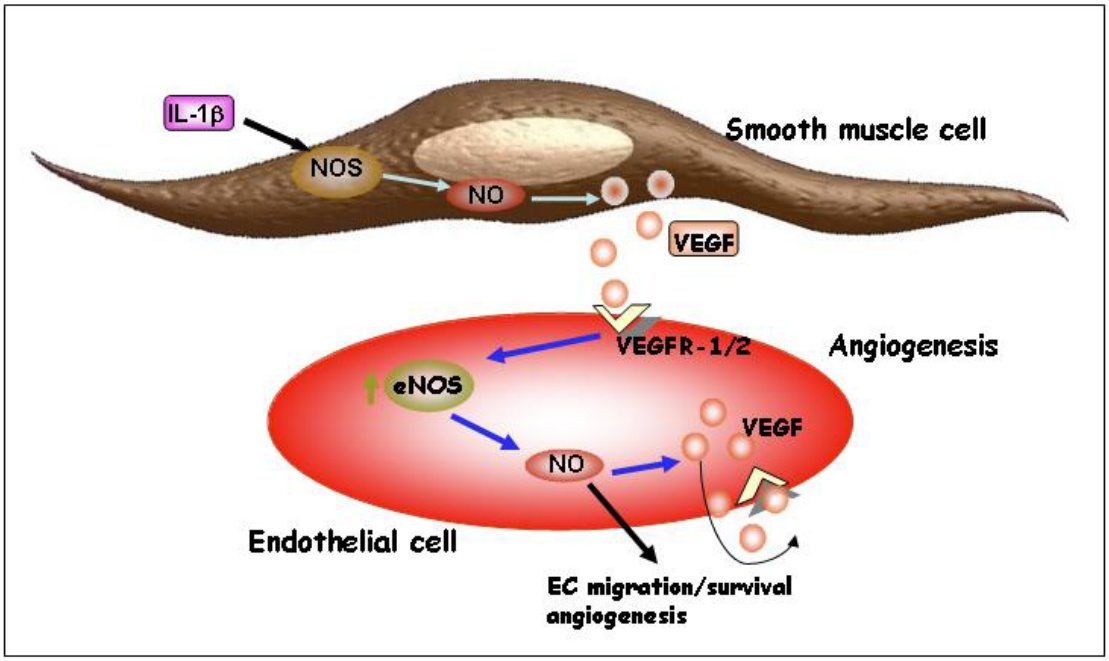

Figure 8.6: Autocrine /paracrine role of nitric oxide in regulation of VEGF in smooth muscle and endothelial cell cross talk

Note that IL-1β induced VEGF production in ASM cells is NOS dependant and that this VEGF may, in turn, be involved in angiogenesis via the nitric oxide pathway in endothelial cells. Adapted from reference (36).

Interestingly, the second part of our study to look at the role of nitric oxide in IL-1β-induced endothelial cell proliferation revealed that IL-1β was potent mitogen for porcine pulmonary artery endothelial cells, similar to the angiogenic molecules VEGF and PlGF. However, the conditioned medium from IL-1β-treated ASM cells did not induce significant endothelial cell proliferation though it contained sufficient VEGF as revealed by ELISA. We believe that super oxide radicals formed as a result of IL-1β treatment of the porcine ASM cells may be the reason for the unaltered cell proliferation. Further studies are required to explore these possibilities. Moreover, IL-1β mediated mitogenesis seen in the pulmonary artery endothelial cells does not depend on the nitric oxide pathway as we have demonstrated by inhibiting NOS using L-NAME.

Taken together, our results suggest that nitric oxide pathway may modulate the synthesis of VEGF during airway inflammation and subsequently result in vascular remodelling in chronic airway diseases. The induction of VEGF synthesis by nitric oxide may be of great importance in the maintenance of vascular homeostasis e.g in the response to endothelial injury as well as during vascular remodelling in the airways. It is clear that two pathways synergistically regulate vascular remodelling and function via enhanced reciprocal synthesis of nitric oxide and VEGF; this interaction may contribute to the bronchial remodelling during chronic airway inflammation.

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9

Cyclically Stretched Airway Smooth Muscle Cells and Angiogenic Molecules

Based on: "Alagappan VK, Firdausi S, de Boer WI, Dekkers DHW, Garrelds IM, Bogers AJJC, Lamers JMJ and Sharma HS. Mechanically Stretched Airway Smooth Muscle Cells Produce Angiogenic Molecules: Role of ERK Pathway"- To be submitted for publication.

Chapter 9: Cyclically Stretched Human ASM Cells and Angiogenic Molecules

Summary
Bronchial hyperresponsiveness associated with chronic inflammation is a key feature in asthma where airway smooth muscle undergoes cyclical strain that may alter its molecular phenotype. Using a cyclical stretch model, we explored the protein expression profile, signalling pathways and the secretion of angiogenic molecules in human cultured airway smooth muscle (ASM) cells. Human ASM cells were isolated from bronchial rings obtained from patients who underwent lobectomy/pneumonectomy. Serum deprived (48 h) human ASM cells, seeded in collagen-I coated 6-well BioFlex plates, were subjected to a pattern of 0.5 s stretch and 0.5 s relaxation (frequency 1 Hz) for 5, 15, 30 min and 1, 2, 4, 8, 24 and 48 h in a Flexer strain unit. Cells were harvested and the conditioned medium as well as cell extracts were collected at each time point and analyzed by antibody arrays, ELISA and Western blotting. Protein profiling by antibody arrays revealed enhanced secretion (2-10 fold) of various direct/indirect angiogenic molecules such as vascular endothelial growth factor (VEGF), angiogenin, interleukin (IL)-6 and IL-8 in the conditioned medium derived from ASM cells subjected to cyclical stretch. VEGF secretion, assessed by ELISA, was significantly higher after 8 h ($p \leq 0.02$) and 24 h ($p \leq 0.001$) as compared to controls. Western blot analysis showed robust phosphorylation of ERK1/2 after 15 min and Akt; P-Thr-Akt ($p \leq 0.001$) and P-Ser-Akt ($p \leq 0.004$) after 30 min of stretch. Pre-treatment of cells with respective blockers for Akt, ERK1/2 and RhoA/ROCK pathways revealed significant inhibition of VEGF release only in case of ERK1/2 inhibitor, U0261 after 8 h. Whereas, both ERK1/2 and RhoA/ROCK pathways inhibition resulted in diminished secretion of IL-6 ($p \leq 0.05$) and IL-8 ($p \leq 0.01$) in cyclical stretched ASM cells after 8 h. Our results demonstrate that hyper contractile human ASM cells secrete pro-angiogenic molecules predominantly via ERK1/2 pathway and cytokines via RhoA/ROCK pathways and subsequently contributing to vascular and airway remodelling during chronic airway diseases

9.1 Introduction

Asthma is a chronic inflammatory disease characterized by bronchial hyper-responsiveness and airway remodelling of which molecular mechanisms are poorly understood. Airway smooth muscle (ASM) cells undergo cycles of stretching and shortening during breathing. Bronchial hyper-responsiveness, a common feature of asthma (1), leads to excessive narrowing of the airways driven mainly by the increased muscle mass and contractility. Mechanical stress experienced by ASM has been implicated in such excessive airway narrowing or remodelling (2, 3); molecular mechanisms underlying this behaviour of ASM remain unclear. Many studies have indicated the importance of periodic deep inspirations in maintaining bronchodilatation in normal subjects (4, 5). Furthermore, prolonged withholding of deep inspiration induces asthma-like bronchoconstriction in normal subjects (6). These findings suggest that strain/stretch has a complex modulatory effect on airway

smooth muscle contractility and it is known that physical forces and changes in the mechanical structure of tissues influence cellular physiology (7). Although it is recognized that significant mechanical distension takes place in the lungs and that several airway diseases, including asthma, involve changes in airway mechanics, little is known about the influence of mechanical stimulation on the function of ASM. To learn more about the effects of these hyper-contractile states, various *in vitro* stretch models using rat bronchial rings, bovine trachea or human ASM cells have been attempted (6, 8, 9). Different methods of *in situ or in vitro* mechanical stretch have been developed to mimic what the ASM cells are exposed *in vivo* (8).

Some *in vitro* studies (9, 11) have suggested the role of mitogen-activated protein (MAP) kinase and protein kinase C (PKC) signalling pathways in human ASM cells in response to mechanical strain. However, there is little information on how different signalling pathways interact with each other and what their role is in mediating the expression of various angiogenic molecules. Kumar et al. (9) suggest that the stretch-induced IL-8 release is mediated via increased activation of activator protein-1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) transcription factors via Extra cellular regulated kinase-1 and 2 (Erk1/2) and p38 MAP kinase signalling pathways. In the same context, the study of Li et al. (12) suggests that stretch-induced transcriptional regulation of IL-8 mRNA and IL-8 production is via activation of AP-1 and NF-κB and dependent on the activation of c-Jun NH_2-terminal (JNK) and NF-κB-inducing (NIK) kinases, respectively. Wang et al. (11) suggest a role for the PKC signalling pathway and its interaction with the RhoA-ROCK-1 (Rho kinase-1) signalling, required for the nuclear localization of serum response factor (SRF) in cyclically stretched human ASM cells. Furthermore, evidence points to role of RhoA and its downstream effector Rho kinase in the enhancement of smooth muscle contractility due to mechanical stress (13).

In view of our previous experience with the stretch model on cardiac myocytes (14) and the available evidence (9, 12), we hypothesized that in response to cyclic mechanical strain, the molecular phenotype of human ASM cells is altered. This may result in the expression and secretion of a number of known and unknown molecules, such as growth factors and cytokines that may be relevant in the airway and vascular remodelling process. Using an

in vitro cyclical stretch model, we investigated the expression profile, signalling pathways and the secretion of direct and indirect angiogenic molecules in cultured human ASM cells.

9.2 Materials and methods

9.2.1 ASM cell culture

In accordance with procedures approved by the Erasmus MC Research Ethics Committee, human ASM cells were obtained from the lobar or main bronchus of patients undergoing lung resection for carcinoma of the bronchus, using cell isolation and culture methods described previously (15). Briefly, after removal of the epithelium, fragments of smooth muscle were dissected free of adherent tissue under aseptic conditions and incubated in HBSS containing bovine serum albumin (BSA, fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml) at 37 °C in a humidified incubator containing 5% CO_2 in air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml). Cells were subsequently seeded in 35 mm dishes and maintained in culture by replacing the medium every 72 h. At confluency, cells were further passaged using trypsin/EDTA solution into 75 cm^2 tissue culture flasks. Experiments were performed on near confluent cells between the 4^{th} and 6^{th} passage. Human ASM cells in culture appeared spindle or ribbon-shaped with central oval nuclei containing prominent nucleoli. Cells were characterized immunocytochemically using α-smooth muscle actin and myosin monoclonal antibodies visualized by using a FITC-conjugated secondary antibody and viewed under a fluorescence microscope.

9.2.2 Cyclical stretch model

The human ASM cells (200.000 cells per well) were seeded on BioFlex® six well plates coated with collagen. Cells were grown in DMEM with 10% foetal calf serum (FCS) for 72 h, followed by serum deprivation for 48 h. Before the start of the experiment, the serum free medium (DMEM) was refreshed in each well. The flexible bottomed (silastic membrane) 6-well plates were placed in the base plate (loading post) of the Flexercell stretch apparatus (model 2000FX), Flexcell International Corporation, Hillsborough, USA).

The loading posts where the 6 well bioflex plates are placed, allow the flexible membrane bottom of each culture well to be stretched in a uniform biaxial pattern. The stretch was induced using a computer controlled vacuum regulator to result in a 15% stretch in a cyclical pattern of 0.5 s stretch and 0.5 s relaxation (frequency 1 Hz) (9). Cyclical mechanical stretch was applied to the human ASM cells for shorter (0, 5, 15, 30, 60 and 120 min) or longer time periods (1, 4, 8 and 24 h). Time-matched control cultures were also plated and grown on collagen coated BioFlex plates with no external mechanical strain applied. The experiments were repeated in duplicates using at least three different batches of cells.

9.2.3 Human cytokine antibody array

Conditioned media were collected from human ASM cells subjected to cyclical stretch for 24 h and the protein expression profiles of multiple cytokines were examined in relation to unstretched cells by using the RayBio® Human Cytokine Antibody Array III (Raybiotech, Inc., Norcross USA). Table.9.1 shows the 42 cytokines and proteins that are detectable using this array; mapped in the same order on the membrane. The membranes were blocked with a blocking buffer for 1 h and were then incubated with 1 ml of the conditioned medium from stretched or unstretched cells for overnight at 4 ºC. The membranes were then washed and incubated with Biotin-Conjugated Anti-Cytokines for 3 h at room temperature. Subsequently, they were washed again and incubated with HRP-conjugated streptavidin for 2 h at room temperature; all incubations and wash steps were performed under gentle rotation. The membranes were visualized using detection reagents and exposed to x-ray films. After developing, the films were scanned and analyzed in the BioRad® GS800 scanner using the Quantity One-4.2.1® program.

9.2.4 Cell signalling

To investigate whether mechanical stretch activates the Erk1/2, phosphoinositide 3-kinase (PI3k)-Akt and Rho/ROCK signalling pathways, human ASM cells were mechanically stretched for 0, 5, 15, 30, 60 and 120 min. Furthermore, ASM cells were pre-treated with the inhibitors of these pathways, followed by 15 min and 1 h stretch. Based on the literature, we chose 30 min pre-incubation time and 20 µM/well inhibitor concentration for wortmannin (PI3k; Sigma-Aldrich, Zwijndrecht, Netherlands) (16-18), U0261 (Erk1/2;

Promega®, Madison, USA) (9, 19) and Y27632 (Rho-A/ROCK-1, Calbiochem, La Jolla, USA) (19, 20). Conditioned medium was collected after 1 h and 8 h, following the 1 h stretch with and without kinase inhibitors to investigate if the activation of these pathways was crucial for the production and secretion of VEGF, IL-6 or IL-8. Cell lysates after each time point were analysed using western blot assay for respective signalling pathways. Basal cell activation of the signalling pathways was noted from cells directly harvested at zero time point. Respective time matched controls were taken to compare the effect of the inhibitors.

