

**ANGIOGENESIS, APOPTOSIS
AND RE-EPITHELIALIZATION AT
THE FOCI OF RECENT INJURY IN
USUAL INTERSTITIAL
PNEUMONIA AND
BRONCHIOLITIS OBLITERANS
ORGANIZING PNEUMONIA**

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2002

Abstract

Idiopathic usual interstitial pneumonia (UIP) and bronchiolitis obliterans organizing pneumonia (BOOP) are fibrous pulmonary disorders in both of which there is newly formed connective tissue in distal air spaces. UIP is a progressive and usually fatal lung disease without any efficient treatment, while the prognosis of BOOP is good. In both diseases, an injury of the alveolar epithelium and its basement membrane (BM) leads to migration of fibroblasts and myofibroblasts into air spaces and production of extracellular matrix by these cells. In UIP, the newly formed intraluminal connective tissue lesions cause fusion of alveolar structures and interstitial remodeling, while in BOOP the newly formed connective tissue may resolve completely.

One of the major aims of the research on pulmonary fibrosis is to define the mechanisms that lead to persistence of the newly formed connective tissue and thus to irreversible fibrosis in UIP. The aim of the present study was to compare the extent of capillarization, apoptotic activity and re-epithelialization of the newly formed connective tissue in BOOP and UIP. The number of capillaries per tissue surface area was measured. Furthermore, the expression of angiogenic growth factors vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF) was evaluated in the same areas, in addition to the expression of Flt-1 and Flk-1, which serve as receptors for VEGF. Apoptotic activity was analyzed using TUNEL-method, and the immunohistochemical expression of apoptosis regulating proteins bcl-2, mcl-1, and bax was studied. Finally, the extent of re-epithelialization was studied with the immunohistochemical and ultrastructural localization of laminin-5 γ 2 chain, and the sites of synthesis of laminin-5 γ 2 chain mRNA.

In BOOP, an efficient repair process with good capillarization along with high expression of VEGF and bFGF, and orderly re-epithelialization of the newly formed connective tissue takes place after lung injury. The apoptotic activity of the newly formed connective tissue is also high, presumably leading to resolution of the intraluminal connective tissue in BOOP. In UIP, the newly formed connective tissue showed poor capillarization, inadequate re-epithelialization and low apoptotic activity. The results suggest disturbed or delayed repair process in UIP, contributing to irreversible interstitial fibrosis and remodeling.

Keywords: angiogenesis, apoptosis, pulmonary fibrosis, re-epithelialization

To my family

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Oulu, December 2002

Elisa Lappi-Blanco

Abbreviations

AIP	acute interstitial pneumonia
bFGF	basic fibroblast growth factor
VEGF	vascular endothelial growth factor
BAL	bronchoalveolar lavage
BIP	obliterative bronchiolitis with interstitial pneumonia
BM	basement membrane
BOOP	bronchiolitis obliterans organizing pneumonia
CFA	cryptogenic fibrosing alveolitis
COP	cryptogenic organizing pneumonia
CTGF	connective tissue growth factor
DAD	diffuse alveolar damage
DEPC	diethylpyrocarbonate
DIP	desquamative interstitial pneumonia
DPLD	diffuse parenchymal lung disease
dpm	disintegrations per minute
ECM	extracellular matrix
EDTA	dinatrium-diamin-tetra-acetic acid
ELF	epithelial lining fluid
FasL	Fas ligand
FGF	fibroblast growth factor
Flk-1	fetal liver kinase-1
Flt-1	fms-like tyrosine kinase
GIP	giant cell interstitial pneumonia
IL	interleukin
ILD	interstitial lung disease
IPF	idiopathic pulmonary fibrosis
kDa	kilodalton
LIP	lymphoid interstitial pneumonia
KDR	kinase domain region
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase

mRNA	messenger RNA
NSIP	nonspecific interstitial pneumonia
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
RBILD	respiratory bronchiolitis interstitial lung disease
TGF- β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases
TNF- α	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UIP	usual interstitial pneumonia
uPA	urokinase-type plasminogen activator

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Lappi-Blanco E, Kaarteenaho-Wiik R, Soini Y, Risteli J & Pääkkö P (1999) Intraluminal fibromyxoid lesions in bronchiolitis obliterans organizing pneumonia are highly capillarized. *Hum Pathol* 30: 1192–1196.
- II Lappi-Blanco E, Soini Y, Kinnula V & Pääkkö P (2002) VEGF and bFGF are highly expressed in intraluminal fibromyxoid lesions in bronchiolitis obliterans organizing pneumonia. *J Pathol* 196: 220–227.
- III Lappi-Blanco E, Soini Y & Pääkkö P (1999) Apoptotic activity is increased in the newly formed fibromyxoid connective tissue in bronchiolitis obliterans organizing pneumonia. *Lung* 177: 367–376.
- IV Lappi-Blanco E, Kaarteenaho-Wiik R, Sormunen R, Salo S, Määttä M, Autio-Harminen H, Soini Y & Pääkkö P (2002). Laminin-5 γ 2 chain is a marker of re-epithelialization in idiopathic UIP and BOOP. Submitted for publication.

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1 Introduction

Usual interstitial pneumonia (UIP) and bronchiolitis obliterans organizing pneumonia (BOOP) are fibrous pulmonary disorders, both of which display newly formed connective tissue in distal airspaces (Katzenstein & Myers 1998). Idiopathic UIP (later called as UIP) is a progressive and usually fatal lung disease without an efficient treatment (Bjoraker *et al.* 1998), while the prognosis of BOOP is good, nearly 85% of patients recovering usually with, or in a few cases without, steroidal therapy (Katzenstein 1997). In both diseases the primary morphological change is an injury of the intraluminal (i.e. alveolar or bronchiolar) epithelium and its basement membrane (BM) leading to migration of fibroblasts and myofibroblasts into air spaces and production of extracellular matrix by these cells (Basset *et al.* 1986, Kuhn *et al.* 1989). In UIP, the newly formed fibromyxoid connective tissue causes fusion of alveolar structures and interstitial remodeling (Basset *et al.* 1986, Kuhn *et al.* 1989), while in BOOP the newly formed connective tissue is thought to form small collagen globules (Usuki & Fukuda 1995) or to resolve completely (Colby 1992).

The conventional treatment of UIP has been based on the concept that inflammation leads to injury and fibrosis (Crystal 1981). However, UIP shows hardly any response to corticosteroids (Bjoraker *et al.* 1998), and mainly because of this the hypothesis of UIP as an inflammatory disease is being questioned. At the moment, one of the major aims of the research on pulmonary fibrosis is to define the mechanisms that lead to persistence of the newly formed connective tissue and thus to irreversible fibrosis in UIP.

The present study was designed to evaluate whether the well characterized phenomena of normal wound healing, namely capillarization, apoptosis and re-epithelialization, are also relevant in the repair process of lung injury. The number of capillaries per tissue surface area was measured in the newly formed connective tissue in BOOP and in UIP. Further, the immunohistochemical expression of angiogenic growth factors, namely vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF), was evaluated in the same areas with the expression of Flt-1 and Flk-1, which serve as receptors for VEGF-A. Apoptotic activity was analyzed using TUNEL-method, and the immunohistochemical expression of apoptosis regulating proteins bcl-2, mcl-1, and bax was also studied. Finally, the extent of re-epithelialization was evaluated with the

expression of laminin-5, which provides adhesive connections for epithelial cells (Carter *et al.* 1991, Rousselle *et al.* 1991). Immunohistochemical and ultrastructural localization of laminin-5 γ 2 chain as well as synthesis of laminin-5 γ 2 chain mRNA were also studied.

2 Review of the literature

2.1 Function and anatomy of the lung

The lung is a unique internal organ exposing itself directly and constantly to the surrounding atmosphere. The main function of the lung is gas exchange, but it also performs many non-respiratory functions. For example, the lung detoxifies inhaled or ingested noxious agents, controls air and vascular flows, produces surfactant, and participates in transportation, degradation and production of diverse biologically active substances. The gross structural arrangement of the lung is analogous to that of a tree; the trachea is the trunk, the bronchi are its branches, and the alveoli its leaves. The trachea and bronchi provide the rigid conducting system, and the major function of gas exchange occurs in the alveoli. The lung has dual blood supplies. The pulmonary arterial circulatory system arises from the right ventricle of the heart, follows the bronchi and bronchioles, and accommodates total systemic venous return. The bronchial arterial circulatory system is a part of the systemic circulation, and nourishes the bronchial tree as far as the respiratory bronchioles. Because of its sturdiness and efficiency, vigilant defense mechanisms, and enormous reserve capacities, the lung frequently becomes irreversibly damaged before the person becomes symptomatic (Dail & Hammar 1994).

2.1.1 Normal structure and function of alveoli

Normal alveoli are lined by flattened type I pneumocytes and cuboidal type II pneumocytes, both of which form tight junctions (Wolff & Crystal 1997). Type II pneumocytes are more numerous than type I pneumocytes, but they cover only 7 % of alveolar surface, while the attenuated type I cells cover the remaining 93 % (Crapo *et al.* 1982). Gas transfer takes place across the alveolar-capillary membrane, which consists of attenuated cytoplasm of type I pneumocyte, the endothelial cell cytoplasm and their irregularly fused basement membranes (BMs). The two constituent cell layers also form the air-blood barrier that keeps the lung “dry” and maintains complete separation of three

fluid media: blood plasma, interstitial fluid, and alveolar lining layer (Schneeberger 1997, Simionescu 1997). The functions of type II cells include synthesis and secretion of surface-active material, maintenance of alveolar epithelium by proliferating and differentiating into type I cells, maintenance of minimal amount of alveolar fluid by sodium transport, and secretion of a variety of growth factors and cytokines, such as transforming growth factor-beta (TGF- β), TGF α , interleukin-8 (IL-8), IL-6, monocyte chemoattractant protein (MCP-1), tumor necrosis factor-alpha (TNF- α), granulocyte macrophage-colony stimulating factor (GM-CSF), endothelin-1 (ET-1), and nitric oxide (Mason & Shannon 1997).

The normal alveolar epithelial and endothelial BMs are thin and continuous, except for localized interruptions under type II cells. The role of BMs is to create structural support and molecular barrier, to induce some cellular functions including cell adhesion and migration and to mediate survival signals for adjacent cells. Type IV collagens, laminins, entactin/nidogen, and heparan sulfate proteoglycan perlecan are the principal components of BMs (Crouch *et al.* 1997). Anchoring fibers containing type VII collagen have been occasionally identified in fibrotic human lung as a minor component of BMs (Kawanami *et al.* 1982).

Capillary endothelial cells make up 30% of the cells in the alveolar region of the lung and they comprise 14% of the alveolar tissue volume (Crapo *et al.* 1982). The endothelium of the alveolar capillaries of the normal lung forms a continuous surface lining the endothelial BM. Endothelial cells are joined to one another by tight junctions, and their permeability properties and resistance to injury are essential for the preservation of the air-blood barrier (Simionescu 1997). Endothelial cells are metabolically active, participating in molecular transport, degradation, and production (Silverman *et al.* 1997).

The role of the interstitium is to separate and bind together the cell layers of the airspace epithelium and the vascular endothelium, and to join different parts of the lung (Weibel & Crystal 1997). Moreover, ordered layering of different cell types and BM maintains normal communication between cells and extracellular matrix (ECM) components. The ECM is a highly specialized, controlled and dynamic complex of macromolecules that lies underneath epithelial cells and surrounds connective tissue cells. In addition to its supportive property it directs many functional processes e.g. signaling, tissue differentiation, adhesion, regeneration and migration (Adams & Watt 1993). In normal lung, the major constituents of the extracellular matrix include collagen types I, III, V and VI, elastic fibers, fibronectin, proteoglycans, and BM proteins. Mesenchymal cells are mainly fibroblasts, myofibroblasts, and pericytes. Undifferentiated mesenchymal cells and smooth muscle cells are relatively uncommon. The inflammatory cells include T and B lymphocytes and natural killer cells, mast cells, as well as rare polymorphonuclear leukocytes and mononuclear phagocytes (Weibel & Crystal 1997).