Total protein extraction and measurement

After the application of mechanical stretch for different time periods, human ASM cells were lyzed in Laemmli loading buffer containing 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, and 0.0025 % bromophenol blue in water for the Western blot analysis. BioRad RC DC protein assay kit and bovine serum albumin as standard were used to measure the amount of protein in the cell lysates.

Western blot analysis

Cell lysates protein samples (10-20 µg) were separated on a 10% acrylamide gel using standard SDS-PAGE procedures and transferred to polyvinylidene difluoride membranes using the Bio-Rad® mini Protean™ II system. The membranes were afterwards blocked with 5% bovine serum albumin (BSA) or 5% milk powder in TTBS buffer (10 mM Tris/HCL, pH 7.6**,** 150 mM NaCl**,** 0.1% (w/v) Tween) supplemented 0.1% Goat serum, for at least 30 min. After blocking, the membranes were incubated for overnight at 4 °C with the rabbit polyclonal antibodies (Cell Signalling®, Danvers, USA) directed against total-Erk 1/2, Phospho-Erk 1/2, total-P38, Phospho-P38, total-Akt or Phospho-Akt (Thr308), or with the mouse monoclonal antibody directed to Phospho-Akt (Ser473). After incubation with the first antibody, the blots were washed with 150 mM TTBS and incubated for at least 3 h with the second antibody, horseradish peroxidase conjugated goat anti-rabbit (GARPO) or HRP conjugated goat anti-mouse (GAMPO) antibodies (PIERCE® Rockford, USA) in TTBS buffer supplemented with 0.5% milk powder. Immunoreactivity was visualized using enhanced chemiluminescene (ECL) detection reagent (PIERCE® Rockford, USA). After developing, the films (Amersham Hyperfilm ECL) were scanned and analyzed in the BioRad® GS800 scanner using the Quantity One-4.2.1® program.

9.2.5 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

Conditioned media were collected from human ASM cells that were unstretched or stretched for 1, 4, 8 and 24 h and assessed for VEGF (ELISA, R & D, Abingdon, UK) and IL-6 and IL-8 (PeliKine Compact ELISA, Sanquin, Amsterdam, NL). Furthermore, conditioned media were collected after 1 h and 8 h following 1 h stretch, with and without the kinase inhibitors, and were assessed using the above human-specific ELISA kits.

9.2.6 Statistical analysis

All data in the figures are given as mean±SEM. Statistical analysis was performed by using two-tailed, independent sample "t"-test. Significance was accepted at $p \leq 0.05$.

9.3 Results

9.3.1 Protein profiling by antibody array

Conditioned media from human ASM cells that were unstreched (control) or mechanically stretched for 24 h were analyzed to investigate the protein release, using the human cytokine antibody array. Figure 9.1 shows blots from control (upper left panel) and stretched (upper right panel) cells. Out of the possible 42 proteins mapped on the membrane, stretching of cells resulted in upregulation of 8 and downregulation of 1 (Figure 9.1). The representative quantative data show that mechanical stretch of human ASM cells increased the expression of angiogenin (ang), vascular endothelial growth factor (VEGF), growth-related oncogene (GRO), regulated on activation, normal T cells expressed and secreted (RANTES), interleukin (IL)-6, IL-8, monocyte chemotactic protein (MCP)-1 and tumour necrosis factor (TNF)-β (Figure 9.1, lower panels A and B; Table 9.1).

9.3.2 Signal transduction pathways: PI3K-Akt, ERK 1/2 and RhoA/ROCK

To investigate the activity of Erk1/2, P38 and Akt kinases in response to mechanical stretch, human ASM cells stretched for shorter time periods were analyzed using Western blots. Data in Figure 9.2 (left panels) demonstrate that the application of cyclical mechanical stretch to human ASM cells significantly activated Erk1/2 phosphorylation as early as 5 min compared with the total Erk 1/2 (stretch 1.19±0.099 vs. control 0.03±0.10 arbitrary densitometric unit (ADU), p=0.000025); it peaked at 15 min (stretch 1.16±0.11 vs. control 0.03±0.099 ADU, p=0.000047) and then significantly decreased after 30 min (stretch

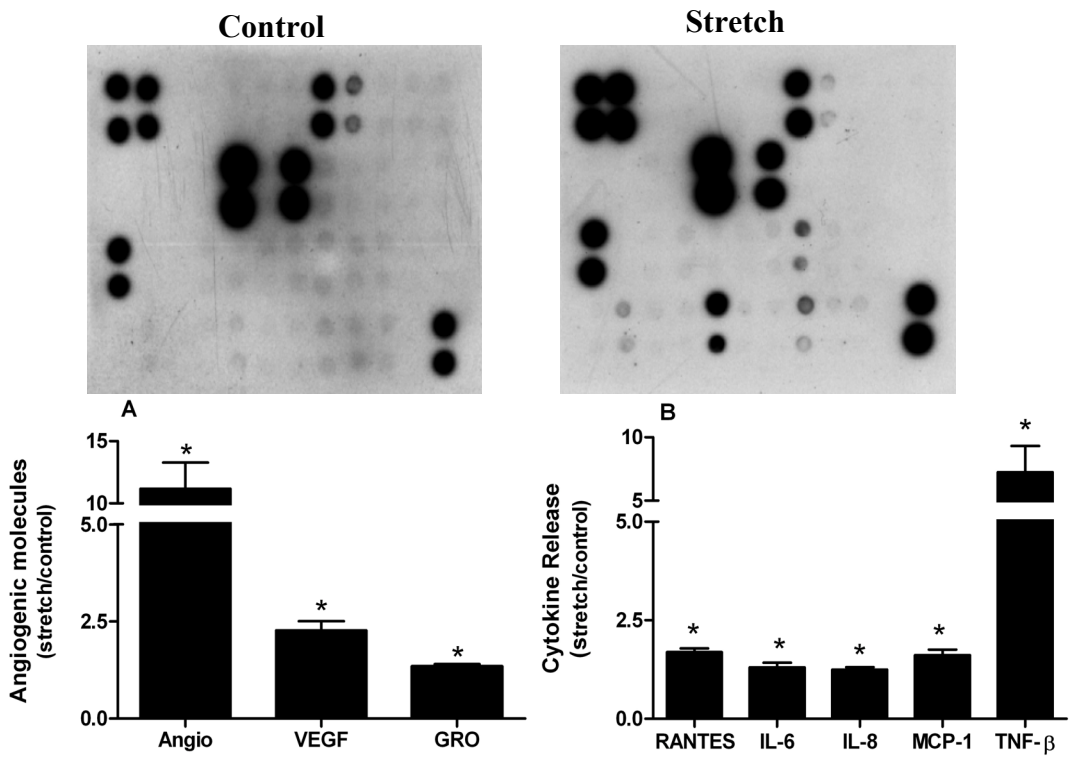

Figure 9.1: Representative blots demonstrating the stretch-induced expression of direct and indirect angiogenic factors in human ASM cells.

*Protein array of supernatant obtained from human ASM cells mechanically stretched for 24 h shows enhanced expression of a number of (A) direct and (B) indirect angiogenic factors compared to control (unstretched) cells. The bar diagram is a quantitative representation of fold change in expression of angiogenic molecules and cytokines between the stretched and unstretched cells. Data represent mean value± SEM of three experiments. Angio=angiogenin, VEGF=vascular endothelial growth factor, GRO=growth-related oncogene, RANTES =Regulated on activation, normal T cells expressed and secreted, IL-6= interleukin-6, IL-8=interleukin-8, MCP-1=monocyte chemotactic protein-1, TNF-β= tumour necrosis factor-β. *P≤ 0.05 vs unstretched ASM cells (control).*

Table 9.1: Cytokines detected (bold letters) out of the possible 42 cytokines using RayBio® Human Cytokine Antibody Array III

A	B	C	D	E	F	G	H	I	J	K	L
Pos	***Pos***	*Neg*	*Neg*	ENA-78	GCSF	GM-CSF	**GRO**	**GRO-α**	I-309	IL-1 α	IL-1β
Pos	***Pos***	*Neg*	*Neg*	ENA-78	GCSF	GM-CSF	**GRO**	**GRO-α**	I-309	IL-1 α	IL-1β
IL-2	IL-3	IL-4	IL-5	**IL-6**	IL-7	**IL-8**	IL-10	IL-12	IL-13	IL-15	IFN-γ
IL-2	IL-3	IL-4	IL-5	**IL-6**	IL-7	**IL-8**	IL-10	IL-12	IL-13	IL-15	IFN-γ
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1	**RANTES**	SCF	SDF-1	TARC	TGF-β_1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1	**RANTES**	SCF	SDF-1	TARC	TGF-β_1
TNF-α	**TNF-β**	EGF	IGF-I	**Ang**	OSM	Tpo	**VEGF**	PDGF	Leptin	*Neg*	***Pos***
TNF-α	**TNF-β**	EGF	IGF-I	**Ang**	OSM	Tpo	**VEGF**	PDGF	Leptin	*Neg*	***Pos***

Pos, Positive; Neg, negative; MCSF, macrophage colony-stimulating factor; IGF-I, insulin-like growth factor I; MDC, macrophage-derived chemotactic factor; Ang, angiogenin; GCSF, granulocyte colony-stimulating factor; MIG, monokine induced by IFN-γ; OSM, oncostatin M; GRO, growth-related oncogene; VEGF, vascular endothelial growth factor; SCF, stem cell factor; PDGF, platelet-derived growth factor; SDF-1, stromal cell-derived factor-1; TARC, thymus- and activation-regulated cytokine; RANTES, Regulated on activation, normal T cells expressed and

secreted; IL, interleukin; MCP,monocyte chemotactic protein; TNF, tumour necrosis factor; TGF-β, Transforming growth factor-β; EGF, Epidermal growth factors; Tpo, Thrombopoietin.

0.53±0.16 vs. control 0.03±0.099 ADU, p=0.023). Cyclical stretching also caused a significant increase in P38 phosphorylation after 5 min (stretch 0.18±0.020 vs. control 0.047±0.09 ADU, p=0.0043) and 15 min (stretch 0.29±0.032 vs. control 0.047±0.09 ADU, p=0.0019) of stretch, with maximum activation being observed after 30 min (stretch 0.60±0.081 vs. control 0.047±0.09 ADU, p=0.0024) of mechanical stretch (Figure 9.2, right panels).

As shown in Figure 9.3 (left panels), Akt phosphorylation (Thr308) significantly increased in response to mechanical stretch after 30 min (p=0.0012), peaking at 60 min (p=0.019) and then reverting slightly at 120 min (p=0.005). The other phosphorylated form of Akt (Ser473) showed significant increases after 30 (p=0.0004), 60 (p=0.024) and 120 min (p=0.003) of mechanical stretch (Figure 9.3, right panels).

The activation of these different signalling mechanisms was also investigated by using respective inhibitors. Data in Figure 9.4 (left panels) demonstrate a significant inhibition of Erk1/2 phosphorylation by U0261 after 15 (p=0.041) and 60 min (p=0.036) mechanical stretch. Also the measurement after 480 min (p=0.015) following the 60 min stretch protocol shows a significant inhibition of phosphorylated Erk1/2 by U0261. The downstream signalling of the RhoA kinase was investigated using the RhoA/Rock inhibitor Y27632. We evaluated stretch-induced P38 phosphorylation in human ASM cells treated with Y27632. As shown in Figure 9.4 (right panels), phosphorylation of P38 was significantly inhibited after 60 min (p=0.0000028) stretch, but there was no inhibition after 15 min stretch or at the 480 min time point.

The role of Akt phosphorylation was investigated using Wortmannin. Figure 9.5 (left panels) shows the time dependent inhibition of Thr308 form, which significantly increased after 15 (p=0.0029) and 60 min (p=0.00043) stretch in controls. This inhibition was also present even at the 480 min (p=0.0039) time point. The Ser473 form was not affected significantly (Figure 9.5, rightt panels).

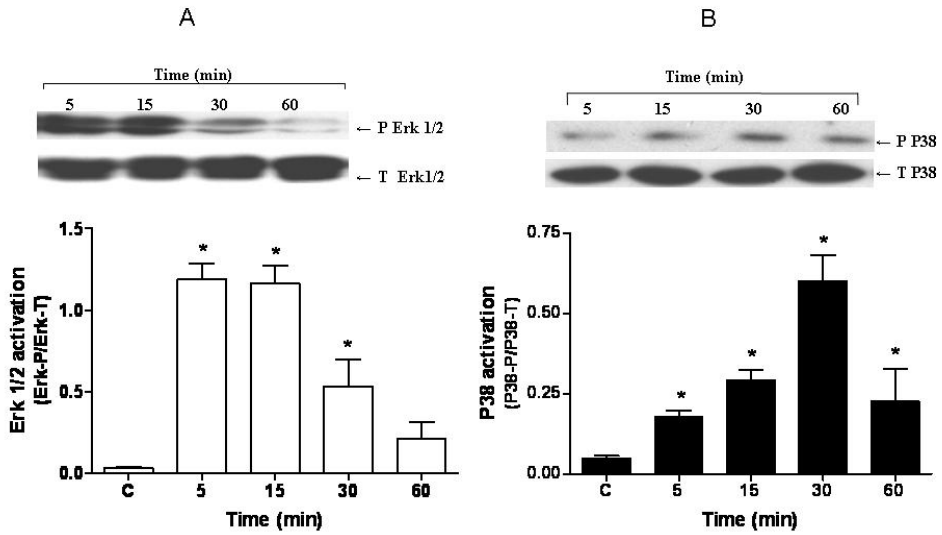

Figure 9.2: Effect of cyclical stretch on Erk1/2 and p38 activation

*Human ASM cells were mechanically stretched for 5, 15, 30 and 60 min, and the activation of (A) Erk1/2 and (B) p38 was determined by the western blot method (upper panels). Unstretched cells were included as control. The bar diagrams depict quantification of phosphorylated Erk1/2 activation as the ratio between the phosphorylated forms of Erk 1/2 or P38 relative to their respective total forms. Values are shown as mean±SEM of three experiments. * P ≤ 0.05*

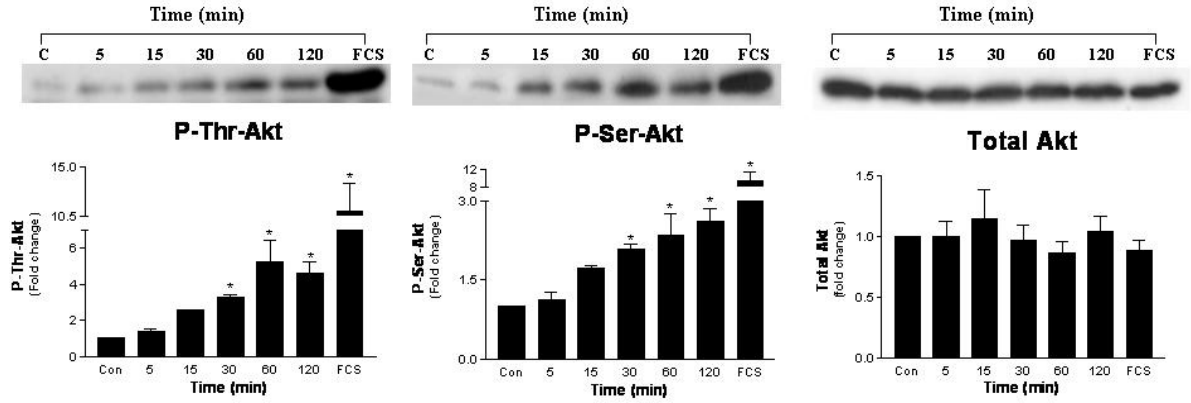

Figure 9.3: Effect of mechanical stretching on the phosphorylation of Akt

*Human ASM cells were mechanically stretched for 5, 15, 30 60 and 120 min, and the activation of Akt was determined by the western blot method with antibodies against phophorylated (Ser473 and Thr308) and non-phosphorylated forms of Akt. Unstretched cells were included as control. The bar diagrams depict the quantification of the respective Akt activation at different time points as fold change from the baseline value. Values are shown as mean±SEM of three experiments. * P≤0.05, C=control (con) FCS=serum (positive control).*

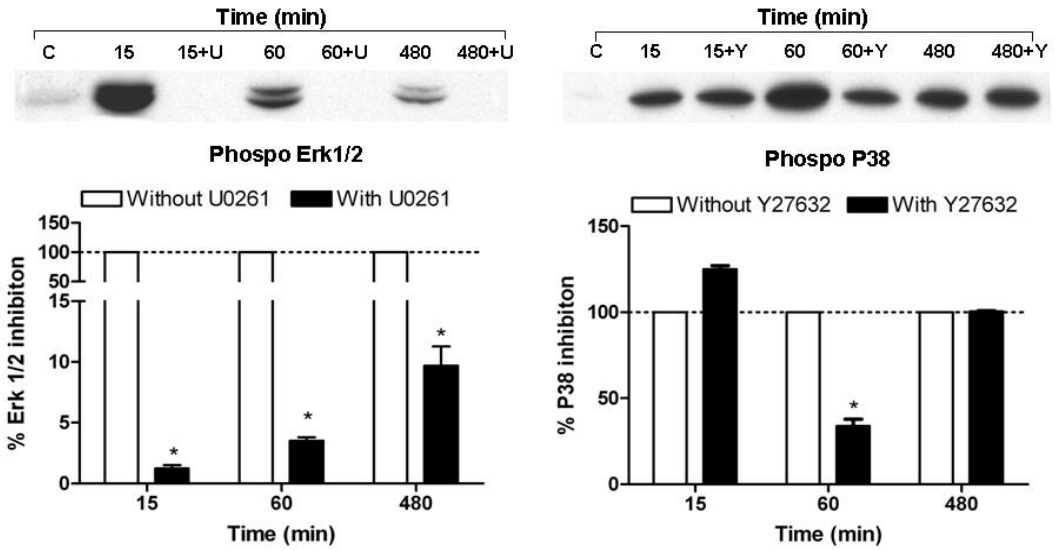

Figure 9.4: Inhibition of Erk1/2 and P38 phosphorylation

*Human ASM cells were pretreated for 30 min with 20 μM U0261 (Erk1/2 inhibitor; left panels) or 20 μM Y27632 (P38 inhibitor; right panels) followed by mechanical stretch for 15 and 60 min. Cells were lyzed and analyzed using Western blot method with respective phospho antibodies after these time points and after 480 min, following the 60 min protocol. The bar diagrams depict the percentage inhibition of Erk1/2 or p38 phosphorylation in relation to the controls without inhibitors. Values are shown as mean±SEM. *p≤0.0.5, C=control; U=U0261, Y= Y27632.*

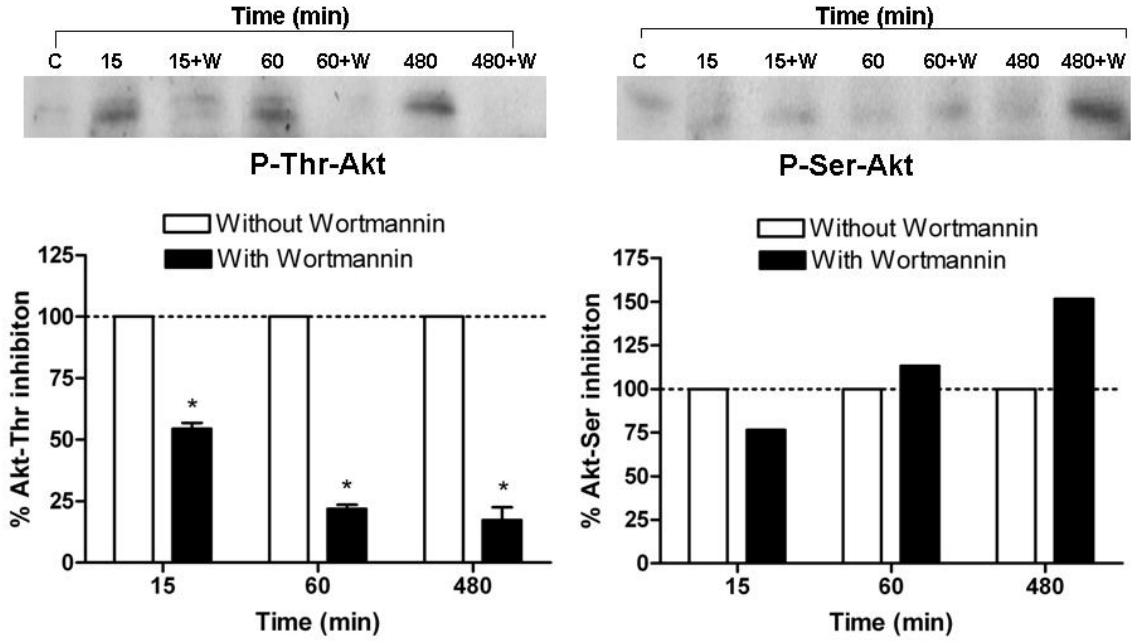

Figure 9.5: Inhibition of Akt phosphorylation

*Human ASM cells were pretreated for 30 min with 20 μM wortmannin (Akt inhibitor) followed by mechanical stretch for 15 and 60 min. Cells were lyzed and analyzed using Western blot method with respective phospho antibodies after these time points and after 480 min, following the 60 min protocol. The bar diagrams depict the percentage inhibition of Akt phosphorylation in relation to the controls without the inhibitors. Values are shown as mean±SEM. *p≤0.05, C=control; W=Wortmannin.*

9.3.3 Measurement of IL-8 and IL-6

By application of the novel human cytokine antibody arrays we obtained a clear-cut evidence for the release of IL-8 as well as IL-6 in human ASM cells subjected to mechanical stretch compared to the unstretched cells (see Figure 9.1). To get real quantitative measurements of these cytokines, we analyzed the same conditioned medium using ELISA. Figure 9.6 demonstrates a significant increase of IL-8 (6782±464 vs. 4029±389 pg/ml, p=0.01) and near significant increase of IL-6 (1436 ±191 vs. 1054 ±40.9 pg/ml, p=0.06) as compared with control, unstretched cells.

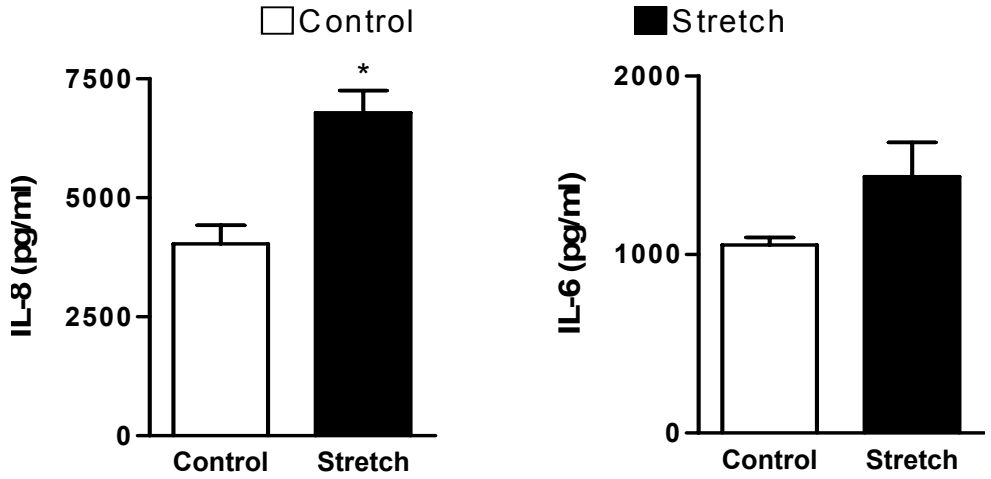

Figure 9.6: Effect of mechanical stretch on the release of IL-8 and IL-6

*Conditioned medium from human ASM cells was collected after 24 h mechanical stretch and controls (unstreched) for the measurement of cytokine release using ELISA. The bar diagrams depict IL-8 and IL-6 release in pg/ml. Values are shown as mean±SEM. * p≤ 0.05.*

To investigate whether IL-8 and IL-6 production is linked with the activation of Erk1/2, RhoA or Akt signalling pathways, we treated the ASM cells with inhibitors of these pathways and measured the release of these cytokines at 8 h after 1 h of stretch. The results were almost the same for both cytokines and are summarized in Figure 9.7. There was a significant decrease of IL-6 release in the presence of Erk1/2 (U0261, p=0.022) or RhoA/Rock (Y27632, p=0.0023), compared with stretched cells without the inhibitors (Figure 9.7A). The IL-8 release was also attenuated by U0261 (p=0.0017) and Y27632 (p=0.0077; (Figure 9.7B). However, the Akt inhibitor Wortmannin did not block the release of IL-6 and IL-8; they rather increased it at 8 h (Figure 9.7).

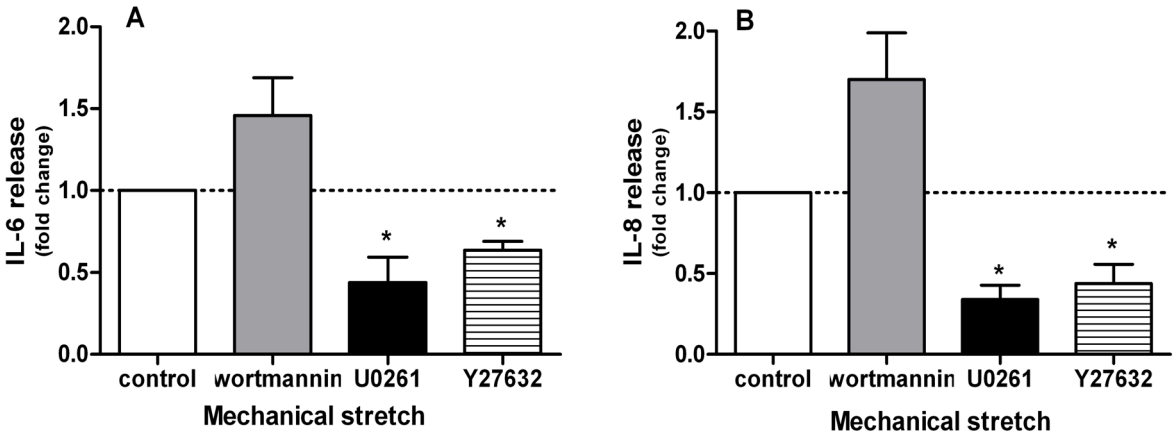

Figure 9.7: Effect of inhibition of Erk1/2, RhoA/Rock and Akt pathway on the release of IL-6 and IL-8

*Human ASM cells were pretreated for 30 min with Wortmannin (PI3k inhibitor), U0261 (ERK1/2 inhibitor) or Y27632 (ROCK inhibitor), followed by 1 h mechanical stretch. The production of IL-6 (A) and IL-8 (B) in condition medium was measured after 8 h following 1 h stretch, using ELISA. The bar diagrams depict cytokine release in fold change between the cells treated with the inhibitor and cells not treated. Values are shown as mean±SEM. *p≤0.05.*