2.2 Pulmonary fibrosis

Fibrotic pulmonary disorders are diseases of the lower respiratory tract characterized by injury to the lung parenchyma and by diffuse replacement of the normal architecture by mesenchymal cells and the connective tissue matrix secreted by these cells. In lung,

fibrosis is a nonspecific reaction to injury occurring when the severity and duration of alveolar wall injury prevents reconstitution of normal structure. Derangement of the alveolar walls and loss of functional alveolar capillary units interferes with normal physiologic blood flow and gas diffusion (Crystal *et al.* 1981, Wolf & Crystal 1997).

Nomenclature and classification of fibrous pulmonary disorders has been multiplex and evolving, and until recent years clinicians and pathologists have used different classification systems and terminology (Katzenstein & Myers 1998, American Thoracic Society 2000). Acute and chronic lung disorders with variable degrees of pulmonary inflammation and fibrosis are collectively referred to as diffuse parenchymal lung diseases (DPLDs) or interstitial lung diseases (ILDs) (American Thoracic Society 2002). The term “interstitial” refers to a condition in which the predominant tissue abnormality is in the alveolar septa, in contrast to intrabronchial and intra-alveolar locations. However, the process usually begins within the airspaces, and the interstitial changes are nearly always accompanied by some airspace abnormalities (Basset *et al.* 1986, Katzenstein 1997).

Fibrotic pulmonary disorders represent a wide variety of diseases with different etiologies, clinical findings, therapies and prognoses. More than 100 causes are suspected to have a role in the pathogenesis of pulmonary fibrosis, including collagen vascular diseases (Lynch & Hunninghake 1992), radiation and chemotherapy (reviewed by Abid *et al.* 2001), infections (Stewart *et al.* 1999, reviewed by Lok & Egan 2000), immunologic mechanisms (Wallace *et al.* 1994, Sauty *et al.* 1997, Kaneko *et al.* 2000), possible genetic alterations (Bitterman *et al.* 1986, Marshall *et al.* 2000, Hodgson *et al.* 2002, Thomas *et al.* 2002), exposure to organic and mineral dust, organic solvents and other occupational environments (Scott *et al.* 1990, Iwai *et al.* 1994, Hubbard *et al.* 1996, Baumgartner *et al.* 2000), smoking (Myers *et al.* 1987, Baumgartner *et al.* 1997, Moon *et al.* 1999), medication (Katzenstein 1997, Hubbard *et al.* 1998, reviewed by Ozkan *et al.* 2001), and other toxic and physical insults. However, the etiology often remains unknown (Katzenstein 1997).

The exact prevalence and incidence of pulmonary fibrosis is not known. In the study of Coultas *et al.* 1994, the prevalence of ILDs/DPLDs was 80.9/100 000 for men and 67.2/100 000 for women. Similarly, the overall incidence of ILDs/DPLDs was slightly more common in males, being 31.5/100 000 for men and 26.1/100 000 for women. The incidence, prevalence and death rate increase with age (Scott 1990, Coultas *et al.* 1994, Iwai *et al.* 1994, Mannino *et al.* 1996).

2.2.1 Idiopathic interstitial pneumonias

Idiopathic interstitial pneumonias (IIPs) represent fulminant or chronic and fibrotic pulmonary disorders without apparent etiology (Katzenstein 1997). IIPs can be distinguished from other forms of ILDs/DPLDs by clinical methods including history, physical examination, chest radiology and laboratory studies, and pathology (American Thoracic Society 2002). Hamman and Rich were the first to describe diffuse interstitial pulmonary fibrosis as a distinct entity (Hamman & Rich 1944). Liebow devised the first detailed pathologic classification of interstitial pneumonias that included five groups:

usual interstitial pneumonia (UIP), desquamative interstitial pneumonia (DIP), obliterative bronchiolitis with interstitial pneumonia (BIP), lymphoid interstitial pneumonia (LIP), and giant cell interstitial pneumonia (GIP) (Liebow 1975) (Table 1). In 1998, Katzenstein and Myers represented a modified classification of idiopathic interstitial pneumonias called “classification of idiopathic pulmonary fibrosis” (Table 1). This classification was remarkable because it emphasized the poor prognosis of UIP and thus the importance of histopathologic diagnosis. Acute interstitial pneumonia (AIP or Hamman-Rich disease) (Katzenstein *et al.* 1986), respiratory bronchiolitis with interstitial lung disease (RBILD) (Myers *et al.* 1987), and nonspecific interstitial pneumonia (NSIP) (Katzenstein & Fiorelli 1994) were added to this classification as new entities. The histologic pattern of BIP, now called bronchiolitis obliterans organizing pneumonia (BOOP) (Epler *et al.* 1985), was excluded for being more of an intraluminal than interstitial disease. LIP and GIP were also excluded for not being idiopathic.

A consensus statement including a revised classification has been recently published (American Thoracic Society/European Respiratory Society 2002) (Table 2). This new classification separates the histologic pattern of a pathologist’s report from the clinical-radiologic-pathologic diagnosis as a foundation for the classification. The diagnostic criteria for UIP are defined and the term idiopathic pulmonary fibrosis (IPF) is suggested for idiopathic UIP. Also, a provisional role for NSIP is suggested. The histologic pattern of BIP/BOOP is again included, but now it is called cryptogenic organizing pneumonia (COP). LIP is also included in the new classification.

Table 1. Previous classifications of idiopathic interstitial pneumonias.

Classification	Type of interstitial pneumonia
Liebow 1975	Usual interstitial pneumonia (UIP) Desquamative interstitial pneumonia (DIP) Obliterative bronchiolitis with interstitial pneumonia (BIP) Lymphoid interstitial pneumonia (LIP) Giant cell interstitial pneumonia (GIP)
Katzenstein & Myers 1998	Usual interstitial pneumonia (UIP) Desquamative interstitial pneumonia (DIP)/Respiratory bronchiolitis with interstitial lung disease (RBILD) Acute interstitial pneumonia (AIP; Hamman-Rich disease) Nonspecific interstitial pneumonia/fibrosis (NSIP)

Table 2. Histologic and clinical classification of idiopathic interstitial pneumonias (ATS/ERS 2002).

Histologic patterns	Clinical-Radiologic-Pathologic Diagnosis
Idiopathic pulmonary fibrosis/cryptogenic fibrosing alveolitis	Usual interstitial pneumonia
Nonspecific interstitial pneumonia (provisional)	Nonspecific interstitial pneumonia
Organizing pneumonia	Cryptogenic organizing pneumonia
Diffuse alveolar damage	Acute interstitial pneumonia
Respiratory bronchiolitis	Respiratory bronchiolitis interstitial lung disease
Desquamative interstitial pneumonia	Desquamative interstitial pneumonia
Lymphoid interstitial pneumonia	Lymphoid interstitial pneumonia

2.2.2 Idiopathic usual interstitial pneumonia

Idiopathic usual interstitial pneumonia (idiopathic UIP; also called idiopathic pulmonary fibrosis i.e. IPF, or cryptogenic fibrosing alveolitis i.e. CFA) is a chronic, progressive, and usually fatal lung disease of no identifiable etiology (Katzenstein 1997). The current classification states that idiopathic UIP/IPF is recognized as a specific form of chronic fibrosing interstitial pneumonia limited to the lung and associated with the histologic appearance of UIP on surgical lung biopsy (American Thoracic Society 2000). Idiopathic UIP is characterized histologically by a variable distribution of patchy interstitial fibrosis, inflammation, honeycomb lung and normal lung. The presence of small aggregates of fibroblasts and myofibroblasts, termed fibroblast foci, are necessary for the diagnosis (Katzenstein 1997, American Thoracic Society 2002). In areas of remodeled interstitium with advanced fibrosis the extracellular matrix includes increased amounts of fibroblasts, collagens, fibronectin, elastic fibers, proteoglycans, and smooth muscle cells (Raghu 1985, Crouch 1990, Wolf & Crystal 1997, Negri *et al.* 2000).

Idiopathic UIP is the most common idiopathic interstitial pneumonia, accounting for over 60 % of cases of interstitial pneumonias (Bjoraker *et al.* 1998). The precise prevalence and incidence of idiopathic UIP is not known. The prevalence estimates vary from 6 to 20.2 cases per 100 000 with a male predominance (Scott *et al.* 1990, Coultas *et al.* 1994), and the estimated prevalence is 16–18 per 100 000 in Finland (Hodgson *et al.* 2002). The estimated annual incidence is 7 cases per 100 000 for women and 10 cases per 100 000 for men (Coultas *et al.* 1994). The prevalence, incidence and death rate increase with age (Coultas *et al.* 1994, Mannino *et al.* 1996). Familiar forms also occur (Marshall *et al.* 2000, Hodgson *et al.* 2002, Thomas *et al.* 2002).

Idiopathic UIP occurs mainly in middle aged adults and begins insidiously. No spontaneous remissions occur, and the median survival is 2.8 years after diagnosis (Bjoraker *et al.* 1998). In a recent study, the only specific histopathologic feature predicting poor survival was the number of fibroblast foci (King *et al.* 2001). The fibroblast foci strongly express tenascin-C, and increased tenascin-C expression is also associated with a shortened survival time in idiopathic UIP (Kaarteenaho-Wiik *et al.* 1996, Pääkkö *et al.* 2000). A recent study reports that high serum levels of surfactant protein-A or -D are both biomarkers in idiopathic UIP and predictive of survival (Greene *et al.* 2002). Other adverse prognostic factors include male gender, advanced disease, and possibly increased release of PGE₂ from macrophages (Schwartz *et al.* 1994). Surprisingly, survival may be extended in patients who are cigarette smokers at the time of diagnosis (King *et al.* 2001).

The conventional treatment of idiopathic UIP has been based on the concept that inflammation leads to injury and fibrosis (Crystal *et al.* 1981). Corticosteroids and immunosuppressive or cytotoxic agents (e.g., azathioprine or cyclophosphamide) have been used to eliminate or suppress the inflammatory component, but the responses have been modest and transient (Bjoraker *et al.* 1998, Katzenstein & Myers 1998, reviewed by Lynch *et al.* 2001).

2.2.3 Bronchiolitis obliterans organizing pneumonia

Bronchiolitis obliterans organizing pneumonia (BOOP) is a nonspecific manifestation of acute lung injury localized to the peribronchiolar parenchyma. Although the disease was first described a long time ago (Lange 1901, Gosink *et al.* 1973), it became well known only after the report by Epler *et al.* in 1985. Liebow classified bronchiolitis obliterans with interstitial pneumonia (BIP) as one type of interstitial pneumonias (Liebow 1975). The same histological pattern was later called cryptogenic organizing pneumonitis (Davison *et al.* 1983). Katzenstein and Myers used the term BOOP, and excluded it from IIPs as a predominantly intraluminal rather than interstitial abnormality (Katzenstein & Myers 1998). The new consensus classification prefers the term COP for idiopathic BOOP because it conveys the essential features of the syndrome and avoids confusion with airway diseases. The corresponding histologic pattern is organizing pneumonia (American Thoracic Society 2002).

BOOP is a flu-like illness with subacute onset, and the patients are usually middle aged with an age range from 21 to 75 years. Men and women are equally affected and there is no association with smoking. The prognosis is good, and nearly 85 % of the patients recover with or without steroidal therapy (Epler 1985, Costabel *et al.* 1992a). Relapses are possible in about half of the cases (Watanabe *et al.* 1998, Lazor *et al.* 2000), and in 10–15 % of patients BOOP remains a progressive disease with a poorer prognosis (Cohen *et al.* 1994, Yousem *et al.* 1997). A wide variety of infectious, toxic, and inflammatory processes commonly produce the histologic pattern of bronchiolitis obliterans organizing pneumonia, although the etiology often remains unknown (Katzenstein 1997).