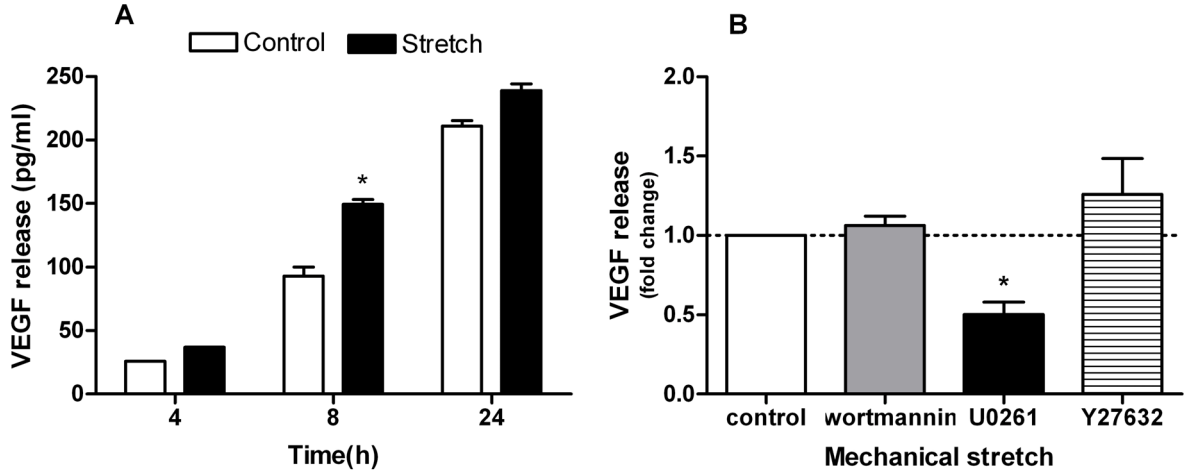

Figure 9.8: Effect of mechanical stretch and inhibition of Erk1/2, RhoA/Rock and Akt pathway on the release of VEGF from human ASM cells

*(A) Human ASM cells were mechanically stretched for 1, 4, 8 and 24 h, and VEGF release was measured in conditioned medium using ELISA. (B) Human ASM cells were pre-treated for 30 min with wortmannin (PI3k inhibitor), U0261 (ERK1/2 inhibitor) or Y27632 (ROCK inhibitor), followed by 1 h mechanical stretch. The production of VEGF in conditioned medium was measured after 8 h following the 1 hr stretch, using ELISA. The bar diagram depicts VEGF release in fold change between cells with and without inhibitor treatment. Values are shown as mean±SEM. *p≤0.05.*

9.3.4 Measurement of VEGF

The human cytokine antibody array showed an increased production of VEGF in stretched human ASM cells compared with unstretched cells (see Figure 9.1). Figure 9.8A

demonstrates the time dependent release of VEGF, which is significantly increased after 8 h (149.27 vs. 93.02 pg/ml, p=0.019) stretch compared with unstretched cells. To investigate whether VEGF production is linked with the activation of Erk1/2, RhoA or Akt signalling pathway, we measured the VEGF release in conditioned medium treated from cells treated with inhibitors for these pathways. Figure 9.8B show a significant (p=0.0030) decrease of VEGF release in the presence of the Erk1/2 inhibitor U0261, compared with stretched cells without inhibitor. However, VEGF release was not blocked in the presence of the RhoA/Rock inhibitor, Y27632 or the Akt inhibitor, Wortmannin.

9.4 Discussion

Our study explored the effects of uniform biaxial cyclic mechanical stretch on the expression and production of direct and indirect angiogenic factors by human ASM cells and identifies the signalling mechanism that leads to the release of these mediators. Protein array of supernatant obtained from human ASM cells shows upregulation of angiogenic molecules such as VEGF, angiogenin and GRO in addition to cytokines such as IL-6, IL-8, RANTES, MCP-1 and TNF-β. Most of these molecules have been implicated in the airway

and vascular remodelling process in chronic airway diseases, such as asthma and COPD (21, 22). Enhanced release of VEGF, IL-6 and IL-8 protein in stretched ASM cells as compared to the unstretched was confirmed by quantitative ELISA assays. In addition, our study provides an insight into the significant role of Erk 1/2 signalling pathway in the production of VEGF, Erk 1/2 and Rho/ROCK signalling pathways in the production of IL-6 and IL-8 by human ASM cells subjected to cyclical stretch.

Given the highly dynamic environment of the airway wall, there have been surprisingly few reports examining the responses of airway cells to mechanical stimuli. Supporting the available evidence (15, 22-24), our data confirms the "synthetic" nature of human ASM cells, particularly in the state of hypercontraction. These results when viewed in the context of airflow obstruction further affirm the plausible role of ASM cell-derived mediators in airway and vascular remodelling. Recently, the potential of ASM cells to produce various cytokines and chemokines in the conditioned medium in response to a variety of mediators has been discussed and demonstrated by employing antibody array technique (21). There is a clear distinct pattern of enhanced expression of molecules in these mechanically stretched cells favouring tissue remodelling. Angiogenin and VEGF, in particular, are documented as

the most potent angiogenic molecules (25, 26). Moreover, the cytokines and chemokines, such as IL-6, IL-8, and MCP-1, are reported to be inducers of angiogenesis (27, 28), though not directly but via induction of VEGF (28-30). Evidence supports the hypothesis that airway pressure and secondary mechanical stretch are the primary stimuli of tracheal occlusion-induced lung growth (31).

With the use of tracheal rings isolated from rats, Fukunga et al. (8) investigated the isometric contractile responses. In another study the smooth muscle strips from bovine tracheas were stretched *in vitro* to investigate the effect of a single rapid stretch on post stretch force and myosin phosphorylation (6) or to investigate that sinusoidal length oscillation and receptor activation interactively regulate the abundance of mRNA encoding smooth muscle actin and myosin isoforms (10). Previous work by Kumar et al. (9) revealed that prolonged uniaxial cyclic mechanical stretch of human ASM cells induced the expression and production of IL-8. The induced production of IL-8 was also found in a study by Li et al. (12). Our data that biaxial cyclical mechanical stretch induced not only the production of IL-8 but also of IL-6, MCP-1, TNF-β and RANTES and, the angiogenic molecules VEGF, angiogenin and GRO confirms and adds onto the reports. Our study based on the stretch model clearly demonstrates a role for the Erk 1/2 pathway in VEGF release in these cells. Additionally, the production of the indirect angiogenic factors, IL-6 and IL-8 is dependant on the RhoA/ROCK pathway and Erk 1/2 pathway as evident from the blockage of release of these proteins with the pre-treatment of the cells with the respective inhibitors. This supports the finding of Kumar et al. (9) wherein the inhibition of ERK1/2 and p38 MAP kinase inhibited the expression and production of IL-8 in these cells. Akt signalling pathway has been implicated in survival response (32, 33) and this pathway is activated under cyclical stretch of cells *in vitro*. Though this pathway is activated in our model, we do not see a role of it in the release of VEGF; a known survival factor. Moreover, inhibition of this pathway with Wortmanin did not block the release of VEGF, IL-6 or IL-8 from stretched cells suggesting that an alternative signal transduction mechanism may be at work. Furthermore, Wang et al. show the importance of RhoA-ROCK signalling for serum response factor (SRF)-dependent transcription of smooth muscle-specific genes, the pathway which plays also a well-defined role in controlling F-actin polymerization and stress fibres formation in cultured cells (11). That study suggests inhibition of activation of RhoA in cultured cells upon stretch in contrast to the reported data indicating that RhoA activation in ASM cells under mechanical strain (13).

Though, we could observe the activation of p38 upon cyclical mechanical stretch our findings show that Y27632 inhibited contraction-induced p38 phosphorylation, suggesting that RhoA/Rho kinase activation was upstream of p38 and induced its activation similar to what has been shown earlier in stretching of rabbit facial vein (34).

Cytoskeletal changes and functional consequences of mechanical strain on human ASM cells have been investigated by a number of groups, we are among the few who have reported on the synthetic phenotype of human ASM cells in culture in relation to different mediators of asthma and now we report under the condition of cyclical strain. Further studies are needed to demarcate the complexity in signalling events and their independent role in ASM phenotype in the conditions such as in asthma. Moreover, the limitations of an *in vitro model* need to be taken into account when extrapolating the results to an *in vivo* diseased condition. Already difference in the data emerging between uniaxial and biaxial stretch (11, 13) have to be properly reconciled. Recently a study (35) has attributed the difference in response to stretch *in vitro* to the fundamental differences in the ASM environment in asthma and in culture. Even the most sophisticated technology cannot mimic the complicated interactions among cells, tissues and organs that occur in humans and animals.

Acknowledgements

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10

General Discussion

Chapter 10: General Discussion

Airflow obstruction in chronic airway disease has been shown to be associated with airway and pulmonary vascular remodelling. Repetitive injury/repair of airway epithelium and associated remodelling processes are common features in asthma and chronic obstructive pulmonary disease (COPD). Regulated repair is a crucial event for restoration of barrier function and reduction of inflammation. A number of growth factors have been implicated in regulating this epithelial repair processes. The data presented in the first half of the thesis focussed on unravelling molecular mechanisms involved in epithelial injury and repair where our results suggests a role for the IGF-I/IGFR-I and FGF/FGFR-1 system in modulating bronchial epithelial repair and hence airway remodelling. Increase in size and number of blood vessels both within and outside the smooth muscle layer as well as hyperaemia of bronchial vasculature are contributing factors in airway wall remodelling in patients with chronic respiratory disease. Results included in this thesis clearly support the notion of ongoing complex mechanisms leading to angiogenesis/vascularisation and vascular dilatation. Emerging evidences indicate that vascular changes directly add to the airway narrowing and hyperresponsiveness by exudation and transudation of pro-inflammatory mediators, cytokines and growth factors (1, 2). This facilitates trafficking of inflammatory cells. We have further shown that specific angiogenic and hyperpermeability factor like VEGF, could directly contribute to pathological events resulting in vascular remodelling in the airways. Moreover, various pro-inflammatory cytokines (IL-1β and TNF-α) and mediators (ANG II and ET-1) synthesized during the disease process enhance expression and secretion of the angiogenic molecule (VEGF) that directly participates in vascular remodelling. In addition, nitric oxide seems to perpetuate the expression and secretion of VEGF in ASM cells for paracrine action. Finally, simulating the *in vivo* state of hypercontractility seen in chronic airway diseases, we established an *in vitro* model of cyclical stretch and observed an enhanced expression of direct and indirect angiogenic molecules in ASM cells clearly advocating for an important role of these cells in ongoing angiogenic processes during airway diseases.

10.1 Epithelial injury/repair and bronchial remodelling

It is now well established that particles from cigarette smoke cause damage to the airways, particularly the lining epithelium (3, 4). Both non-symptomatic smokers and patients with

COPD show signs of damage and repair to the epithelial surface in the form of a denuded epithelium and squamous metaplasia (2). Epithelial injury is normally followed by a complex repair process that comprises subsequent epithelial migration, proliferation, and differentiation and cell death (5). The primary aim is to rapidly restore the denuded epithelium and this process has been reported to be mediated via growth factors, such as Transforming Growth Factors-β (TGF-β), Keratinocyte Growth Factor (KGF), Epidermal Growth Factor (EGF) and their receptors (6-8). The processes of normal and abnormal wound healing as a response to injury have been studied extensively (9-13). The environment of cytokines and growth factors, (myo)fibroblast-derived extracellular matrix and adequate capillaries facilitate epithelial cell proliferation and migration, leading to wound closure (13). Within the airways, the bronchial epithelium, subepithelial myofibroblasts and ASM cells are the major cell types involved in tissue repair processes.

Our results add up to the available evidence that indeed IGFs and FGFs have the potential to stimulate epithelial repair similar to EGFs (14, 15) and defensins (5), which are innate immune factors present in airway surface liquid and make up part of the lung's natural defence. The increased bronchial IGF-I expression in COPD patients and its receptor localization in epithelium in addition to the wound repair potential of IGF-I advocate for its crucial role in tissue repair mechanisms during smoke-induced lung injury. Considering our data in this context, we conclude that ASM cell-derived IGF-I acts in a paracrine fashion on the IGFR-I positive epithelial cells resulting in their proliferation and thus contributing to the airway remodelling during COPD. Various studies suggest a differential localization and expression pattern of IGF-I and IGF-II in foetus, infants and adults (16-19) and with advancing gestational age the presence of IGF-I decreases in the mesenchyme and cuboidal epithelium (19) but IGF-I is markedly increased in neonates with bronchopulmonary dysplasia and respiratory distress syndrome (19). This indicates that there may be an re-emergence of foetal pattern of IGF-I expression during intense repair after lung injury, as also reported in hyperoxic rat lung injury (20). Additional support for the involvement of IGF-1 comes from our observation that levels of IGF-I were inversely correlated with the disease severity (as reflected by lung function), suggesting for the repair mechanisms in analogy to those during foetal development. Similarly, in our previous study (21) we found up-regulated FGF-1, FGF-2 and FGFR-1 expression in bronchial epithelium indicating that such compensatory mechanisms are also active in COPD.

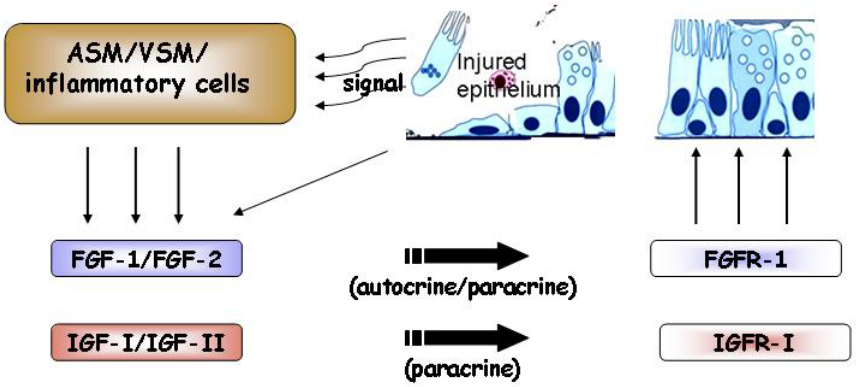

Figure 10.1: Autocrine and paracrine mechanisms of FGFs and IGFs mediated epithelial wound closure

Note that the injured or activated epithelium signals the other resident and infiltrating cells to release FGF-1,2 and IGF-I,II, which could act in autocrine or paracrine fashion on the injured epithelium and enhance the repair process.

Our current results support the notion that increased bronchial expression of FGF-1, FGF-2 and FGFR-1 in patients with COPD could participate in regulating the process of airway remodelling. The correlation of clinical data (lung function) with the epithelial Ki-67 staining suggests an initiation of the repair process, which in turn may be successful depending upon the availability of the necessary factors and host conditions. Apart from stopping smoking, modulation of these pathways should be considered in the development of therapeutic interventions aimed at restoring the epithelial integrity and hence prevent or even reverse chronic airflow limitation in COPD. Figure 10.1 is a schematic representation of the possible mechanisms involved in bronchial epithelial repair mediated through growth factors.

10.2 Vascular remodelling and role of airway smooth muscle derived mediators

The current thesis supports the hypothesis that ASM cells are active sources of mediators relevant to the pathogenesis of airway disease. Pro-inflammatory cytokines such as IL-1β and TNF-α that are associated with chronic inflammation of the airways drive production of VEGF by ASM cells (Chapter 4). Similarly, vasoactive peptides, ANG II and ET-1 (Chapter 7), or cyclic mechanical stretch (Chapter 9) enhance VEGF release which via paracrine mechanisms in the vicinity of bronchial endothelial cells may perpetuate the

bronchial vascular remodelling that has been documented in asthma and COPD. The relative importance of each cell type for VEGF release under pathophysiological conditions is unknown. However, we and others have demonstrated that human ASM cells express VEGF both *in vitro* and *in vivo* (Chapter 5 and 6) in the intact myobundles of patients with COPD (22-24). These latter findings suggest that VEGF release by human ASM cells in culture is not an artefact. Rather, ASM represents a possible pathophysiologically important pool of VEGF available during airway inflammation that may be amenable to therapeutic intervention.

Increasing experimental evidence suggests that inhibition of VEGF activity via blockade of its receptors has a potential therapeutic value by decreasing angiogenesis and vascular remodelling (25). This approach reverses pathophysiological symptoms including airway hyperresponsiveness and inflammation in a mouse model of asthma (26). One likely mechanism for the effectiveness of this approach could be prevention of VEGF-induced vascular permeability, thereby suppressing inflammation by reducing vascular leakage and migration of cells and mediators into the airways. However, the inhibition of VEGF could be beneficial or detrimental depending on the type of chronic airway disease. For example, some reports suggest that increased levels of VEGF are associated with airflow limitation in bronchitis and decreased levels with airflow limitation and alveolar destruction in patients with emphysema (27, 28). Likewise, long-term glucocorticoids treatment in patients with asthma significantly affects airway remodelling by reducing basement membrane thickness, and reducing indices of submucosal vascularity, including blood vessel number and total vascular area (29, 30). Our findings that dexamethasone completely inhibits the expression and release of the angiogenic factor VEGF from cultured ASM cells would support this notion. The effect of glucocorticoids on airway remodelling, however, remains controversial. Inhibition of VEGF production by ASM cells may be one mechanism by which glucocorticoids might affect tissue remodelling.

Current *in vivo* and *in vitro* data indicate that interactions between various airway cells like smooth muscle cells, endothelium, myofibroblasts and inflammatory cells via growth factors and cytokines and other mediators contribute in vascular remodelling during different pathophysiological conditions (31-34) as its clearly depicted in a schematic diagram (Figure 10.2). Undoubtedly, more research focus has been recently directed towards unravelling molecular events underlying the bronchial vascular remodelling that

may directly or indirectly participate in the pathogenesis of chronic lung diseases. Though, the precise nature of these events is far from clear, our study sheds light on potential mechanisms of vascular remodelling that could take place in chronic airway diseases.

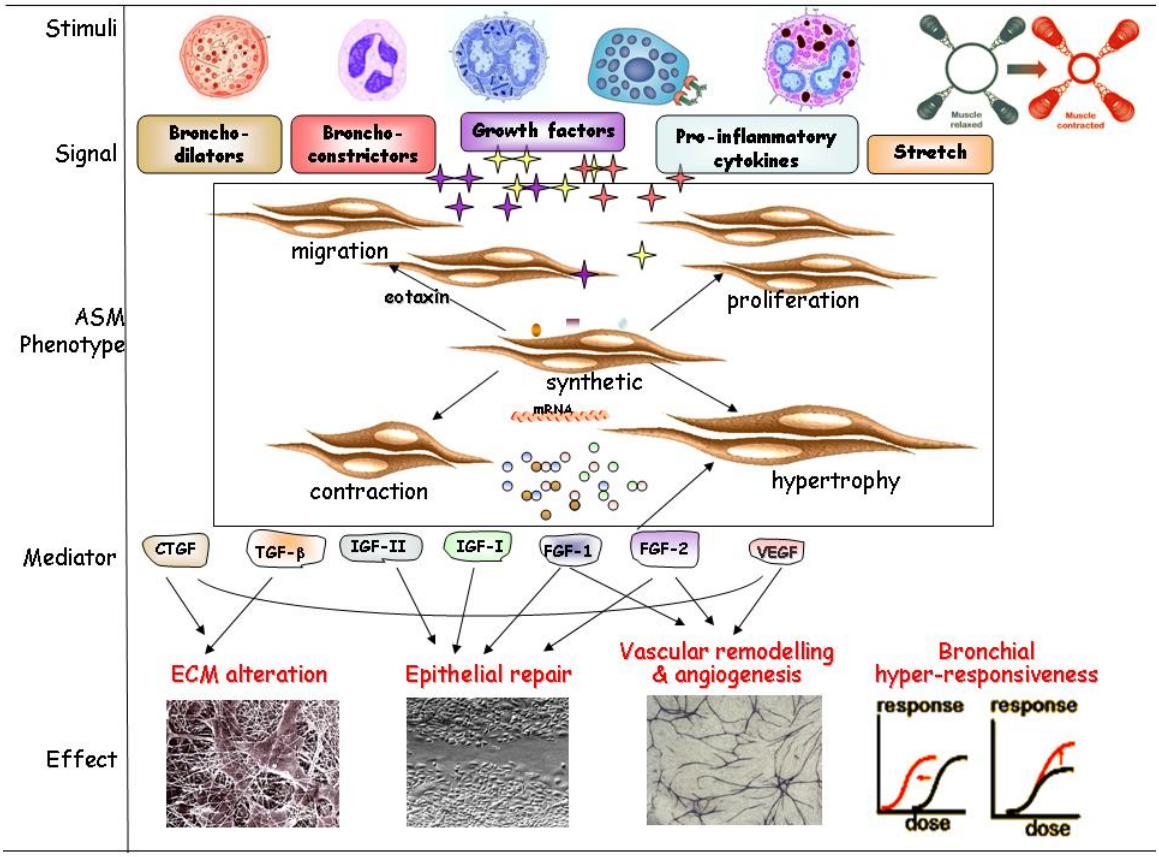

Figure 10.2: Central dogma of airway smooth muscle cells in bronchial and vascular remodelling

Schematic illustration that depicts various cellular interactions and underlying molecular events, where human ASM cells are the central mediators of the airway remodelling in chronic airway diseases. Note the flow of events from stimuli to ECM alteration, epithelial repair, vascular remodelling and bronchial hyperresponsiveness.

Moreover, our results (Chapter 8) suggest that the nitric oxide pathway may modulate the synthesis of VEGF during airway inflammation and subsequently vascular remodelling in chronic airway diseases. The induction of VEGF synthesis by nitric oxide may be of great importance in the maintenance of vascular homeostasis and in the response to endothelial injury and during vascular remodelling in the airways. Because nitric oxide and VEGF reciprocally enhance their synthesis, this interaction may play a significant role. A variety of signalling pathways have been implicated in the upregulated production of VEGF in ASM cells. Prostaglandin $(PGE)_2$-stimulated VEGF production has been proposed to be mediated via cAMP activation (35). Knox et al. reported that bradykinin activates PKC,

which then induces COX-2. The major cyclo-oxygenase (COX) product, PGE_2 then activates VEGF via cAMP that in its turn activates e.g. PKA and subsequently activate MEK1/2, ERK1/2, CREB pathways in human ASM cells (35). Our study (Chapter 9) based on the stretch model clearly demonstrates a role for the Erk 1/2 pathway in VEGF release in these cells. Additionally, the production of the indirect angiogenic factors IL-6 and IL-8 is dependant on both RhoA/ROCK and Erk 1/2 pathways. Furthermore, we observed Akt phosphorylation in our cyclic stretch model, but we did not see the role of Akt signalling pathway in the expression and release of VEGF in human ASM cells. Therefore, it is quite possible that Akt signalling pathway being implicated in survival responses (36, 37) is also involved in stress/survival responses under cyclical stretch conditions in our *in vitro* model.

10.3 Limitations of *in vitro* models

Cells in culture-based *in vitro* models are cost effective and they are important tools to understand the underlying molecular events in a variable-controlled environment. However, there are limitations to these cultured cell based models due to several inherent conditions. Monolayers of cells, such as H292 and primary human ASM cells in culture may loose their phenotype due to culture conditions, coated matrix and due to lack of tissue microenvironment. The number of passages would also affect cells in culture. Although intrinsic characteristics of the individual cell type in relation to disease may be retained (38, 39) if obtained from asthmatics or other airway diseases, many of them may loose this property over a long period of time in culture.

Caution should be exercised when extrapolating the results from a study based on cell lines or even primary cell in relation to the *in vivo* situation. The cell line NCI-H292, derived from a human lung mucoepidermoid carcinoma cells, has growth characteristics in culture that could influence the response to growth factors. However, NCI-H292 cells have been successfully used in many studies (5, 40) and we do not believe that using other cell line/cells would change the results significantly; at least the repair experiments have been conducted in different epithelial cells and the results were not significantly different from each other (40). *In vitro* wound repair model that we have developed and used in this thesis was primarily based on NCI-H292 cells and it was used only to demonstrate the potential of IGFs/FGFs in repair in relation to controls using the same cell line. Additionally, primary

bronchial epithelial cells could be used in future studies to confirm our data obtained with this cell line.

We studied the angiogenic potential of the stimulated ASM conditioned medium using a cell-based system of pulmonary artery endothelial cells or HUVEC. This is a monolayer cell system, which could be used to assess the proliferation of endothelial cells. However, angiogenesis involves cell migration, proliferation and then tube formation. Future studies using a sophisticated model reflecting *in vitro* or *in vivo* angiogenesis needs to be employed to investigate further the role of these ASM-secreted mediators. Recently, a 3D *in vitro* model of angiogenesis has been developed in this area of research (41).

Our *in vitro* cyclic mechanical stretch model based on human ASM cells is a novel approach to extend the hypercontractile responses observed *in vivo* conditions in patients with chronic airway diseases and in this regard few other studies have also been attempted before (42-45). However, being realistic is to understand that this model is an isolated system of cell culture wherein the rate and force of contraction is regulated via a computer controlled apparatus. This system cannot guarantee a true representation of *in vivo* conditions existing in disease states, such as asthma. However, the interesting part of this model is its dynamic environment in which there is an alternate phase of contraction and relaxation of the cells under study. This helps us to at least have an idea of the possible underlying mechanisms in these conditions. Recently, the difference in response to stretch *in vitro* has been attributed to the fundamental differences in the ASM environment in asthma and in culture (46). Even the most sophisticated technology cannot mimic the complicated interactions among cells, tissues and organs that occur in humans and animals. Scientists must understand these interactions before confirming a new hypothesis or treatment. The final test, however, has to be done in a whole, living system.

10.4 Clinical relevance and implications for future research

There has been a constant search for effective treatment strategies to prevent the progression of airflow obstruction or to treat debilitating and distressing symptoms of chronic respiratory conditions. Novel, possibly broad-acting, anti-inflammatory compounds include specific inhibitors of phosphodiesterase 4 (PDE 4), 5-lipoxygenase (leukotriene synthesis blockers), COX (prostaglandin synthesis blockers) and leukotriene receptors (47, 48).

However, more specific therapies directed against inflammation and remodelling without adverse effects are needed. Current therapies offer symptom control only but not an effective cure. A significantly growing area of unmet medical research where there is a constant need to develop strategies in disease modifying and curative treatments.

Different levels of intervention could be explored. The currently examined interventions include:

1. Scavenging and neutralisation of proteins by binding to soluble receptors or neutralising antibodies,
2. Inhibition of protein binding to its receptor by small compounds or incomplete and non-activating cytokines,
3. Inhibition of protein activation (processing of preprotein into mature protein), and
4. Inhibition of signal transduction and transcription via inhibition of receptor-dependent tyrosine kinases, MAPK, or nuclear factor κB (NFκB) and its inhibitors IKK and IκB and via antisense mRNA or small interference (si) RNA molecules for growth factors or cytokines.

A number of potential drugs are still in preclinical development or in clinical trial.