Histologically, BOOP is characterized by organizing pneumonia involving alveolar ducts and alveoli with or without bronchiolar intraluminal polyps. All deposited connective tissue throughout the lesion is the same age, and the majority of changes center on small airways. The surrounding alveolar septa are mildly thickened by a chronic inflammatory cell infiltrate, and there is also accumulation of foamy macrophages in air spaces (Katzenstein 1997, American Thoracic Society 2002). The cases with progressive BOOP were accompanied by scarring and remodeling of the background lung parenchyma and the presence of alveolar exudate more often than the cases with good outcome (Yousem *et al.* 1997, Chang *et al.* 2002).

2.2.4 Pathogenesis of pulmonary fibrosis

In 1986, Basset *et al.* showed that the basic morphological defect in various acute and chronic interstitial lung diseases is injury in the intraluminal epithelium and its BM leading to migration of fibroblasts and myofibroblasts from the interstitial compartment into air spaces. This leads to formation of intraluminal fibrosis, which in advancing and chronic forms of fibrosis mediates fusion of adjacent alveolar structures (i.e. alveolar collapse) and further remodeling of interstitium (Basset *et al.* 1986). Similar observations had already been suggested earlier, and they have been confirmed in later studies in both humans and animal models (Brody & Craighead 1976, Kawanami *et al.* 1983,

Katzenstein 1985, Myers & Katzenstein 1988a,b, Kuhn & McDonald 1991, Usuki & Fukuda 1995). In advanced pulmonary fibrosis there is finally disorder of the tissue, distorted matrix deposition, mesenchymal cell proliferation and alteration to normal lung structure, with compromised gas exchange function (Wolff & Crystal 1997).

It has long been believed that IIPs result from persistent inflammation with attendant activation of inflammatory cells, i.e. alveolar macrophages, neutrophils, eosinophils, T-cells, B-cells, basophils and mast cells. This concept has been the basis for anti-inflammatory treatment and also for attempts to estimate the prognosis of certain diseases, such as idiopathic UIP (Crystal *et al.* 1981, Wolff & Crystal 1997). Inflammatory cells are known to release various oxidants, proteases, cytokines and growth factors that further modulate the inflammatory response. Also epithelial, endothelial and mesenchymal cells act as chemoattractants and further release polypeptides contributing to the inflammatory milieu (Wolff & Crystal 1997).

Matrix metalloproteinases (MMPs) comprise a family of matrix-degrading proteinases, the activities of which are highly regulated by tissue inhibitors of metalloproteinases (TIMPs) (reviewed by Winkler & Fowlkes 2002). MMPs are involved in the pathogenesis of pulmonary fibrosis by contributing to the breakdown and remodeling that occurs during lung injury (Pardo *et al.* 1992, Hayashi *et al.* 1996, Fukuda *et al.* 1998, Selman *et al.* 2000, Suga *et al.* 2000, Ramos *et al.* 2001, Choi *et al.* 2002). MMPs may also modulate inflammatory response by releasing growth factors and cytokines known to influence growth and differentiation of target cells within the lung. The function of MMPs also allows migration of inflammatory cells into damaged lung tissues (reviewed by Winkler & Fowlkes 2002).

So far, the best known and most studied growth factors implicated in the pathogenesis of pulmonary fibrosis are TGF- β , TNF- α , platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-binding proteins, interleukin-4, interferons, ET-1 and connective tissue growth factor (CTGF). Growth factors modulate the emergence and persistence of myofibroblasts, synthesis of ECM proteins, production of metalloproteinases and their inhibitors, expression of adhesion molecules, chemotaxis, angiogenesis, and probably apoptosis of parenchymal cells (reviewed by Allen & Spiteri 2002). Modulation of effects of growth factors is also a basis for new therapeutic inventions for treatment of pulmonary fibrosis (Ziesche *et al.* 1999, Kolb *et al.* 2002, reviewed by Allen & Spiteri 2002).

2.2.5 Pathogenesis of intraluminal fibrosis in BOOP and UIP

Ultrastructurally, both in BOOP and UIP, injury of the alveoli leads to necrosis and sloughing of alveolar lining cells resulting in denuding of epithelial BM. The denuded BM is either disrupted or forms infoldings and deep invaginations (Myers & Katzenstein 1988 a and b). Interstitial fibroblasts and myofibroblasts migrate into air space through focal defects in the alveolar epithelium and underlying BM (Basset *et al.* 1986). The modified fibroblasts and myofibroblasts continue proliferating and producing extracellular matrix in the alveolar spaces, and the resulting newly formed connective tissue lesions are called fibroblast foci in UIP and Masson bodies in BOOP (Epler *et al.*

1985, Kuhn *et al.* 1989, Kuhn & McDonald 1991, Katzenstein 1997). Fusion of alveolar structures via intraluminal fibrosis together with synthesis of extracellular matrix leads to interstitial remodeling in UIP (Basset *et al.* 1986, Kuhn *et al.* 1989). In BOOP, the Masson bodies are thought to form small collagen globules (Usuki & Fukuda 1995), or they may resolve completely (Colby 1992).

The fibroblast foci and Masson bodies bear striking similarities both at light microscopic level and ultrastructurally. Both contain fibroblasts and myofibroblasts aligned parallel to another (Kuhn & McDonald 1991). The extracellular matrix is edematous and contains fibronectin (Kuhn & McDonald 1991) and tenascin-C (Kaarteenaho-Wiik *et al.* 1996), and varying amounts of glycosaminoglycans versican, decorin, biglycan and hyaluronan (Bensadoun *et al.* 1996). The fibroblasts/myofibroblasts express mRNAs of collagens I, III and VI, and these proteins are also detected in the extracellular matrix to various extents depending upon stage of organization (Specks *et al.* 1995, Fukuda 1998). Collagen type IV has been detected as a component of BM, which is partly disrupted or thickened (Basset 1986, Kuhn 1989). Myofibroblasts of fibroblast foci and Masson bodies express alpha smooth muscle actin (α -SMA), which evidently contributes to the contractile capacity of these cells (Kuhn & McDonald 1991, Kapanci *et al.* 1995, Yoshinouchi *et al.* 1999).

Differences between fibroblast foci and Masson bodies have been observed in the amount and distribution of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Although somewhat contradictory, the results suggest that imbalance between collagenases and TIMPs favors interstitial remodeling in UIP (Pardo *et al.* 1992, Hayashi *et al.* 1996, Fukuda *et al.* 1998, Selman *et al.* 2000, Suga *et al.* 2000, Ramos *et al.* 2001, Choi *et al.* 2002). There are also studies suggesting increased local procoagulant and anti-fibrinolytic activity in UIP (Kotani *et al.* 1995, Fujii *et al.* 2000), but it is unclear whether this is a disease-related phenomenon or a physiologic reaction that accompanies alveolar epithelial damage and tissue repair (Imokawa *et al.* 1997). It is also suspected that in UIP failure of re-epithelialization of fibroblast foci maintains fibroblast/myofibroblast activity and extracellular matrix synthesis (Adamson *et al.* 1990, Uhal *et al.* 1995, Uhal *et al.* 1998, Kuwano *et al.* 1999, Barbas-Filho *et al.* 2000).

2.3 Angiogenesis

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is fundamental for normal embryological development and tissue repair. Pathologic angiogenesis is characteristic of the progression of neoplastic and chronic inflammatory diseases. Regulation of angiogenesis *in vivo* is complex and it is controlled by a variety of soluble factors, extracellular matrix proteins, and differences in endothelial cell phenotype and function (reviewed by Swerlick 1995).

2.3.1 *Vascular endothelial growth factor-A*

Vascular endothelial growth factor-A (later called VEGF), belonging to the platelet-derived growth factor (PDGF)/VEGF family of growth factors, is a key regulator of angiogenesis. VEGF is a heparin-binding glycoprotein of about 45 kDa molecular weight that stimulates proliferation, migration, and proteolytic activity of endothelial cells (Ferrara & Henzel 1989, Leung *et al.* 1989, Pepper *et al.* 1991, Unemori *et al.* 1992, Lamoreaux *et al.* 1998). VEGF is also necessary for the survival of endothelial cells due to its ability to inhibit apoptosis and capillary regression (Gerber *et al.* 1998). Through its capacity to induce nitric oxide, VEGF may mediate vasodilatation and increase blood flow that precede angiogenesis (Liu *et al.* 2002, reviewed by Ziche & Morbidelli 2000). VEGF is also a potent mediator of increased vascular permeability; hence its other name, vascular permeability factor (Senger *et al.* 1983).

VEGF is synthesized by numerous cell lines (reviewed by Grützkau *et al.* 1998) and secreted through conventional pathways (Leung *et al.* 1989). To date, six human VEGF mRNA species, encoding VEGF isoforms of 121, 145, 165, 183, 189 and 206 amino acids, are produced by alternative splicing of the VEGF mRNA. An important biological property that distinguishes the different VEGF isoforms is their heparin and heparan-sulphate-binding ability. VEGF₁₂₁ is the most soluble isoform and does not bind to heparin or extracellular matrix (ECM), whereas VEGF₁₈₉ and VEGF₂₀₆ are almost completely sequestered in the ECM. VEGF₁₆₅ is a heparin-binding protein, and 50–70% of VEGF₁₆₅ remains bound to cell surface and ECM. VEGF₁₂₁, VEGF₁₄₅ and VEGF₁₆₅ induce angiogenesis *in vivo*, but VEGF₁₄₅ is found mainly to be expressed in cells derived from reproductive organs, as is apparently also VEGF₂₀₆. VEGF proteins may become available to target cells as freely diffusible proteins (VEGF₁₂₁ or VEGF₁₆₅) or following protease activation and cleavage. VEGF isoforms in the ECM constitute a reservoir of growth factors that can be slowly released by exposure to heparin, heparan sulphate and heparinases, or more rapidly mobilized by specific proteolytic enzymes such as plasmin and urokinase-type plasminogen activator (uPA). The bioactive VEGF consisting of the first 110 NH₂ terminal amino acids is generated in the extracellular compartment (reviewed by Neufeld *et al.* 1999, Ferrara 2001, and Robinson & Stringer 2001). Alternative splicing of VEGF may have an important role in the regulation of VEGF activity in developing and injured lungs (Watkins *et al.* 1999).

In the lung of human fetus, VEGF has been expressed in bronchial epithelium and in smooth muscle cells and pericytes of vessels (Shifren *et al.* 1994, Acarregui *et al.* 1999). Similarly, in normal adult human lung, VEGF has been expressed in bronchial epithelial cells, type II pneumocytes, smooth muscle cells of arterioles and bronchioles, and in alveolar macrophages (Fehrenbach *et al.* 1999a). The major site of VEGF mRNA expression in the human lung is probably alveolar type II epithelial cells (Boussat *et al.* 2000). Also, the normal human epithelial lining fluid (ELF) contains high concentrations of VEGF (Kaner & Crystal 2001). These results suggest that in lung, VEGF is involved not only in angiogenesis, but also in the maintenance and regulation of existing vessels with a paracrine mechanism of action between endothelial and nearby cells. Hypoxia stimulates VEGF gene expression in human endothelial and vascular smooth muscle cells

and in fibroblasts *in vitro* (Brogi *et al.* 1994, Namiki *et al.* 1995, Jackson *et al.* 1997). In animal models, both acute and chronic hypoxia increase lung tissue gene expression for VEGF and its receptors Flt-1 and Flk-1 (Tuder *et al.* 1995, Christou *et al.* 1998).

Recently, there have been observations suggesting that VEGF could also have actions not related to angiogenesis: VEGF may have nonendothelial target cells in testis regulating male fertility (Korpelainen *et al.* 1998) and act as a direct autocrine growth factor for tumor cells in non-small cell lung carcinomas (Decaussin *et al.* 1999), renal tubular cells (Kanellis *et al.* 2000) and regenerating myocytes (Rissanen *et al.* 2002).

2.3.2 Flt-1 and Flk-1

VEGF has at least two receptors, called Flt-1(fms-like tyrosine kinase)/VEGFR-1 and Flk-1(fetal liver kinase-1)/VEGFR-2/KDR(kinase domain region) (deVries *et al.* 1992, Terman *et al.* 1992). Flt-1 and Flk-1 are transmembrane tyrosine kinase receptors, which are both upregulated by hypoxia (Tuder *et al.* 1995, Christou *et al.* 1998). When activated, Flt-1 and Flk-1 promote angiogenesis, but with somewhat different functions and signal transduction cascades (Waltenberger *et al.* 1994). Flt-1 is more closely associated with cell differentiation, while Flk-1 is thought to have a more important role in VEGF-mediated endothelial cell proliferation (Shibuya *et al.* 1990). Originally, Flt-1 and Flk-1 were thought to be endothelial cell-specific. Subsequently, however, trophoblast cells, monocytes, renal mesangial and tubular cells, hematopoietic stem cells, megakaryocytes, retinal progenitor cells, pancreatic duct cells, non-small cell lung carcinoma cells, lung fibroblasts and muscle cells have also been shown to express one or both of these receptors (reviewed by Neufeld *et al.* 1999, Öberg *et al.* 1994, Decaussin *et al.* 1999, Kanellis *et al.* 2000, Ishida *et al.* 2001, Rissanen *et al.* 2002). According to recent studies it seems possible that VEGF has autocrine actions both on endothelial and non-endothelial cells via Flt-1 or Flk-1 (Namiki *et al.* 1995, Decaussin *et al.* 1999, Kanellis *et al.* 2000, Rissanen *et al.* 2002).

2.3.3 Basic fibroblast growth factor

Basic fibroblast growth factor (later called bFGF; also called FGF-2) is a well-documented angiogenic growth factor and induces endothelial cell replication, migration and extracellular proteolysis (Gospodarowicz *et al.* 1983, Montesano *et al.* 1986, Tsuboi 1990). bFGF is produced by several normal and tumor cells, endothelial cells included, and has autocrine activities on angiogenesis (Moscatelli *et al.* 1986, Schweigerer *et al.* 1987, Sato & Rifkin 1988). bFGF may promote angiogenesis both by a direct effect on endothelial cells and indirectly by the upregulation of VEGF in endothelial cells (Stavri *et al.* 1995), and bFGF and VEGF have a synergistic effect in the induction of angiogenesis both *in vitro* (Pepper *et al.* 1992) and *in vivo* (Mattern *et al.* 1997). Also, induction of bFGF induced angiogenesis is partly dependent on the activation of VEGF (Tille *et al.* 2001).

bFGF belongs to the FGF superfamily, which contains at least twenty distinct FGFs. Four different bFGF polypeptides of 18–24.2 kDa can be formed from the one *fgf-2* gene (reviewed by Powers *et al.* 2000). bFGF does not code for a signal sequence required for vectorial translocation into endoplasmic reticulum and can be released through yet unknown mechanism of exocytosis independent of the ER-Golgi pathway (Abraham *et al.* 1986, Mignatti *et al.* 1992). Mechanical damage such as wounding has also been proposed as one mechanism for release of biologically active bFGF from endothelial cells (McNeil *et al.* 1989). In the ECM and on the cell surface, bFGF is bound to heparan-like glycosaminoglycans (HLGAGs) of the ECM and is present in BMs *in vivo* (Folkman *et al.* 1988). The association to HLGAGs may afford bFGF protection from proteolysis, besides creating a localized and persistent reservoir of the growth factor. bFGF is released from ECM by enzymatic cleavage of proteolytic enzymes, or by binding to a carrier protein, which can then deliver bFGF to the tyrosine kinase transmembrane receptors (reviewed by Powers *et al.* 2000).

Like VEGF, bFGF also inhibits apoptosis of endothelial cells (Karsan *et al.* 1997). bFGF is also a well-characterized fibroblast growth factor inducing mitogenic and chemotactic activity and differentiation of some other cell types of mesodermal and neuroectodermal origin (reviewed by Mignatti & Rifkin 1991).

In normal adult human lung, bFGF has been expressed in the BMs of blood vessels, epithelial cells lining trachea and major bronchi, and variably in endothelial cells. In bleomycin-induced acute lung injury in the rat, there was strong bFGF positivity in vascular media and endothelial cells, mast cells, and in fibrotic areas (Liebler *et al.* 1997). On the other hand, in an animal model with oxygen stress induced fibrosing alveolitis, bFGF was upregulated after injury and synthesized by type II pneumocytes (Sannes *et al.* 1996).

2.3.4 Angiogenesis in pulmonary fibrosis

There are only a few studies on angiogenesis in human pulmonary fibrosis, and the results must be evaluated in the light of former and current knowledge on pathogenesis of pulmonary fibrosis. When summarized, the results suggest not only loss of gas exchanging capillaries, but also focal increase in the amount of capillaries in fibrous areas. In 1953, Golden & Bronk observed a variable morphology of alveolar vasculature in diffuse pulmonary fibrosis (Golden & Bronk 1953). However, with current knowledge, some of the changes interpreted by them as alveolar wall hypertrophy probably represent collapse of alveolar walls. A decrease in the number of septal capillaries and in the effective gas exchanging surface of capillaries has been shown in fibrotic human lung (Gracey *et al.* 1968). Based on morphometric, angiographic, light microscopic and ultrastructural studies, Bignon *et al.* showed in 1974 that in diffuse pulmonary fibrosis, the number of capillaries was decreased in areas with severe fibrosis, but intensively increased in fibrous areas at some distance from air space surface. The results also suggested that these capillaries originate from bronchial circulation (Bignon *et al.* 1974). This was in line with the study of Turner-Warwick, who showed the presence of systemic pulmonary microvasculature anastomoses in pulmonary fibrosis (Turner-Warwick 1963). From

studying the biopsy specimens of patients who underwent open lung biopsy for fibrosing alveolitis, Coalson (1982) found alteration of the capillary structure together with evidence for both endothelial cell death and regeneration.

The extent of capillarization in newly formed connective tissue has been unclear so far. The number of capillaries within newly formed connective tissue has been reported to be small despite the underlying disease (Kawanami *et al.* 1983, Basset *et al.* 1986). The observations of an early phase of the fibrous process with prominent proliferating capillaries (Anderson & Foraker 1960, Hasleton 1983) or a stereotyped response in lung injury with a granulation tissue response and budding of capillaries (Snider 1986) have been treated with caution after the emergence of the concept of the collapse of the alveolar walls. However, in a profound study Peyrol *et al.* showed that in BOOP there are intraluminal protrusions of altered capillaries in the areas of intra-alveolar fibrosis (Peyrol *et al.* 1990).

Despite the active research on growth factors in pulmonary fibrosis, little interest has been focused on angiogenic growth factors. In surgical wounds, functional VEGF is a key mediator in wound angiogenesis, fluid accumulation, and granulation tissue formation (Howdieshell *et al.* 2001). In human lung, bFGF inducing granulation tissue formation has been identified from bronchoalveolar lavage fluid obtained from patients suffering from an acute lung injury, and alveolar macrophages were identified to be the cellular source bFGF (Henke *et al.* 1991, Henke *et al.* 1993). Keane *et al.* showed in 1997 that human UIP fibroblasts induced an angiogenic response partly caused by a CXC chemokine, IL-8. They also observed that areas associated with IL-8 immunolocalization demonstrated significant vascular remodeling in UIP. In a rat model of bleomycin-induced pulmonary fibrosis, an increased number of VEGF-positive type II alveolar epithelial cells and myofibroblasts were identified in fibrotic lesions (Fehrenbach *et al.* 1999a).

2.4 Apoptosis

Apoptosis is a biochemically regulated and active cell death program whereby individual cells die without injuring neighboring cells or causing inflammatory reaction. During apoptosis, the dying cell separates from its neighbors and undergoes a period of membrane blebbing, condensation of cytoplasm and increase in cell density. Simultaneously the nuclear chromatin becomes compact, segregates and forms sharply delineated masses along the nuclear envelope. The nucleus splits into discrete fragments and finally, the cell splits into a cluster of membrane bound apoptotic bodies, each containing a variety of organelles. Apoptotic bodies are ingested by nearby cells and macrophages before they cause an inflammatory reaction (Kerr *et al.* 1972, Kerr *et al.* 1994). The morphologically visible process of apoptosis takes a few hours, and the majority of the time is spent on the degradation within the phagocytic cells (Wyllie 1997a). Apoptosis contrasts with necrosis, which is a passive and accidental form of cell death. In necrosis the cell swells, the cell membrane is disrupted, and the nuclear and cytosolic structures are demolished, causing an inflammatory reaction in the neighboring cells (Kerr *et al.* 1972, Wyllie 1997a).

Apoptosis takes place in a variety of biologically significant situations including embryogenesis, organogenesis and the maintenance of homeostasis as well as normal function of the immune system (reviewed by Cohen *et al.* 1992, Wyllie 1997a, and Cummings *et al.* 1997). Abnormal regulation of apoptosis has been implicated in the onset and progression of diseases both in the form of inhibited and excessive apoptosis (reviewed by Thompson 1995 and Antonsson 2001). In the lung, as in other organs, apoptosis plays a critical role in organogenesis and alveolarization by reducing the number of fibroblasts and type II epithelial cells (Schittny *et al.* 1998). The normal resolution of inflammation in the lung occurs also through the regulated removal by apoptosis of unwanted cells such as granulocytes without the release of damaging histotoxins. Controlled and localized apoptosis is a prominent feature in the resolution of pneumonia (reviewed by Haslett 1999). According to current knowledge, abnormal regulation of apoptosis also plays a role in the pathogenesis of asthma and COPD (Kasahara *et al.* 2000, Dorscheid *et al.* 2001, Melis *et al.* 2002).

2.4.1 Pathways of apoptotic signaling

Apoptosis can be induced by a variety of physiologic, damage-related and therapy-associated agents (reviewed by Thompson 1995, and Wyllie 1997b). Two major apoptosis pathways have been identified, namely the death receptor pathway (also called extrinsic pathway) and the mitochondrial (intrinsic) pathway. The death receptor pathway involves at least five transmembrane receptors belonging to the TNF (tumor necrosis factor)/NGF (nerve growth factor) -receptor superfamily (reviewed by Timmer *et al.* 2002). Fas is a type I cell surface protein belonging to the TNF/NGF receptor family (Itoh *et al.* 1991). FasL is a type II membrane protein that belongs to the TNF family and is expressed predominantly in activated T lymphocytes and in tissues including small intestines, kidney, testis, and lung (Suda *et al.* 1993). The mitochondrial pathway is mediated by mitochondrial membrane permeabilization and the release of cytochrome c (reviewed by Antonsson 2001). In both of these pathways, the final result is the activation of the so-called caspase cascade, which leads to proteolysis of structural and regulatory proteins and cell death, bcl-2 family proteins and p53 regulating apoptosis (reviewed by Antonsson 2001; Timmer *et al.* 2002). p53 is a nuclear protein important in cell cycle regulation and homeostasis. p53 is up-regulated in response to DNA damage and functions either by inhibiting cellular division through G1 arrest or by facilitating apoptosis. p21^{Waf1/Cip1} is a cyclin-dependent kinase inhibitor that is induced by p53 and can activate both G1 and G2 cell cycle arrests (reviewed by Bálint & Vousden 2001)

2.4.1.1 Bcl-2 family

The bcl-2 family is a group of apoptosis-regulating genes which are able to inhibit or promote apoptosis. At least 20 bcl-2 family members have been identified in mammalian cells (Table 3). All members possess at least one of the four bcl-2 homology domains

(BH1-BH4). Current data suggest that there are two potentially independent mechanisms for promoting cell death. One mechanism is based on dimerization, which is essential for the function of pro-apoptotic BH3 subfamily and provides an important mechanism for controlling the activity of bcl-2 and bax. The other, an intrinsic, heterodimerization-independent function is probably related to the ability of these proteins to insert into membranes (reviewed by Antonsson 2001).