Table 10.1: Examples of VEGF, VEGFR and FGFR1 antagonists in clinical trial potentially useful for the treatment of chronic airway disease*

Name	Type of compound	Function
Bevacizumab (*Avastin*®)	Humanized mouse IgG1 anti VEGF antibody	inhibits binding VEGF to VEGFR
VEGF Trap	VEGFR1 and VEGFR2 binding domains linked to IgG1 constant region	Neutralisation VEGF
CEP-7055	indenopyrrolocarbazole derivate	inhibits pan-VEGFR kinase
SU 6668	IgG2a fusion protein	inhibits VEGFR2, FGFR1, PDGFRb tyrosine kinase
PTK 787		

Based on reference (49)

In this context, this thesis and our published articles shed light towards the development of a novel therapeutic target in chronic respiratory conditions. Several studies, including ours (22, 50), have pointed out to the involvement of VEGF/VEGFR in pulmonary and vascular remodelling and inflammation. VEGF transgenic mice showed alveolar vascular and airspace remodelling as well as increased numbers of alveolar macrophages, or a Th2-type airway inflammation in a murine asthma model (51). An *in vitro* study demonstrated that VEGF induces monocyte migration via VEGFR1 expressed by monocytes (52). Therefore, such inhibitors may be of potential use for the treatment of vascularisation as seen in asthma or specific subtypes of COPD, like chronic bronchitis but not in emphysema as pointed out in early chapters (chapter 4). Several drugs have been developed to reduce tumour growth and metastasis by impairing the neovascularisation. Antagonists include those which have entered in clinical trials and in preclinical investigations (Table 10.1). Among those in clinical trial are bevacizumab (Avastin®), CEP-7055, VEGF Trap and PTK787 (53). Many other small molecules as VEGFR inhibitors are being developed for clinical therapy (54, 55). SU6668 is a IgG2a fusion protein inhibiting tyrosine kinase activity of FGFR1, VEGFR2 and platelet growth factor receptor-B. SU6668 was demonstrated to inhibit tumour growth, lung cancer metastasis and tumour vascularisation (56). Its broader spectrum of inhibitory effects may be more effective in treating airway remodelling in COPD. Despite these data, not much has been published on such treatments in asthma or COPD. Also the redundancy of the VEGF-VEGFR system may temper applicability and efficacy of VEGF/VEGFR inhibitors (57). In addition, for example, in patients with emphysema, inhibitors of apoptosis are needed where, in contrast to chronic bronchitis, expression of hypoxia inducible factor VEGF and VEGFR2 may be stimulated to overcome endothelial and epithelial cell death and improve vascular restoration (58-60). Hence, treatment with VEGF/VEGFR inhibitors should be further investigated and may be restricted to specific subtypes of chronic lung diseases. Furthermore, keeping in view of the role of IGFs and FGFs in bronchial epithelial repair as demonstrated in this thesis, it would be tempting to explore the possibilities to extend the therapeutic potential of these growth factors in chronic airway diseases, such as in COPD.

10.5 Concluding remarks

Airway remodelling in chronic respiratory diseases has been extensively documented while the focus lately has been also towards examining vascular remodelling in chronic airway

diseases. This thesis is among the first few to explore the underlying mechanisms in this aspect. New data in this area are very exciting and will continue to provide input into the development of new medications, anti-inflammatory drugs and drugs directed at the pathophysiology of these chronic respiratory conditions. Alternative therapeutic approaches are needed at this time where we have reached a ceiling in the improvement of symptoms with the current therapies. Physiologic outcomes such as FEV1 are long established and widely accepted standard but currently debated. Appropriate endpoints to assess the clinical efficacy needs to be developed while novel and complex endpoints will need to be assessed. Newer outcomes that include a broader range of parameters will be more suitable in case of COPD. For example, the BODE index that also includes physical strength in the 6 minutes walking test would take into account that COPD is a systemic disease. Such measures will be better to test the effectiveness of a drug that improves all aspects of the disease. Otherwise, the new drug should affect a specific and essential part of the disease, and in combination with one or more other drugs, address all aspects of the disease.

Cigarette smoking and exposure to air pollution is associated with extensive injury and reparative changes alongside the inflammatory response. The capacity of repair declines with age. We need to probe whether enhancement of the reparative mechanism is a suitable therapeutic goal. The first part of the thesis harps on the possible role of the growth factors such as IGFs and FGFs in bronchial epithelial repair after injury. Future studies in the direction of wound repair would determine novel therapeutic approaches in this area.

Over the years, the role of ASM cells in airway remodelling has been established. In this thesis, we extend the ASM biology to include it in the perpetuation of angiogenesis and vascular remodelling via enhancing mediators, such as VEGF involved primarily in these processes. We show that ASM cells play a central role by responding to mediators of asthma and COPD and expressing angiogenic molecules. Recent investigations also point to the potential molecules involved in these processes, including growth factors and cyto- or chemokines. Specific antagonists capable of reducing growth factor and cytokine expression or signalling pathways are already available or being developed. Some of these drugs seem to be effective in different inflammatory diseases or in reducing tumour growth. However, these antagonists have not yet been tested extensively with regard to reducing the remodelling processes in chronic inflammatory lung diseases. Experimental models and expression studies suggest that anti-VEGFR strategies could be of importance to use in

patients with emphysema. Feedback on the respiratory impact from current therapies using anti-VEGF measures in other diseases would be crucial. This suggests the need of stratification of antagonist therapy. However, a complete block of cytokine-driven or growth factor-dependent repair mechanisms may be disadvantageous as defence reactions to infection being very crucial may be impaired. Furthermore, other drugs like retinoids and even growth factors may provide restoration of lung tissue structure. Such approaches, however, will have to wait for the initial results based on growth factor or cytokine antagonist therapy available for chronic lung diseases.

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Summary & Samenvatting

Summary

Summary in English

The work embodied in this thesis mainly focuses on the molecular mechanisms underlying bronchial angiogenesis and tissue remodelling that eventually contribute to the airway remodelling, a hallmark in the pathogenesis of chronic lung diseases. The thesis constitutes of two main parts; the first two major chapters deals with bronchial epithelial injury and repair mechanisms during airway remodelling and the second part spanning over six chapters covers the aspects of bronchial vascular remodelling studied using both human lung tissue *in situ* (immunohistochemistry) as well as *in vitro* (ASM cells). The data presented in this thesis illustrate the role of a number of cytokines and growth factors in airway remodelling and can be summarised as below:

Chapter 1, being the general introduction covers the review of current literature and introduces the two main sections: 1. Bronchial remodelling and 2. Vascular remodelling. It provides an overview of the clinical characteristics, pathogenesis of chronic airway diseases, COPD and asthma in relation to airway remodelling. The structural abnormalities as observed in epithelial repair, bronchial remodelling and vascular changes are described. Documented evidence regarding microvascular changes and altered bronchial vessels are presented. The role of cytokines and growth factors in airway as well as vascular remodelling and pulmonary angiogenesis in airways are introduced. The role of ASM beyond its established profile in the process of airway vascular remodelling and cellular interaction is discussed. *In vitro* models used in this project to decipher the bronchial and vascular remodelling are explained. The role of growth factors such as insulin like and fibroblast growth factors in bronchial repair and the role of mediators of chronic airway disease such as TNF-α, IL-1β, ANG II, ET-1 or stretch in vascular remodelling is hypothesized. An extensive review of literature has been included on the topic of angiogenesis and vascular remodelling in relation to mainly VEGF and its receptors but other cytokines are also included in detail. Finally, in view of the current literature, a hypothesis was proposed to examine the expression pattern and the role of various key growth factors and cytokines in tissue repair and remodelling processes in the airways.

In **Chapter 2,** we focussed on the role of the IGF-I/IGFR-I system in bronchial epithelial repair and hence airway remodelling in COPD patients. We investigated the bronchial expression pattern of IGF-I and IGFR-I in COPD patients and correlated the expression levels with corresponding lung function data. Using an *in vitro* epithelial wound repair model in NCI-H292 cells, we further assessed the mitogenic effects of IGFs. Enhanced expression of IGF-I was localized in airway smooth muscle and vascular smooth muscle cells in patients with COPD as compared to those without COPD. IGFR-I was localized in epithelial cells and alveolar macrophages and its expression levels did not differ between the two groups. Expression of IGF-I in ASM and VSM cells inversely correlated with the respective lung function data (FEV_1/FVC) in two groups. *In vitro* experiments revealed that both IGF-I and IGF-II induced cell proliferation (2-5 folds) and growth (2-4 folds) of ASM as well as NCI-H292 cells. Our wound repair experiments demonstrated accelerated and significant closure of a mechanically injured confluent monolayer of NCI-H292 cells at 72 h of IGF treatment. Taken together, our results suggest that the IGF-I/IGFR-I system play a pivotal role in epithelial repair processes and hence contribute to the airway remodelling in COPD.

In **Chapter 3**, we explored the proliferative activity of bronchial epithelial cells in COPD (by Ki-67 staining in airway epithelium) in relation to lung function (FEV_1 values) and examined the mitogenic role of FGF-1 and FGF-2 and their wound closure potential in an *in vitro* wound repair model, using the bronchial epithelial cell line (NCl-H292). Ki-67 Labelling Index values showed significant doubling in COPD patients as compared to non-COPD and inverse correlation with FEV_1 values ($r = -0.43$; $p<0.03$). Both FGF-1 and FGF-2 increased cell proliferation (3-7 folds) after 24 h and cell counts (2 folds) after 48 h. Both FGF-1 and FGF-2 also increased proliferation (3-7 folds) as assessed by [^{3}H]thymidine incorporation and cell counts (up to 2 fold, after 48 h of incubation). Analysis of images of the wounds taken at different time points (24, 48, 72 h) revealed that injured epithelial monolayers showed enhanced wound closure with FGF-1 (67%) or FGF-2 (61%) as compared to controls (38%) after 72 h. Specific inhibitors of ERK1/2 (U0126) and FGFR-1 (SU5402) completely blocked FGF-1- and FGF-2-induced ERK1/2 activation after 15 min. FGF-1 stimulates wound closure by 1.8 times, which was inhibited over 90% by U0126 or over 60% by SU5402. FGF-2 stimulates wound closure by 1.6 times which was inhibited over 75% by U0216 or 50% by SU5402. Similarly, the phosphatidylinositol-3-kinase inhibitor, LY294002 blocked epithelial repair in FGF-1 (28%) and FGF-2 (22%) treated

cells. The increased bronchial epithelial expression of fibroblast growth factors and their receptor, FGFR1, in COPD and the higher number of proliferating bronchial epithelial cells in COPD suggest a potential role for the FGF/FGFR-1 system in bronchial airway remodelling and repair in patients with COPD; this is further supported by the mitogenic response of NCI-H292 cells to FGFs and the wound repair potential of FGF-1 *in vitro.*

We further explored in **Chapter 4** the vascular remodelling component in chronic bronchitis and emphysema. Based on the available evidence, we hypothesize that in chronic bronchitis, expression of VEGF and VEGFR-2 expression leads vascular remodelling, which is inefficiently compensated by the low expression of VEGFR-1. In subjects with emphysema, however, VEGFR2 expression was lower. Preferential activation of VEGFR-1 resulted in higher MMP activity, alveolar destruction and endothelial apoptosis. Hence, the balance between VEGF, VEGFR-1 and VEGFR-2 are critical in the pathogenesis of COPD subtypes.

In **Chapter 5,** we described the expression of VEGF and its receptors VEGFR-1 (flt-1) and VEGFR-2 (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. VEGF expression was enhanced in VSM cells of microvessels in the bronchial mucosa and submucosa as well as in ASM cells in COPD patients as compared to non-COPD. VEGF expression was more intense in COPD in the intimal and medial VSM of the peripheral pulmonary arteries associated with the bronchiolar airways. KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM of the peripheral pulmonary arteries, whereas Flt-1 expression was higher 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 FEV_1 values. Our results implicate VEGF and its receptors, Flt-1 and KDR/Flk-1, in peripheral vascular and airway remodelling processes in COPD.

Chapter 6 assesses the role of proinflammatory mediators such as IL-1β and TNF-α towards airway vascular remodelling. We investigated IL-1β and TNF-α-induced secretion and expression of VEGF by human ASM cells in culture. IL-1β (0.5 ng/ml) and TNF-α (10 ng/ml) each increased VEGF mRNA expression in human ASM cells, reaching maximal levels between 16-24 h and 4-8 h, respectively. Both cytokines also induced a time dependent release of VEGF, which was not associated with increased ASM cell proliferation. Preincubation of cells with 1 μM dexamethasone abolished the enhanced release of VEGF by TNF-α. These data suggest that human ASM cells express and secrete VEGF in response to pro-inflammatory cytokines and that VEGF may participate in paracrine inflammatory mechanisms of vascular remodelling in chronic airways disease.

To further explore the role of ASM in bronchial angiogenesis and remodelling, we examined in **Chapter 7** the effects of conditioned media from ASM cells on the proliferation and growth of pulmonary artery endothelial cells. Angiotensin II (ANG II) and endothelin-1 (ET-1) are potent vasoconstricting circulatory hormones implicated in asthma. ET-1, but not ANG II, induced ASM cell proliferation and DNA biosynthesis as indicated by increased [^{3}H]thymidine incorporation. Northern blot hybridization showed that both ET-1 and ANG II induced the expression of two mRNA species of 3.9 and 1.7 kb encoding VEGF in cultured ASM cells reaching maximal levels between 4-8 h of incubation. Induced expression and release of VEGF declined after 8 h of ANG II incubation and the levels remained elevated in the case of ET-1. Furthermore, conditioned medium from ASM cells treated for 8 h with ET-1 resulted in pulmonary artery endothelial cell proliferation as assessed by the [^{3}H]thymidine incorporation assay, and cell counts after 24 h. Our results suggest that proliferating ASM cells produce angiogenic factors, like VEGF that could induce pulmonary artery endothelial cell growth resulting in bronchial angiogenesis and remodelling.