Table 3. The bcl-2 protein family. Modified from Antonsson 2001 and Zhang et al. 2001.

Anti-apoptotic	Pro-apoptotic
Bcl-2	Bax
Bcl-X _L	Bak
Bcl-w	Bok
Mcl-1	Bcl-X _S
BOO/DIVA	Bid
A1/Bfl-1	Bad
NR-13	Bik/Nbk
Bcl2-L-10	Bim/Bod
	Blk
	Hrk
	Nix
	BNip3
	Noxa
	PUMA
	Bcl-rambo

2.4.2 Analysis of apoptosis

There are many approaches to analyze apoptosis, but none can exceed morphologic examination. Nuclear shrinkage and budding, loss of cell shape, and eventual cytoplasmic blebbing are hallmarks of an apoptotic cell. Although these are features of late-stage apoptotic cells, they are the gold standard in the recognition of apoptotic morphology (Kerr *et al.* 1972, Kerr *et al.* 1994). Additional methods include the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for identification of DNA fragmentation, and methods based on detection of apoptosis-related proteins or cell membrane changes. These techniques also identify later stages of apoptosis, and their detection abilities vary considerably (reviewed by Soini *et al.* 1998, and Stadelmann & Lassmann 2001). Combining a plain morphological evaluation of apoptosis with the 3'-end labeling method forms a reliable tool for analysis of apoptosis (Soini *et al.* 1998).

2.4.3 Apoptosis in pulmonary fibrosis

Polunovsky *et al.* (1993) showed that bronchoalveolar lavage fluid obtained from patients suffering from adult respiratory distress syndrome (ARDS) induced cell death of fibroblasts and endothelial cells. The mode of cell death for endothelial cells was apoptosis. Fibroblast death was distinct from necrosis, but also differed from typical apoptosis (Polunovsky *et al.* 1993). Since then research on apoptosis in pulmonary fibrosis has focused on apoptosis of epithelial cells in order to understand the mutual interaction between mesenchymal and epithelial cells. It has been shown that altered fibroblasts isolated from fibrotic human or rat lung release soluble factors capable of inducing cell death and net loss of alveolar epithelial cells (Uhal *et al.* 1995). These factors have later been identified as angiotensin peptides (Wang *et al.* 1999). Apoptosis has been shown to be the major pathway responsible for the resolution of type II pneumocytes in acute lung injury (Bardales *et al.* 1996, Guinee *et al.* 1996). In acute lung injury, apoptosis has also been observed in interstitial and endothelial cells, but to lesser extent than in epithelial cells (Guinee *et al.* 1996).

Similarly to acute lung injury, increased apoptosis of alveolar epithelial cells has been observed in advanced fibrotic lung of idiopathic UIP (Kuwano *et al.* 1996, Uhal *et al.* 1998, Barbas-Filho *et al.* 2000). Apoptosis of epithelial cells occurs both in areas of BM injury in proximity to underlying myofibroblasts (Uhal *et al.* 1998) and in normal alveoli (Barbas-Filho *et al.* 2000). p21 and p53 have been observed to be upregulated in bronchial and alveolar epithelial cells in patients with idiopathic UIP (Kuwano *et al.* 1996). There is also evidence that apoptosis of epithelial cells is at least partly mediated by Fas-Fas ligand pathway. By immunohistochemistry Fas has been detected in bronchiolar and alveolar epithelial cells and FasL in lymphocytes and granulocytes (Kazufumi *et al.* 1997, Kuwano *et al.* 1999). Kuwano *et al.* (2000) also observed that the levels of soluble FasL in BAL were increased in patients with idiopathic UIP, while there was an elevation of soluble Fas in patients with BOOP, suggesting a pathogenetic role for epithelial cell apoptosis and activated T-cells in both diseases. Recently it was reported that the immunoreactivity for the Fas-associated death domain protein as well as for caspase-1 and caspase-3 were significantly increased in alveolar epithelial cells of idiopathic UIP compared with normal controls (Maeyama *et al.* 2001).

2.5 Re-epithelialization

Re-epithelialization of the newly formed connective tissue is supposed to be a crucial event in the pathogenesis of pulmonary fibrosis (Adamson *et al.* 1988, Adamson *et al.* 1990, Selman *et al.* 2001). Epithelial injury targets type I pneumocytes, but the recovery phase is characterized by proliferation of type II pneumocytes (Adamson *et al.* 1974, Kawanami *et al.* 1982, Myers & Katzenstein 1988a and b, Adamson *et al.* 1990). The epithelial-fibroblast control may be mediated by direct contacts of epithelial cells with fibroblasts of surrounding ECM (Adamson 1990), or by prostaglandin E₂ secreted by type II pneumocytes (Taylor *et al.* 1979, Goldstein & Polgar 1982, McAnulty *et al.* 1997). Type II pneumocytes are capable of differentiating into type I pneumocytes

(Adamson *et al.* 1974), but cuboidal cells of probably bronchiolar origin can also serve as progenitor cells in alveolar injury (Kawanami *et al.* 1982). The metaplastic squamous- and bronchiolar-type cells often detected in UIP are probably also of bronchiolar origin (Kawanami *et al.* 1982, Chilosi *et al.* 2001).

2.5.1 Laminins

Laminins are a group of large heterotrimeric glycoproteins, forming large polymerizing networks and providing binding sites for different matrix molecules. The laminin molecule is composed of three chains; the α , β , and γ chain, which are linked together by disulfide bonds to form either a cruciform or T or Y shaped structure. Currently at least 11 different laminin chains and twelve different laminin heterotrimers are known to exist in mammals. Biological functions of laminins are mediated by specific or non-specific receptors present on cell membranes, such as integrins, membrane-bound proteoglycans (e.g. dystroglycan) and other membrane-bound glycoproteins. With collagen IV, laminins form a stable structural framework to which other BM proteins are bound. However, the binding interactions among individual laminins, nidogens, collagens, and other components not only serve as anchors that target BM deposition, but also lead to changes in matrix organization, receptors and cortical cytoskeletal components. Thus, laminins exhibit several essential functional properties in cell adhesion, migration, differentiation, tissue development and mitogenic modulation (reviewed by Colognato & Yurchenco 2000). Cell adhesion is essential for the cellular differentiation and also for the prevention of apoptosis, and laminins and type IV collagen are known to mediate survival signals for epithelial cells (Mooney *et al.* 1999, Esco *et al.* 2001).

2.5.1.1 Laminin-5 ($\alpha3\beta3\gamma2$)

$\alpha3$, $\beta3$, and $\gamma2$ chains form laminin-5 (previously called kalinin, epiligrin, nicein or ladsin) heterotrimer. $\alpha3$ is additionally present in laminins-6 and -7, but there is no knowledge so far that $\beta3$ or $\gamma2$ chains form laminin heterotrimers other than laminin-5. A unique property of the $\alpha3$ and $\gamma2$ chains is that they are processed in ECM space after secretion, which has not been detected for any other laminin chains. The laminin $\alpha3$ chain, initially 190 kDa in size, is cleaved into a 160 kDa sized molecule and can be further cleaved into a 145 kDa sized laminin chain by tissue-type plasminogen activator and plasminogen (Goldfinger *et al.* 1998). The $\gamma2$ chain is synthesized and secreted as a high molecular weight precursor of 155 kDa, which is processed extracellularly to 105 kDa weight (Marinkovich *et al.* 1992). Recently, matrix metalloproteinase-2 (MMP-2) and membrane type-1 matrix metalloproteinase (MT1-MMP) have been shown to cleave laminin $\gamma2$ chain specifically to generate its truncated form (Giannelli *et al.* 1997, Koshikawa *et al.* 2000).

Biological functions of laminin-5 are mediated via integrins $\alpha_3\beta_1$ (Carter *et al.* 1991), $\alpha_6\beta_1$ (Delwel *et al.* 1993), and $\alpha_6\beta_4$ (Niessen *et al.* 1994). Laminin-5 was first found from skin, where it was shown to be located in the anchoring filaments mediating the attachment of keratinocytes to the BM (Rousselle *et al.* 1991). In skin, the adhesion between epithelial and mesenchymal cells is mediated through the chain of proteins consisting of hemidesmosomal proteins, $\alpha_6\beta_4$ integrin, laminin-5 and type VII collagen (Rousselle 1991 and 1997, reviewed by Jones *et al.* 1998). Laminin-5 also interacts with laminins-6 or -7 by forming disulfide-bonded complex, and this association may provide a mechanism for linking laminin-5 with BM proper (Champlaud *et al.* 1996). Mutations in all three laminin chain-coding genes cause detachment of cells from BM and blister formation (Pulkkinen *et al.* 1994a,b, Kivirikko *et al.* 1995). Laminin-5 is also highly expressed especially by migrating keratinocytes in wounded skin in establishing a new BM-zone (Pyke *et al.* 1994). Thus, laminin-5 has important roles in the attachment of epithelial cells.

In addition to the role of laminin-5 in re-epithelialization and BM integrity, it induces cell migration (Giannelli *et al.* 1997, Goldfinger *et al.* 1998, Salo *et al.* 1999). This functional conversion may be modulated by the extracellular processing of laminin chains (Giannelli *et al.* 1997, Goldfinger *et al.* 1998, Koshikawa *et al.* 2000). Laminin-5 is also known to modulate T cell proliferation, activation and apoptosis (Vivinus-Nebot *et al.* 1999, Sato *et al.* 1999), and it may prevent hypoxia-mediated apoptosis of human corneal epithelial cells (Esco *et al.* 2001).

Laminin-5 has been observed in BMs of both squamous and glandular epithelia in several locations, e.g. skin, lung, breast, intestine and prostate (Rousselle *et al.* 1991, Kallunki *et al.* 1992, Pyke *et al.* 1994, Virtanen *et al.* 1995, Hao *et al.* 1996). Recently, expression of laminin-5 has also been found in vascular smooth muscle cells (Kingsley *et al.* 2002). By *in situ* hybridization the γ_2 chain was found to be highly expressed in epithelial cells of fetal bronchi and alveoli (Kallunki *et al.* 1992). In normal adult lung, laminin-5 γ_2 chain is localized in the BMs of bronchiolar and alveolar epithelium (Mizushima *et al.* 1998).

No previous studies comparing the extent of re-epithelialization in BOOP and in UIP were available. Neither were there studies on the role of laminin-5 in re-epithelialization of the newly formed connective tissue in pulmonary fibrosis.

2.6 Evolving theory of the pathogenesis of idiopathic UIP

One of the major aims of the research on pulmonary fibrosis is to define the mechanisms that lead to persistence of fibroblast foci and thus to irreversible fibrosis and interstitial remodeling in UIP. The reason for this is that idiopathic UIP is a progressive and usually fatal lung disease still without efficient treatment (Bjoraker *et al.* 1998, Katzenstein & Myers 1998). UIP responds to corticosteroids much more poorly than the other histopathologic subgroups of interstitial pneumonias, except for acute interstitial pneumonia (Bjoraker *et al.* 1998, Daniil *et al.* 1999, Nicholson *et al.* 2000). Mainly because of this the prevailing hypothesis of UIP as an inflammatory disease is being questioned. Parallel with the classification of idiopathic interstitial pneumonias

represented by Katzenstein & Myers (1998), it became evident that part of the cases included in earlier studies as UIP actually represented other subgroups of interstitial pneumonias with different histological characteristics and different clinical behavior. In fact, in UIP the inflammatory component is usually mild, occurs mainly in areas of collagen deposition of honeycomb change, and rarely involves otherwise unaltered alveolar septa (Katzenstein & Myers 1998). On the other hand, interstitial lung diseases in which inflammation is a prominent feature of early disease, for example desquamative interstitial pneumonia and hypersensitivity pneumonitis, often do not progress to end-stage fibrosis (Katzenstein & Myers 1998). Adamson *et al.* showed in 1988 that epithelial injury in the absence of ongoing inflammation is adequate to stimulate the development of fibrosis. In recent years a few animal model studies have been published also suggesting that it is possible to dissociate the inflammatory response from the fibrotic response (Huaux *et al.* 1998, Munger *et al.* 1999, Sime *et al.* 1997). In histologic tissue samples, the extent of cellularity or alveolar space cellularity (i.e. amount of inflammatory cells) did not affect survival. Instead, the only histologic finding with prognostic significance was the number of fibroblast foci, which had an adverse correlation with survival (King *et al.* 2001). However, both the number of fibroblast foci and the amount of interstitial mononuclear cells have been shown to correlate with pulmonary function (Nicholson *et al.* 2002).

In 2001, Selman *et al.* published a review presenting a theory of an abnormal wound healing model for UIP. According to this theory, fibrosis in UIP is suspected to result from a failure of re-epithelialization of injured areas combined with prolonged survival of active myofibroblasts and deficient vascularization of fibroblast foci. The authors have compared morphological changes of fibroblast foci of UIP to those seen in normal wound healing model in skin and in Masson bodies of BOOP (Selman *et al.* 2001). In the present study an analogous approach has been applied to the subject by comparing vascularization, apoptosis and re-epithelialization of intraluminal fibrosis in UIP and BOOP.

3 Aims of the study

1. To assess the extent of capillarization in the newly formed intraluminal connective tissue in BOOP and UIP (I).
2. To study whether VEGF and bFGF and the receptors of VEGF, Flt-1 and Flk-1, are expressed in BOOP and UIP, and whether their expression correlates with the extent of capillarization in the newly formed intraluminal connective tissue (II).
3. To study the extent of apoptotic activity and the expression of apoptosis regulating proteins bcl-2, mcl-1 and bax in the newly formed intraluminal connective tissue in BOOP and UIP (III).
4. To study the extent of re-epithelialization, and determine the immunohistochemical expression and ultrastructural location of laminin-5 γ 2 chain protein and expression of laminin-5 γ 2 chain mRNA in the newly formed intraluminal connective tissue, and compare the expression of laminin-5 γ 2 chain protein with the extent of epithelialization in BOOP and UIP (IV).

4 Materials and methods

4.1 Tissue specimens, patients and follow-up information

All open or thoracoscopic lung biopsies diagnosed histologically as BOOP or UIP were selected from the files of the Department of Pathology, Oulu University Hospital, from 1977 to 2001. Additional cases of BOOP were collected from the Kuopio and Tampere University Hospitals and the Kokkola, Kemi and Jyväskylä Central Hospitals. The biopsies were taken from different parts of the left or right lung before the patients had received any medication therapy. The biopsy material had been fixed in 10% formalin under vacuum to expand the tissue and remove air bubbles (Wagenvoort 1980) or perfused by injecting the fixative by a small syringe into bronchioles as described by Dail and Hammar (1994). Histological slides of 4–5 μm sections prepared for diagnostic purposes were stained with hematoxylin and eosin (H&E), Giemsa, Verhoeff, van Gieson, periodic acid-Schiff alcian blue, and periodic acid-Schiff stains; staining for acid-fast bacilli and *Pneumocystis carinii* was done in selected cases. In part of the cases, 1–2mm³ pieces of the tissue were processed for electron microscopy (EM) for primary diagnostic purposes. Part of the tissue material was used for microbiological analysis for *Mycobacterium tuberculosis*, other bacteria and fungi. All these cultures were negative.

After re-evaluating the histological slides, 47 patients (16 women and 31 men) were selected and classified into two groups: those fulfilling the histological and clinical criteria for BOOP (n=17), and those showing UIP with fibroblast foci in distal air spaces (n=30). The diagnosis of all cases was based on a light microscopic evaluation using the histological criteria presented by Katzenstein (1997). The UIP cases were selected from a much larger cohort of UIP cases by the presence of several fibroblast foci, which may be few in advanced cases of UIP (Katzenstein and Myers 1988). In addition to the histological diagnosis, we also confirmed that the clinical data, the pulmonary function tests, the bronchoalveolar lavage findings, the chest X-ray and high-resolution computed tomography (HRCT) findings, and the follow-up information obtained from the hospital patient records were consistent with the diagnoses. Two UIP patients selected for study I had a connective tissue disease, and these cases were excluded from further studies. Two

patients had BOOP suspected to be caused by *Chlamydia pneumoniae*; in all the other cases, no etiological agent was found. Thus, the majority of the cases represent idiopathic BOOP and UIP.

4.2 Methods

4.2.1 Immunohistochemistry for paraffin sections (I–IV)

The proteins and their respective antibodies used in these studies are listed in Table 4. The staining procedures for VEGF (C-1) and Flk-1 were done using the Histostain-Plus bulk kit (Zymed, San Francisco, CA, USA) according to the instructions provided by the manufacturer. For all the other stainings, the avidin-biotin-complex kit (ABC-kit; I: Dako, Carpinteria, CA, USA; II, III and IV: Dakopatts, Copenhagen, Denmark) was used according to the manufacturer's instructions. Briefly, adjacent 4 or 5 μm thick sections of the lung tissue were first deparaffinized in xylene and rehydrated in graded ethanol. After this, the antibodies requiring an antigen retrieval step were processed as shown in Table 4. The quenching of endogenous peroxidase activity was performed for all specimens in 0.1 % hydrogen peroxide diluted in methanol (H_2O for laminin-5 $\gamma 2$ chain immunohistochemistry), and non-specific binding was blocked by incubating them in 20 % fetal calf serum diluted in phosphate buffered saline (PBS). The incubation times used for different antibodies are shown in Table 4. All specimens were then overlaid with the suitable secondary antibodies followed by Histostain-Plus or ABC-kit. Diaminobenzidine was used for color reaction, and methyl green or hematoxylin was used for counterstaining. All steps were followed by washes with PBS. Negative controls for all immunostainings were obtained by substituting the primary antibody with PBS.

4.2.2 Immunohistochemistry for frozen sections (Mab 6C12) (IV)

4 μm sections were fixed in acetone in -20°C for 10 min. Non-specific binding was blocked with 1 % bovine serum albumin (Roche, IN, USA) in PBS for 30 min at RT. After incubation with primary antibody to monoclonal rabbit anti-human laminin-5 $\gamma 2$ chain (Mab 6C12; 27 $\mu\text{g}/\text{ml}$) at 37°C for 1 h, secondary anti-mouse IgG antibody with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, CA, USA) was added according to the instructions of the manufacturer. The process was completed in a similar manner as the process for paraffin sections, and every step was followed by washes with PBS.

Table 4. Antigens and their respective antibodies used in the studies.

Antigen	Antibody	Dilution, incubation time and possible antigen retrieval treatment	Source
7S domain of human type IV collagen	rabbit anti-human 7S	1:50, overnight at 4°C, pepsin at 37°C 30 min	gift from Dr Juha Risteli
P1 fragment of human laminin	rabbit anti-human P1	1:25, overnight at 4°C, pepsin at 37° 30 min	gift from Dr Juha Risteli
CD34	mouse monoclonal anti-human	1:25, 30 min at RT, 0.4 % pepsin at 37°C 30 min	Novocastra Laboratories, Newcastle upon Tyne, UK
von Willebrand factor (FVIII)	rabbit polyclonal anti-human	1:250, 30 min at RT, 0.4 % pepsin at 37°C 30 min	Dako, Glostrup, Denmark
Bcl-2	mouse monoclonal anti-human bcl-2 (clone 124)	1:50, overnight at RT, CB 5 min	Dako, Glostrup, Denmark
Bax	rabbit polyclonal anti-human bax	1:1000, overnight at RT, CB 5 min	Pharming, San Diego, CA, USA
Mcl-1	rabbit polyclonal anti-human mcl-1	1:1500, overnight at RT, CB 5 min	Pharming, San Diego, CA, USA
VEGF (A-20), splice variants 121, 165, 189	rabbit polyclonal anti-human	1:25, overnight at RT, CB 2 + 10 min	Santa Cruz Biotechnology, Santa Cruz, CA, USA
VEGF (C-1), amino acids 1–140	mouse monoclonal anti-human	1:250, overnight at RT, 0.4 % pepsin at 37°C 30 min	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Flt-1	rabbit polyclonal anti-human	1:100, overnight at RT, CB 2 + 10 min	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Flk-1	mouse monoclonal anti-human	1:250, overnight at RT, CB 2 + 10 min	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Laminin-5 γ 2 (anti-LAM γ 2-III)	rabbit polyclonal anti-human	14 μ g/ml, overnight at 4°	gift from Sirpa Salo, PhD
Laminin-5 γ 2 (Mab 6C12)	rabbit monoclonal anti-human	27 μ g/ml, 1 h at 37°C	gift from Sirpa Salo, PhD
cytokeratin (clone MNF 116)	mouse monoclonal anti-human	1:100, 1 h at RT	Dako, Glostrup, Denmark

Abbreviations: RT= room temperature. CB 5 min = citrate buffer (pH 6) treatment in microwave oven, incubating at room temperature for 20 minutes after the microwave. CB 2 + 10 min = citrate buffer (pH 6) treatment in microwave oven (2 min with 850 W and 10 min with 300 W), incubating at room temperature for 20 minutes after the microwave

4.2.3 Assessment of vascular density in newly formed intraluminal connective tissue (I)

During the analysis, the division of the newly formed intraluminal connective tissue into intraluminal buds, obliterative fibrosis, and mural incorporating fibrosis as described by Basset *et al.* (1986) was followed, and only the intraluminal buds of young connective

tissue were recorded. The number of the cross sections of the capillaries per square millimeter of the newly formed connective tissue was counted from the slides stained for laminin, von Willebrand factor, and CD34. Depending on the slide, between 5 and 28 (average, 11) lesions were counted per slide. The area of each lesion was calculated using an ocular micrometer, with which two dimensions of each lesion were measured. Only capillary structures with a lumen were accepted. Intraluminal fibrous lesions reminiscent of collapsed alveoli were excluded.

One observer (E.L.-B.) counted all specimens (I). This was followed by determining the possible intraobserver and interobserver variations by recounting all of the specimens stained for laminin independently by two observers (E.L.-B. and R.K.-W.) 6 months later (I).

4.2.4 Analysis of the results of the immunostainings (II–IV)

The results were evaluated on the basis of both quantitative and qualitative immunostainings as follows: – = negative, + = weakly positive, ++ = moderately positive, and +++ = strongly positive. Only intraluminal connective tissue lesions described by Basset *et al.* (1986) were evaluated. The positively stained cells within the newly formed intraluminal connective tissue lesions were counted for VEGFs, bFGF, Flt-1, Flk-1, bcl-2, mcl-1, and bax (II and III). For the evaluation of VEGFs and bFGF, the number of positively stained cells is given as a percentage of the total number of the cells, and epithelial cells covering the intraluminal lesions were excluded (II). In cases of BOOP, we counted 420 fields with an average of 97 cells per field, and on average, the total number of evaluated cells per slide was 1,041 in BOOP. In cases of UIP, a total of 325 fields were evaluated with an average of 30 cells per field. In UIP, the total number of cells evaluated per slide was on average 255 (II). For bcl-2, mcl-1 and bax, the number of positively stained mesenchymal cells as a percentage of the total number of mesenchymal cells was evaluated (III). The extent of re-epithelialization of the intraluminal connective tissue lesions was evaluated based on cytokeratin-staining (IV). The extent of re-epithelialization was given as a percentage of surface area of intraluminal connective tissue lesions covered with cytokeratin-positive cells. Expression of the laminin-5 γ 2 chain was then identified in the same lesions from adjacent sections, and correspondingly, the extent of the laminin γ 2 chain was analyzed as a percentage of the surface area of intraluminal connective tissue lesions covered with laminin-5 γ 2 chain positive cells. Based on these results, a percentage of laminin-5 γ 2 chain positive epithelial cells was counted for both BOOP and UIP. In cases with BOOP, a total of 289 intraluminal fibromyxoid lesions was evaluated, and a total of 134 fibromyxoid lesions was evaluated in UIP. As a control, part of the material was evaluated using an ocular micrometer. The evaluation of the extent of re-epithelialization based on ocular micrometer measurements and routine microscopic evaluations gave repeatable results, Pearson's correlation coefficient being 0.9 ($p < 0.001$) (IV).