In **Chapter 8,** we investigated the effects of nitric oxide pathway on the pro-inflammatory cytokine IL-1β-induced expression and secretion of VEGF and PlGF from cultured porcine ASM cells. IL-1β did not affect PlGF release, but augmented VEGF release at 24 h from 399±11 pg/ml (control) to 968±123 pg/ml. This VEGF release was inhibited by L-NAME (532±52 pg/ml) and restored by L-arginine (1529±287 pg/ml). IL-1β induced expression (1.8 fold vs. control) of VEGF mRNA at 4 h, but no increase was detected at 24 h. IL-1β

induced expression of VEGF mRNA was attenuated by L-NAME and augmented by L-arginine at 4 h. Restoring the nitric oxide pathway by L-arginine in L-NAME-treated cells elevated expression of VEGF. However, the IL-1β-induced endothelial cell proliferation was not affected by interference with nitric oxide pathway. Our findings suggest that the nitric oxide pathway modulates the synthesis of VEGF and this may subsequently result in vascular remodelling in chronic airway diseases.

Chapter 9 employs a cyclical stretch model to investigate the expression profile of proteins, signalling pathways and the secretion of angiogenic molecules in human ASM cells in culture. This *in vitro* model revealed that hypercontractile human ASM cells release VEGF, angiogenin and cytokines, such as interleukin (IL)-6, IL-8, monocyte chemotactic protein MCP-1, RANTES, TNF-β and growth-related oncogene. VEGF secretion, as assessed by ELISA, was significantly higher after 8 h and 24 h, as compared to controls. Western blot analysis showed robust phosphorylation of ERK 1/2 after 15 min and P-Thr-Akt and P-Ser-Akt after 30 min of stretch. Respective blockers of Akt (wortmannin), ERK 1/2 (U0261) and Rho/ROCK (Y27632) pathways revealed significant inhibition of VEGF release only with U0261 after 8 h. Furthermore, cyclical stretch induced significant release of IL-6 and IL-8 after 24 h that was inhibited by blockers of ERK 1/2 and RhoA/ROCK pathways. Our results demonstrate that the hypercontractile human ASM cells secrete angiogenic molecules via activation of the ERK1/2 pathway, which, in turn, could contribute to vascular and airway remodelling.

Finally in **chapter 10,** a comprehensive discussion has been put forward in the light of our findings and the current literature on molecular interactions that possibly underlie the pathogenesis of bronchial and vascular remodelling during chronic airway diseases.

In conclusion, we have demonstrated that; 1) IGF-I/IGFR-I and FGF/FGFR-1 systems play a pivotal role in epithelial repair and airway remodelling during chronic airway diseases, 2) the enhanced expression of VEGF and its receptors (Flt-1 and KDR/Flk-1) contribute to bronchial angiogenesis in COPD, 3) ASM cells express and release biologically active VEGF in response to pro-inflammatory cytokines as well as vasoactive peptides, 4) Nitric oxide donors augment interleukin-1β induced VEGF expression in human cultured ASM cells and 5) mechanically stretched airway smooth muscle cells produce pro angiogenic molecules via ERK1/2 signalling pathway. It is anticipated that our findings may be useful

in selectively developing therapeutic strategies to achieve regulated bronchial angiogenesis and diminished tissue remodelling in patients with chronic airway diseases, like COPD and asthma.

Samenvatting (Summary in Dutch)

De studies beschreven in dit proefschrift richten zich met name op de moleculaire mechanismen van bronchiale angiogenese en veranderingen in het luchtwegweefsel. Deze processen liggen mogelijk ten grondslag aan de luchtwegveranderingen die een kenmerk zijn van de pathogenese van chronische luchtwegziekten. Het proefschrift bestaat uit twee delen. Het eerste deel bestaat uit twee hoofdstukken waarin schade en herstelmechanismen in bronchiaal epitheel zijn bestudeerd die kunnen optreden tijdens luchtwegveranderingen. Het tweede deel beslaat de volgende zes hoofdstukken waarin aspecten van bronchiale vasculaire veranderingen zijn bestudeerd in zowel menselijk longweefsel door middel van immunohistochemie, en in gekweekte luchtweg gladde spiercellen (*in vitro* ASM cellen). De in dit proefschrift gepresenteerde gegevens illustreren de rol van een aantal cytokinen en groeifactoren bij luchtwegveranderingen. In de volgende paragrafen worden de resultaten beschreven.

Hoofdstuk 1 is onderverdeeld in twee secties namelijk 1) bronchiale herstructurering en 2) vasculaire herstructurering. Het geeft een overzicht van de klinische kenmerken, pathogenese van de chronische luchtwegaandoeningen COPD en astma in relatie tot luchtwegherstructurering. De structurele veranderingen die plaatsvinden door epitheliale herstelprocessen, bronchiale herstructurering en vasculaire veranderingen worden beschreven. De recente literatuur aangaande micro-vasculaire veranderingen en vaatveranderingen in de bronchiale wand wordt besproken. Verder wordt de rol van cytokines en groeifactoren in luchtweg- en vasculaire herstructurering, en pulmonaire bloedvatvorming in de luchtwegen geïntroduceerd. Nieuwe inzichten op het gebied van luchtweg gladde spier (ASM) biologie in het proces van luchtweg- en vasculaire herstructurering worden uitgelegd. De *in vitro* modellen die centraal staan in dit project worden ingeleid. Het doel van dit proefschrift wordt aan het eind van dit hoofdstuk gegeven. De rol van groeifactoren zoals “insulin-like” en “fibroblast growth factors” die betrokken zijn bij bronchiale herstelprocessen alsmede de rol van mediatoren zoals TNF-α, IL-1β, ANG II en ET-1 met betrekking tot chronische luchtwegziekten worden belicht in de

hypothese. Tenslotte wordt mechanische rek van de bloedvatwand als hypothese onderzocht voor vasculaire herstructurering.

In **hoofdstuk 2** ligt de nadruk op de rol van het IGF-I/IGFR-I systeem in bronchiale, epitheliale herstelprocessen in luchtwegherstructurering in COPD patiënten. Hier onderzochten we de bronchiale expressie van IGF-I en IGFR-I in patiënten met en zonder COPD en correleerden deze expressie niveaus met de corresponderende longfunctie data van de patiënten. De mitogene effecten van IGFs werden met behulp van een epitheliaal wondherstelmodel *in vitro* in NCI-H292 cellen verder onderzocht. Verhoogde expressie van IGF-I was aanwezig in ASM en "vasculaire gladde spier, vascular smooth muscle (VSM)" cellen in patiënten met COPD vergeleken met controle patiënten. IGFR-I was aanwezig in het bronchiale epitheel en alveolaire macrofagen, maar de expressie was niet verschillend tussen de twee experimentele groepen. De expressie van IGF-I in ASM en VSM cellen was omgekeerd evenredig met longfunctie data (FEV_1/FVC) in de twee groepen. Verder toonde we in *in vitro* experimenten aan dat zowel IGF-I als IGF-II de groei van ASM en NCI-H292 cellen bevorderden. Ons model liet ook zien dat IGF stimulatie leidt tot significant versnelde heling van een mechanische verwonde NCI-H292 cellaag na 72h. Samengevat wijzen onze resultaten erop dat het IGF-I/IGFR-I systeem een belangrijke rol speelt bij epitheliale herstelprocessen en dus mogelijk bijdraagt aan de pathogenese van COPD.

In **hoofdstuk 3** bekeken we de proliferatieve activiteit van bronchiale epitheelcellen in COPD (d.m.v. Ki-67 kleuring in het luchtwegepitheel) in relatie tot longfunctie (FEV_1 waarden). Tevens worden de mitogene rol van FGF-1 en FGF-2 en hun capaciteit op wondheling in hetzelfde *in vitro* model met de bronchiale epitheelcellijn (NCl-H292) onderzocht. Ki-67 tellingen lieten een significante verdubbeling van het aantal delende cellen in COPD patiënten zien vergeleken met non-COPD patiënten, die tevens omgekeerd evenredig was met de FEV_1 waarden ($r = -0.43$; $p<0.03$). FGF-1 en FGF-2 verhoogden de proliferatie gemeten door celtellingen na 24 uur en 48 uur. Zowel FGF-1 en FGF-2 verhoogden ook de proliferatie zoals gemeten door incorporatie van radioactief ^{3}H-thymidine. Verder laat de analyse van foto's van beschadigd epitheel zien dat FGF-1 (67%, $P <0.01$) of FGF-2 (61%, $P<0.01$) op verschillende tijden (24, 48, en 72 uur) gunstig waren voor wondheling, tegenover 38% in afwezigheid van deze groeifactoren. Specifieke antagonisten van ERK1/2 (U0126) en FGFR-1 (SU5402) leidden tot complete blokkade van

ERK1/2 activatie door FGF-1 of FGF-2 na 15 minuten. De stoffen U0126 en SU5402 blokkeerden ook de door FGF-1 gestimuleerde wondheling met 90% c.q. 66%, en de door FGF-2 gestimuleerde wondheling met 85% c.q. 50%. Tevens blokkeerde LY294002, een fosfatidylinositol-3-kinase antagonist, de epitheliale wondheling door FGF-1 (28%) en FGF-2 (22%) behandelde cellen. Samengevat, de verhoogde bronchiale expressie van FGFs en hun receptor, FGFR-1 en verhoogde proliferatie in COPD suggereren een rol voor het FGF/FGFR-1 systeem in bronchiale luchtwegherstructurering in COPD, verder ondersteund door onze *in vitro* data.

We beschreven in **hoofdstuk 4** de rol die vasculaire herstructurering speelt in chronische bronchitis en emfyseem door literatuur studie aangevuld door data die op ons eigen lab gegenereerd zijn. De huidige kennis aangaande luchtweg- en vasculaire herstructurering in COPD is verre van volledig. Vermeerderde infiltratie van ontstekingscellen in de adventitia, voornamelijk $CD8^+$ T-lymfocyten, in de spieren rondom pulmonaire en bronchiolaire arterieën is beschreven. Vele groeifactoren, waaronder VEGF en FGF, spelen een essentiële rol in het handhaven van cellulaire homeostase en in weefselherstel. Gebaseerd op het beschikbare bewijs postuleren wij dat de expressie van VEGF en VEGFR-2 leidt tot vasculaire herstructurering in chronische bronchitis, dat in geringe mate gecompenseerd wordt door de lage expressie van VEGFR-1. In patiënten met emfyseem, echter, is VEGFR-2 expressie veel lager. De activatie van VEGFR-1 leidt, op zijn beurt, tot hogere activiteit van enzymen die alveolaire schade en apoptose in het endotheel veroorzaken. Samenvattend, de balans tussen VEGF, VEGFR-1 en VEGFR-2 is van groot belang voor de pathogenese van COPD subtypes.

In **hoofdstuk 5** werd de pulmonale expressie beschreven van VEGF en zijn twee receptoren VEGFR-1 (flt-1) en VEGFR-2 (KDR/flk-1) in de longen van COPD patiënten. Het proteïne expressie patroon van VEGF, VEGFR-1 en VEGFR-2 werd gekwantificeerd in perifeer longweefsel en in de centrale bronchi van (ex-)rokers met en zonder COPD. VEGF, VEGFR-1 en VEGFR-2 kwamen tot expressie in bloedvat en luchtweg gladde spiercellen, bronchiale, bronchiolaire en alveolaire epitheelcellen en macrofagen. Bovendien, brachten endotheelcellen door de gehele longen VEGFR-1 and VEGFR-2 in sterke mate tot expressie. In de bronchiale luchtwegen was VEGF expressie verhoogd in bloedvat gladde spiercellen van microbloedvaten in de bronchiale mucosa en submucosa lagen in de luchtweg gladde spier cellen vergeleken met patiënten zonder COPD. De expressie van

beide receptoren VEGFR-2 and VEGFR-1 was onveranderd tussen beide groepen in de bronchiale luchtwegen. Ter hoogte van het longparenchym was VEGF expressie toegenomen voor COPD patiënten in de intimale en mediale bloedvatgladde spiercellen van pulmonaire arteriën die geassocieerd zijn aan de bronchiolaire luchtwegen alsmede in de kleinere parenchymale bloedvatvertakkingen. Bovendien was in COPD de expressie van VEGFR-2 verhoogd in endotheelcellen, intimale en mediale bloedvatgladde spiercellen van pulmonaire arteriën alsmede in de kleinere alveolaire bloedvatvertakkingen. VEGFR-1 expressie was voor COPD toegenomen in endotheelcellen van beide bovenstaande bloedvatcategorieën. VEGF kleuring was significant toegenomen in bronchiolaire en alveolaire epitheelcellen alsmede in bronchiolaire macrofagen, terwijl de VEGFR-1 receptor alleen in het bronchiolaire epitheel was verhoogd. Tenslotte, werd een negatieve correlatie gevonden van de FEV_1 met de expressie van VEGF in zowel de bronchiale microbloedvaten in de mucosa, alsmede in de bronchiale luchtweg gladde spiercellen en het bronchiolaire epitheel, wanneer de totale patiëntengroep onderzocht werd. Samengevat, wijzen deze resultaten uit dat VEGF en de twee receptoren, VEGFR-1 en VEGFR-2, betrokken zijn bij bloedvat- en luchtwegherstructurering in zowel de perifere long als in de centrale bronchustakken van COPD patiënten.

Hoofdstuk 6 beschrijft de rol van pro-inflammatoire mediatoren zoals IL-1β en TNF-α in relatie tot luchtweg- of vasculaire herstructurering. We hebben de secretie en expressie van VEGF na IL-1β en TNF-α stimulatie in humane ASM cellen in kweek onderzocht. Zowel IL-1β (0.5 ng/ml) als TNF-α (10 ng/ml) verhoogde de VEGF mRNA expressie in humane ASM cellen met een maximum tussen respectievelijk 16-24 uur en 4-8 uur. Beide cytokines leidden ook tot een tijdsafhankelijke secretie van VEGF dat niet toegewezen kon worden aan toename van de hoeveelheid ASM cellen. Voorbehandeling met 1 μM van het glucocorticosteroïde dexamethason onderdrukte deze VEGF toename na TNF-α volledig. Deze data tonen het belang van bovenstaande cytokines voor ASM cellen aan en versterken zo hun bijdrage aan inflammatoire mechanismen in chronische longaandoeningen.