For VEGFs, Flk-1, Flt-1 and bFGF, a skin biopsy with a healing wound was used as a positive control (II). As a positive control for bcl-2, mcl-1, and bax immunostaining, a lymph node with follicular hyperplasia was used (III). Uninvolved peripheral lung tissue obtained from patients operated on for a malignant lung tumor was also used as a control in studies II and IV.

4.2.5 3'-end labeling of DNA in apoptotic cells and assessment of the apoptotic index (III)

To detect apoptotic cells, TUNEL (i.e. *in situ* labeling of the 3'-end of the DNA fragments generated by apoptosis-associated endonucleases) was used. The 3'-end labeling of DNA was performed with the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA) following the instructions of the manufacturer. After dewaxing and rehydration, the sections were incubated with 20 µg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature for 15 minutes. Endogenous peroxidase activity was quenched by 2 % hydrogen peroxide in phosphate buffered saline (PBS), pH 7.2. Terminal transferase enzyme was used to catalyze the addition of digoxigenin labeled nucleotides to the 3'-OH ends of the fragmented DNA. The reaction was stopped after 60 minutes with a buffer provided by the manufacturer. Thereafter, antidigoxigenin-peroxidase solution was applied on the specimens. The slides were lightly counterstained with methyl green. For control purposes we used tissue sections from hyperplastic lymph nodes showing an increased number of apoptotic B lymphocytes within germinal centers, whereas the interfollicular T-lymphocytes showed no or only minimal apoptotic activity.

Cells were defined as apoptotic if the whole nuclear area of a cell, or the peripheral ring of the nuclei as a sign of the early stage of apoptosis, labeled positively. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the cells. In cases in which several apoptotic bodies were found in a group, but clearly located within one cell, the group of apoptotic bodies was counted as one. To estimate the percentage of apoptotic events in a given area (i.e. the apoptotic index) the number of cells of the intra-alveolar connective tissue lesions was also calculated. In cases of BOOP, an average of 26 fields per case in which the total number of cells varied from 40 to 800 cells per field were counted. The total number of evaluated cells per case in BOOP was thus on average 4,860. In cases with UIP, an average of 11 fields per case were evaluated, with a total number of cells from 50 to 300 per field. The total number of evaluated cells per case in UIP was on average 950. The number of apoptotic cells is given as a percentage of the total cell number.

4.2.6 *In situ* hybridization for laminin-5 γ 2 chain mRNA (IV)

For the laminin-5 γ 2 chain, a PstI-EcoRI fragment of clone L15 (bases 2995–3840) was subcloned into pSP64 and pSP65 vectors in the sense and antisense orientation (Kallunki *et al.* 1992). ^{35}S -UTP-labeled antisense and sense RNAs were obtained by means of Sp6 RNA polymerase, using an *in vitro* transcription kit (Promega, Madison, WI, USA). The probe in the sense orientation served as a control for non-specific hybridization.

For the preparation of tissue sections for *in situ* hybridization, 4 μm thick sections from paraffin-embedded lung biopsies were collected on clean SuperFrost Plus glass slides (Erie Company, Portsmouth, NH, USA). Paraffin was removed from the sections with xylene and the slides were dehydrated. Then the sections were treated with 0.2 M HCl for 20 min at RT and washed in DEPC- H_2O for 5 min. For proteolysis the sections were incubated with proteinase K (1mg/ml) (Roche Diagnostics, Indianapolis, USA) for 30 min at 37°C and the reaction was stopped with 0.2% glycine in DEPC-treated phosphate buffered saline (DEPC-PBS). After that the sections were washed twice in DEPC-PBS for 30 sec. The sections were fixed with 4% paraformaldehyde in PBS for 20 min and washed in PBS. Acetylation was done in 0.25% acetic anhydride in 0.1 M trietanolamine (pH 8) for 10 min. The sections were washed in DEPC-PBS, dehydrated and air-dried overnight at RT.

For prehybridization the sections were then treated for 2 h in a moistured incubation chamber with a mixture containing 10 mM dithiothreitol (DTT) (Sigma, St. Louis, MO, USA), 10 mM Tris-HCl, 10 mM NaPO_4 , 5mM EDTA, 0.3 M NaCl, 1 mg/ml yeast tRNA, deionized formamide 50% and dextran sulphate 10% (w/v), 0.02% (w/v) Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.02% (w/v) polyvinylpyrrolidone, and 0.02 mg/ml bovine serum albumin (BSA), and washed twice in PBS and dehydrated. In the hybridization step the probes were first denatured by boiling for 1 min and placed on ice. 1.2×10^5 dpm of the ^{35}S -labeled antisense or sense probe in prehybridization buffer was applied on each section and the hybridization was carried out at +50°C overnight. The posthybridization washes were performed as follows: twice at + 50°C for 1 h in prehybridization mixture except for dextran sulphate and tRNA, 15 min in 0.5 M NaCl/TE (NaCl with EDTA and Tris-HCl) at +37°C, 30 min incubation in 0.5 M NaCl/TE containing 40 $\mu\text{g}/\text{ml}$ RNAase A (Sigma) at +37°C, 15 min in 0.5 M NaCl/TE at + 37°C, once for 15 min in 2 x standard saline citrate (SSC) and twice for 15 min 1 x SSC, both at 50°C. The sections were dehydrated in ethanol containing 300 mM ammonium acetate and air-dried at RT for 1 h.

In autoradiography the slides were dipped into NTB-2 film emulsion (Kodak, New York, USA) and then placed in light-tight boxes for 10–14 days. The slides were developed with D-19 (Kodak), fixed in Unifix (Kodak) and counterstained in hematoxylin and eosin. All the solutions were treated with 0.1 % depc solution (Fluka, Buch, Switzerland), and corresponding sense probes were always used as negative controls.

The hybridized tissue sections of lung biopsies were examined by light microscopy, and the number of grains over the cells was evaluated in general, and particularly in locations where laminin-5 γ 2 chain immunoreactivity was located. Cells hybridized with

the ^{35}S -labeled antisense laminin-5 $\gamma 2$ probe were considered positive if they contained more grains than the corresponding cells that had been hybridized with the ^{35}S -labeled sense laminin-5 $\gamma 2$ chain probe.

4.2.7 Immunoelectron microscopy of laminin-5 $\gamma 2$ chain (IV)

Immuno-EM was used for studying the distribution of laminin-5 $\gamma 2$ chain in two cases of UIP and in one tissue sample from a normal bronchus. Fresh samples from a bronchus and lung tissue were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer with 2.5 % sucrose, pH 7.4, for 2 hours, immersed in 2.3 M sucrose and frozen in liquid nitrogen. Thin cryosections were cut with Leica Ultracut UCT microtome. For the immunolabeling, the sections were first incubated in 0.05 M glycine in PBS followed by incubation in 5% BSA with 0.1% CWFS (cold water fish skin) gelatin (Aurion, Wageningen, The Netherlands) in PBS. Antibodies and gold conjugate were diluted in 0.1 % BSA-C (Aurion) in PBS. All washings were performed in 0.1% BSA-C in PBS. Sections were then incubated with polyclonal antibody to human laminin-5 $\gamma 2$ chain (14 $\mu\text{g}/\text{ml}$) for 60 min followed by protein A-gold complex (size 10 nm) for 30 min, made after Slot and Geuze (1985). The controls were prepared by carrying out the labeling procedure without primary antibody. The sections were embedded in methylcellulose and examined in Philips CM100 transmission electron microscope.

4.2.8 Statistical analyses

All analyses were performed using SPSS software (Chicago, IL). Data are presented as mean \pm standard deviation (SD). The significance of the association was determined using Mann-Whitney U test or the *t*-test. Intraobserver and interobserver variations as well as correlations between different variables were determined using the correlation analysis of Pearson. P-values less than 0.05 were considered statistically significant.

5 Results

5.1 Capillarization in the newly formed connective tissue (I)

5.1.1 *General findings*

Well-formed and vital-looking capillaries with a diameter of 8 to 16 μm and with tubular configurations within intraluminal lesions were a common finding in BOOP. The number of the capillaries within the lesions varied from a few capillaries to the histological picture of granulation tissue with abundant capillarization. The capillarization also seemed to be an early event and took place already when only a fibrin clot consisting of a few cells had been formed. In contrast to this, there were hardly any well-capillarized intraluminal connective tissue lesions in UIP. In a few cases of UIP, well-formed capillaries were seen in the middle of the connective tissue lesions, but usually there were only short capillaries peripherally in the stalk of an intraluminal lesion close to the interstitium. In UIP, there were also numerous intraluminal lesions without any capillaries. Instead, in UIP there was an increased number of capillary structures in the areas of mural incorporating fibrosis.

The capillaries of the intraluminal lesions were randomly scattered without a connecting BM, unlike in the alveolar walls, where the BMs of the capillaries and epithelial cells formed a continuous linear staining pattern well demonstrated by the laminin and type IV collagen staining. In most BOOP cases, it was clearly demonstrated that the capillaries did not only run along the stalk into the fibrous lesion, but there were many capillary protrusions from the surrounding alveolar walls into the lesion as well. Sometimes the capillaries heading toward the center of the lesion formed an asterisk-shaped figure.

In BOOP and in UIP, both plump and slender endothelial cells were seen inside the connective tissue lesions. The immunoreactivity of the endothelial cells was stronger for von Willebrand factor than for CD34. The BMs of the capillaries were of variable thickness. At light microscopic level, there were no differences in the size, configuration, endothelial cells, or BMs of the capillaries between BOOP and UIP.

5.1.2 Number of capillaries in newly formed connective tissue

For the statistical analysis, the number of the capillary cross sections per square millimeter was counted, and the results are shown in Table 5. For all the stainings, counts were higher in BOOP than in UIP, and statistically significant ($p < 0.003$ for laminin, <0.001 for von Willebrand factor, and <0.001 for CD34). All the stainings used in the study (laminin vs. von Willebrand factor; laminin vs. CD34, CD34 vs. von Willebrand factor) correlated with each other ($p < 0.001$ for all three correlations).

The intraobserver ($p < 0.001$) and interobserver correlations ($p < 0.002$) were highly significant.

Table 5. The number of capillary cross sections per square millimeter (no/mm^2) of the newly formed connective tissue in BOOP and in UIP.

Antigen	BOOP	UIP
Laminin	0–229 (mean 107, SD \pm 74)	0–43 (mean 14, SD \pm 15)
Von Willebrand factor	54–158 (mean 103, SD \pm 46)	0–43 (mean 11, SD \pm 14)
CD34	19–119 (mean 63, SD \pm 36)	0–16 (mean 6, SD \pm 6)

5.2 Immunohistochemical expression of VEGF and bFGF (II)

5.2.1 VEGF and bFGF expression in intraluminal connective tissue

The percentage of VEGF and bFGF positive cells of the total number of cells is shown in Table 6. The cells of the newly formed connective tissue were more strongly positive for both VEGF antibodies in BOOP than in UIP ($p < 0.001$ for VEGF (C-1) and <0.004 for VEGF (A-20)). The staining pattern with both antibodies was mostly intracytoplasmic and granular, and particularly strong positivity was found for VEGF (C-1) in BOOP. The positive reaction was often pronounced in the fusiform cells underneath the epithelium. There was a positive correlation between the two VEGF stainings, Pearson's correlation coefficient being 0.375 ($p < 0.05$).

The staining intensity of bFGF was mostly moderate and the location of the immunoreactivity was intracytoplasmic. The staining pattern in the intraluminal connective tissue lesions of BOOP and UIP varied greatly; some cells stained strongly, while others remained negative. Especially in BOOP there were cases in which fibroblasts remained negative, but the capillaries of the newly formed intraluminal connective tissue lesions stained positively. As a whole, expression of bFGF was wider in BOOP than in UIP ($p < 0.02$).