In **hoofdstuk 7** beschreven we de effecten van geconditioneerd medium van ASM cellen op de proliferatie en groei van pulmonaire arteriële, endotheelcellen om een link te vinden tussen factoren uit ASM naar VSM in bronchiale angiogenese. Angiotensin II (ANG II) en Endothelin-1 (ET-1) staan bekend om hun potente vaatvernauwende werking en zijn mogelijk

betrokken bij astma. ET-1, maar niet ANG II, leidt tot proliferatie van ASM cellen (zoals gemeten middels [^{3}H]-thymidine incorporatie). Northern blot hybridisatie toonde twee mRNAs voor 3.9 en 1.7 kb VEGF aan in ASM cellen in kweek. ANG II en ET-1 induceerden VEGF mRNA expressie (2-3 keer) en secretie (1.8-2.8 keer) na 4-8 uur stimulatie en deze inductie daalde weer na 8 uur voor ANG II, maar niet na ET-1. Verder kon geconditioneerd medium van ASM cellen die gedurende 8 uur geïncubeerd waren met ET-1 proliferatie van endotheelcellen veroorzaken (gemeten via ^{3}H-thymidine incorporatie en celtellingen) na 24 uur. Deze data suggereren dat ASM cellen factoren kunnen maken, mogelijk VEGF, die betrokken zijn bij bronchiale angiogenese en vasculaire herstructurering.

In **Hoofdstuk 8** onderzochten we de effecten van de stikstofmonoxide (NO) signaaltransductie route op de expressie van VEGF en PlGF en de secretie door gekweekte ASM cellen door middel van stimulatie met het pro-inflammatoire cytokine, interleukine-1β (IL-1β). IL-1β had geen effect op PlGF secretie maar verhoogde wel de secretie van VEGF na 24 uur (van 398.6±11 pg/ml in de controle naar 968.4±123 pg/ml na IL-1β behandeling). Deze VEGF secretie was onderdrukt door L-NAME (531.8±52 pg/ml) en hersteld na behandeling met L-arginine (1529±287 pg/ml). Verder, induceerde IL-1β een VEGF mRNA expressie (1.8 keer t.o.v. de controle) na 4 uur maar niet na 24 uur. De expressie van VEGF mRNA na IL-1β stimulatie was verlaagd door L-NAME en juist verhoogd door L-arginine na 4 uur. Suppletie van de NO signaaltransductie route met L-arginine in L-NAME behandelde cellen leidt tevens tot een verhoogde expressie van VEGF. Celproliferatie was niet beïnvloed door NO na IL-1β stimulatie. Samengevat, onze resultaten geven aan dat NO signaaltransductie belangrijk is voor de VEGF synthese wat grote gevolgen kan hebben voor het begrip van chronische luchtwegaandoeningen.

In patiënten met astma treedt regelmatig een overmatige contractie van de luchtwegspieren op. In **hoofdstuk 9** gebruikten wij een repetitief "rek en strek" model om de expressie van contractiele eiwitten en signaaltransductie routes, en de secretie van bloedvatvormende moleculen te onderzoeken in gekweekte humane ASM cellen. Dit *in vitro* model toonde aan dat hypercontractiele humane ASM cellen vele factoren zoals VEGF, angiogenin en cytokines inclusief IL-6, IL-8, MCP-1, RANTES, TNF-β (lymphotactin), en GRO uitscheidden. VEGF secretie, gemeten met ELISA, was significant hoger na 8 uur ($p<0.02$)

en 24 uur ($p<0.001$) vergeleken met controle cellen. Met Western blot analyse toonden we na 15 minuten ASM "strekken" fosforylering van ERK1/2, en na 30 minuten P-Thr-Akt ($p<0.001$) en P-Ser-Akt ($p<0.004$) aan. Antagonisten van Akt en Rho hadden verder geen effect op VEGF uitscheiding maar een antagonist van ERK1/2 (U0261) na 8 uur wel. Verder, leidt repetitief strekken van ASM cellen tot een significante secretie van IL-6 ($p<0.05$) en IL-8 ($p<0.01$) na 24 uur wat weer geblokkeerd werd door antagonisten van ERK 1/2 en RhoA/ROCK route. Onze resultaten laten zien dat de hypercontractiele, humane ASM cellen factoren uitscheiden die betrokken kunnen zijn (via ERK1/2 signaaltransductie) bij vasculaire- en luchtweg herstructurering.

Conclusie: onze resultaten tonen aan dat 1) IGF-I/IGFR-I en FGF/FGFR-1 systemen een belangrijke rol spelen bij epitheliaal herstel en luchtwegveranderingen in chronische longziekten, 2) de verhoogde expressie van VEGF en zijn receptoren (Flt-1 en KDR/Flk-1) bijdragen aan bronchiale angiogenese in COPD, 3) ASM cellen biologisch actief VEGF tot expressie brengen en uitscheiden als reactie op blootstelling aan pro-inflammatoire cytokinen en vasoactieve peptiden, 4) stikstofoxidedonoren de door interleukine 1β geïnduceerde VEGF expressie verhogen in gekweekte menselijke ASM cellen, en 5) mechanisch gestrekte luchtweg gladde spiercellen pro-angiogene moleculen maken via de ERK1/2 signaaltransductie route. Deze resultaten kunnen bijdragen aan de ontwikkeling van specifieke therapeutische strategieën om bronchiale angiogenese te reguleren en een vermindering van weefselveranderingen teweeg te brengen bij patiënten met chronische luchtwegziekten als astma en COPD.

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About the author

Vijay Kumar Thyagarajan Alagappan was born on August 3, 1973 in Madras, India. He completed his high school (Central Board of Secondary Education) in 1991 with distinction and certificate of merit in Chemistry. He began his studies of medicine in 1991 at Madras Medical College & Research Institute, India. After successful completion of his internship training at the Government General Hospital, Madras, India he obtained his bachelors degree in Medicine (MBBS) in 1997. He then worked as a senior resident at the Department of Rheumatology and Immunology, Government General Hospital, Madras and actively pursued clinical practice and research during 1997-2001. In August 2001, he moved to The Netherlands on a NUFFIC (Netherlands Organisation for International Co-operation in Higher Education) fellowship to pursue Master's degree in Medical Informatics at The Netherlands Institute for Health Sciences (NIHES), Erasmus Medical Centre, Rotterdam. After completing his MSc degree in 2002, he joined the Department of Pharmacology, Erasmus MC under the direct supervision of Dr. Hari S. Sharma and the promotorship of Prof. Pramod R. Saxena, for his PhD thesis. During his tenure of 4 years he actively collaborated with various scientists at Erasmus MC and LUMC, Leiden. He is now looking forward to pursue his career further in clinical medicine.

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- **Alagappan VK**, McKay S, Widyastuti A, Garrelds IM, Bogers AJ, Hoogsteden HC, Hirst SJ, and Sharma HS. Proinflammatory cytokines upregulate mRNA expression and secretion of vascular endothelial growth factor in cultured human airway smooth muscle cells. Cell Biochem Biophys 2005; 43(1): 119-30.
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List of Abbreviations

°C: degrees Celsius
2-ME :2-mercaptoethanol
aa: amino acid (s)
Ab: antibody (ies)
Ag: antigen
AIDS: acquired immunodeficiency syndrome
ANG II: angiotensin II
Ang: angiopoietin
ASM: airway smooth muscle
ATP: adenosine triphosphate
bp: base pair(s)
BSA: bovine serum albumin
cAMP: cyclic adenosine monophosphate
CD: cluster of differentiation (in combination with numbers, CD1, CD4 etc.)
$CD8^{+ve}$ T: CD8 positive T-lymphocyte
cDNA: complementary DNA
CI: confidence interval
Ci: curie(s)
COPD: chronic obstructive pulmonary disease
COX: cyclo oxygenase
cpm: counts per minute
CTGF: connective tissue growth factors
d: day
Da: dalton(s)
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethylsulfoxide
DNA: deoxyribonucleic acid
DNase: deoxyribonuclease
dNTP: deoxyribonucleoasid triphosphate
dpm: disintegrations per minute
ds: double-strand (ed) (as dsDNA)
DTT: dithiothreitol
EC: endothelial cell
ECM: extra cellular matrix
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
ELISA: enzyme-linked immunosorbent assay
Epi: epithelial cell
ET: endothelin
EtdBr: ethidium bromid
FACS: flourescence-activated cell sorter
Fas: FS7-associated cell surface antigen
FBS: fetal bovine serum
Fc: crystallizable fragment
FCS: foetal calf serum
FEV_1: forced expiratory flow in one second
FGF: fibroblast growth factor
Flk-1: fetal liver kinase-1 (VEGF receptor 2)
Flt-1: fms-like tyrosine kinase (VEGF receptor 1)
FN: fibronectin
FVC: forced vital capacity
g: gram(s)
G: guanosine
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
G-CSF: granulocyte colony-stimulating factor
GINA: The Global initiative for Asthma
GM-CSF: granulocyte-macrophage colony-stimulating factor
GOLD: The Global initiative for Chronic Obstructive Lung Disease
H&E: hematoxylin and eosin stain
h: hour(s)
HBSS: Hank's balanced salt solution
HEPES: N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HGF: hepatocyte growth factor
HIF: hypoxia inducible factor
HIV: human immunodeficiency virus
HIV-tat: HIV-transactivating regulatory protein
HLA: human leucocyte antigen
HPLC: high-performance liquid chromatography
IC_{50}: inhibitory concentration of 50%
ICAM: intercellular adhesion molecule
ID_{50}: 50% infective dose or 50% inhibiting dose
IEF: isoelectric focusing
IFN: interferon (e.g., IFN-g)
Ig: immunglobulin (also IgE, IgG etc.)
IGF: insulin-like growth factor
IL: interleukin (IL-10, IL-12 etc.)
INF-γ: interferon gamma
IP-10: interferon-gamma-inducible 10 kD protein
IR: insulin receptor
IU: international unit
kb: kilobase(s)
$K_{co:}$ carbon mono-oxide constant
Kd: dissociation constant
kDa: kilodalton(s)
KDR: kinase insert domain receptor
KGF: keratinocyte growth factor
Ki: inhibition constant
Km: Michaelis constant
l (L): liter(s)
LAK: lymphokine-activated killer
LD50: 50% lethal dose
LFA-1: leukocyte function-associated antigen-1
LIF: leukemia inhibitory factor
LN: lymph node
LPS: lipopolysccharide
LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
m: meter(s)
M: molar
mAb: monoclonal antibody
MAP: mitogen activated protein
MCP: monocyte chemotactic protein
M-CSF: macrophage colony-stimulating factor
MEM: minimum essential medium
min: minute(s)
ml: milliliter(s)
MMP: metalloproteinase
mo: month(s)
mol wt: molecular weight
mol: mole(s)
mRNA: messenger ribonucleic acid
MW: molecular weight

Mφ: macrophage
n: number of subjects in a study group
ND: not determined
Neu: neutrophil
NF-κB: nuclear factor κB
NF: nuclear factor
NGF: nerve growth factor
NIDDM: non-insulin-dependent diabetes mellitus
NIH: National Institute of Health (Bethesda, Maryland, USA)
NK cell: natural killer cell
no.: number
NS: non-smoker without emphysema
OD: optical density
Oligo: oligodeoxyribonucleotide
osM : osmolar
osmol :osmole
OVA: ovalbumin
P: probability
PAGE: polyacrylamide gel electrophoresis
PAI: plasminogen activator inhitor
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PD-ECGF: platelet-derived endothelial cell growth factor
PDGF: platelet-derived growth factor
PEDF: pigment-epithelium derived factor
PF4: platelet factor 4
PlGF: Placental growth factor
PMN: polymorphonuclear leukocytes
PMSF: phenylmethylsulfonyl floride
Ppm: points per million
Q-PCR: reverse transcriptase-real-time polymerase chain reaction
R: receptor (e.g., IL-2R)
RANTES: regulated upon activation, normal T cell expressed and secreted
RAS: renin-angiotensin system
RBC: red blood cell
RIA: radioimmunoassay
RNA: ribonucleic acid
RNase: ribonuclease
ROS: reactive oxygen species
rpm: revolutions per minute
RPMI: Roswell Park Memorial Institute
rRNA: ribosomal ribonucleic acid
RTK: receptor tyrosine kinase
RT-PCR: reverse transcriptase polymerase chain reaction
RV: residual volume
s.c.: subcutaneous
s: second(s)
SD: standard deviation
SDS: sodium dodecyl sulfate
SE: standard error
SEBM: surface epithelial basement membrane
SEM: standard error of mean
SMA: smooth muscle actin
Ss: single-strand (ed) (e.g., ssDNA)
SSC: standard saline citrate
STAT: signal transducer and activator of transcription
SU5402: 3-[3-(2-Carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone
T 1/2: half-life
TBS: Tris-buffered saline
TCA: trichloroacetic acid
TCR: T cell receptor
TGF: transforming growth factor
Th cell: T helper cell
TIMP: tissue inhibitor of metalloproteinases
TLC: total lung capacity
TNF: tumor necrosis factor
TP: thymidine phosphorylase
Tris: Tris(hydroxymethyl)-aminomethane
tRNA: transfer ribonucleic acid
Ts cell: T suppressor cell
TSP-1: thrombospondin-1
U: unit(s), uridine
U0126: 1, 4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene
UV: ultraviolet
V: volt(s)
VCAM: vascular cell adhesion molecule
VEGF: vascular endothelial growth factor
VEGI: Vascular endothelial growth inhibitor
vol: volume
vs: versus
VSM: vascular smooth muscle
W: watt(s)
WHO: World Health Organization
wk: week
wt: weigth
yr: year
α1-AT: α1-antitrypsin
µl: microliter(s)
µm: micrometer