Table 6. The number of VEGF and bFGF positive cells as percentage of the whole cell number in the newly formed connective tissue in BOOP and in UIP.

Diagnosis	VEGF (C-1)	VEGF (A-20)	bFGF
BOOP	89 % (SD ±10)	60 % (SD ±25)	59 % (SD ±24)
UIP	28 % (SD ±20)	28 % (SD ±22)	36 % (SD ±22)

5.2.2 VEGF and bFGF expression outside the intraluminal connective tissue

The staining pattern of VEGF is shown in table 7. For bFGF, the localization of the immunohistochemical expression was parallel to the expression of VEGF, but as a whole, the staining intensity was somewhat stronger. The strongest expression for bFGF was seen in the basal cells of the bronchioles.

Table 7. Staining pattern of VEGF in BOOP and in UIP.

Extent of localization	Diagnosis	
	BOOP	UIP
Inflammatory cells	++ in macrophages and mast-cells (+) in lymphocytes and plasma cells	(+) in macrophages (+) in lymphocytes and plasma cells
Normal pneumocytes	(+)	(+)
Bronchiolar epithelium	+	+
Regenerating type II pneumocytes	+++	+ / +++
Metaplastic epithelium of squamous or bronchiolar type		+ / +++
Endothelial cells	(+)	(+)
Smooth muscle cells	(+)	(+)
Interstitial	-	++ in incorporating fibrosis
Fibroblasts/myofibroblast of newly formed connective tissue	+++	+

Explanation: () = focal staining

5.3 Immunohistochemical expression of Flt-1 and Flk-1 (II)

5.3.1 Flt-1 and Flk-1 expression in intraluminal connective tissue lesions

Wide positivity was found for Flt-1 and Flk-1 in the intraluminal connective tissue lesions both in BOOP and in UIP, although some of the lesions remained negative in both diseases. In BOOP, some of the newly formed capillaries of the intraluminal connective tissue lesions were visualized by Flt-1 and Flk-1. The Flt-1 and Flk-1 positive staining often localized into fusiform cells underneath the epithelium. We were not able to find any significant difference between BOOP and UIP in the percentage of Flt-1 and Flk-1 positive cells in the intraluminal connective tissue.

5.3.2 Flt-1 and Flk-1 expression outside intraluminal connective tissue

Flt-1 and Flk-1 were constantly positive in the vascular endothelium. There was moderate or strong positivity in regenerating type II pneumocytes, metaplastic bronchiolar- or squamous-type epithelium, and normal bronchiolar epithelium. There was occasional Flt-1 and Flk-1 positivity in normal alveolar pneumocytes. Alveolar macrophages were also widely positive for Flt-1, but the staining for Flk-1 was weak and occasional. In UIP, the incorporating fibrosis showed positivity for Flt-1 and Flk-1. Some of the smooth muscle cells were positive for Flt-1 and Flk-1. The staining pattern was exceptionally strong for Flk-1 in randomly scattered interstitial cells, which showed the morphology and distribution of mast cells.

5.4 Apoptotic index (III)

The mean apoptotic index of the intraluminal connective tissue lesions in BOOP varied from 0.32 to 2.13 % (mean, 0.70 %; SD, ± 0.51 %), and of those in UIP from 0 to 0.34 % (mean, 0.13%; SD ± 0.14 %; $p < 0.003$ by Mann-Whitney U test). Apoptotic bodies were easily recognized. Some of the apoptotic cells had preserved their shape and were easily identified as fibroblasts/myofibroblasts, endothelial cells, or pneumocytes. Apoptotic inflammatory cells were partly identified on the basis of their location among an inflammatory infiltrate. Some of the apoptotic cells had contracted and shrunk so that cell type could not be identified.

5.5 Immunohistochemistry for bcl-2 family proteins (III)

Staining of the bcl-2 family proteins used in the study is shown in Table 8. Briefly, all stainings were positively expressed in epithelia and mostly negative in the normal interstitium and remodeled interstitium in UIP. Also, all stainings were positive in the newly formed connective tissue, but to varying degrees. Bcl-2 showed faint immunoreactivity in 0–4 % of the mesenchymal cells of the newly formed connective tissue. The staining patterns for mcl-1 and bax were more widespread and more intense. No statistical difference was found between BOOP and UIP.

Table 8. Staining for bcl-2 family proteins in BOOP and UIP used in the study. No statistical difference was found between BOOP and UIP and therefore the staining patterns for BOOP and UIP were combined.

Protein	Localization and intensity of the staining						
	Inflammatory cells	Normal alveolar pneumocytes	Regenerating pneumocytes type II	Bronchiolar epithelium	Metaplastic epithelium of UIP	Interstitialium	Fibro/myofibroblasts of newly formed connective tissue
bcl-2	+++ in lymphocytes	–	(+/+++)	(+/+++)	+	(+) in UIP	(+)
mcl-1	+++ in macrophages	–	+++	++	+++	–	+
bax	+++ in macrophages	(+) in type II pneumocytes	+++	++	+++	–	+ / ++

Explanation: () = focal staining

5.6 Immunohistochemical expression of cytokeratin (clone MNF 116): re-epithelialization (IV)

As expected, cytokeratin-staining was strongly positive in all epithelial cells; i.e. in normal type I and type II pneumocytes and bronchiolar epithelial cells, regenerating type II pneumocytes seen both in BOOP and UIP, and in metaplastic squamous- and bronchiolar-type epithelium seen in UIP. Based on the amount of cytokeratin-positive cells covering the intraluminal connective tissue lesions, the extent of re-epithelialization was higher in BOOP when compared to UIP. The mean extent of re-epithelialization of intraluminal connective tissue lesions was significantly higher in BOOP (78 %; SD \pm 27 %) than in UIP (54 %; SD \pm 23 %; $p < 0.016$).

In BOOP, regenerating type II epithelial cells were usually morphologically uniform, and sometimes had a flat shape reminiscent of type I pneumocytes. In BOOP, disarray of epithelial cells was slight or non-existent, and exfoliation of epithelial cells positive for both cytokeratin and laminin-5 $\gamma 2$ chain were seen in two cases out of 15. In UIP, re-epithelialization was more heterogenous, poorly and well epithelialized intraluminal

lesions appearing simultaneously. In UIP, a remarkable finding was the morphological variation of epithelial cells. The finding was well characterized with the stainings both for cytokeratin and the laminin-5 γ 2 chain. There was variation in cell size and shape as well as in the morphology of nuclei. Some of the epithelial cells appeared swollen with vacuolated cytoplasm suggestive of degenerative changes. The epithelium showed disordered layering, and piling of epithelial cells was sometimes seen at the leading edge of the migrating epithelium. Also, exfoliation of laminin-5 γ 2 chain-positive epithelial cells into the alveolar lumen was seen in 11 cases out of 15 in UIP. The mural incorporating lesions were widely or totally re-epithelialized.

5.7 Immunohistochemical expression of laminin γ 2 chain (IV)

Wide positivity for laminin-5 γ 2 chain in paraffin sections (anti-LAM γ 2-III) was found both in BOOP and in UIP. The strictly epithelial location of laminin-5 γ 2 chain immunohistochemistry was clearly defined with adjacent sections stained with cytokeratin. The staining pattern was mostly intracytoplasmic with occasional positive staining along BMs. Staining intensity was mostly moderate or strong, and the staining pattern was diffuse or slightly granular. In normal controls there was weak positivity along BMs.

In BOOP, 73 % (SD \pm 31 %) of regenerating type II epithelial cells covering intraluminal connective tissue lesions were positive for laminin-5 γ 2 chain. Correspondingly, in UIP positivity was seen in 90 % (SD \pm 13 %) of regenerating type II pneumocytes. No statistical difference was seen in the number of laminin-5 γ 2 chain positive type II pneumocytes between BOOP and UIP.

In both diseases, positivity was also seen in nearby alveolar walls lined by regenerating type II pneumocytes. In UIP, wide positivity was also found in remodeled air spaces lined by regenerating type II pneumocytes or metaplastic squamous-type epithelium. The metaplastic bronchiolar-type epithelium of UIP usually stained negatively, or there was only a faint string-like staining pattern along BMs. Bronchiolar epithelial cells stained negatively except for a few minor foci of basal cells in terminal bronchioli containing loose connective tissue in BOOP. In some bronchioles, there was a strong string-like staining pattern along the BM. In most bronchioles and in normal alveoli, the staining pattern along BMs was similar to that in normal controls, showing a faint string-like staining pattern along BMs of bronchioles and alveoli. Frozen section material (Mab 6C12) also demonstrated staining pattern of laminin-5 γ 2 chain along BMs of normal alveoli and bronchioli. No intraluminal connective tissue lesions were seen in frozen sections.

5.8 *In situ* hybridization for laminin-5 γ 2 chain mRNA (IV)

Positive signals for the laminin-5 γ 2 chain mRNA were observed in 12 cases out of 14 in BOOP, and in 10 cases out of 15 in UIP. One BOOP case was excluded for not being representative. The positive signals located on the surfaces of the intraluminal connective tissue at leading edge of the migrating epithelium. There were usually only few positive cells in an intraluminal lesion. In BOOP, positive signals were seen only in intraluminal connective tissue lesions. In UIP, a few positive signals were also found in areas of metaplastic epithelium.

5.9 Immunoelectron microscopy of laminin-5 γ 2 chain (IV)

In a normal bronchus, labeling for laminin-5 γ 2 chain was seen at the outer zone of the lamina densa without any definite intracytoplasmic labeling. In UIP, labeling for laminin-5 γ 2 chain was seen both at the inner and outer zone of lamina densa in type I and II pneumocytes. The labeling was somewhat stronger in BMs of type I pneumocytes when compared to BMs of regenerating type II pneumocytes. No typical hemidesmosomes or definite intracytoplasmic labeling was observed in alveolar epithelial cells.

6 Discussion

6.1 Capillarization of newly formed intraluminal connective tissue in BOOP and UIP

The present study shows that there is clear capillary formation within the newly formed intraluminal connective tissue in BOOP resembling granulation tissue. Moreover, the capillarization seems to be an early phenomenon, taking place already when only a fibrin cloth with just a few cells has been formed. In UIP, the capillarization of the fibroblast foci was sparse whereas capillarization was pronounced in the areas of mural incorporating fibrosis, suggesting a delayed capillary response. The results are in line with the study of Fukuda *et al.* (1998), which is the only other study comparing vascularization of the newly formed connective tissue in BOOP and UIP. Fukuda *et al.* observed that in BOOP there are newly formed capillaries with well developed BMs positive for collagen VII, but in UIP capillaries were not found.

The capillary structures could be sufficiently demonstrated only by special stainings showing either the capillary BMs or the endothelial cells. It is difficult to identify capillaries from the newly formed connective tissue intermingled with variable amounts of inflammatory cells with HE-staining alone. The number of the capillaries also sometimes varied considerably in adjacent sections, which probably correlates with the winding structure of newly formed vessels. These aspects may partly explain why little attention has been paid on the capillarization of the newly formed intraluminal connective tissue. Special stainings and adjacent sections are needed for revealing capillaries in this kind of tissue.

It has been ultrastructurally proved that endothelial injury of different stages occurs at sites of lung injury (Bachofen & Weibel 1974, Adamson & Bowden 1976, Coalson 1982, Myers & Katzenstein 1998b), and endothelial cells have also been shown to be able to regenerate after lung injury (Adamson & Bowden 1974, Coalson 1982). In addition, endothelial cell injury is apparently necessary for the induction of angiogenic growth factors (Maitre *et al.* 2001). In BOOP, the capillaries of the newly formed connective tissue seemed to originate both from the stalk and from the surrounding alveolar walls, suggesting wide regeneration potential, and thus maybe also wide injury of endothelia.

There are not enough data to compare the stage and extent of endothelial injury in BOOP and UIP. However, it is possible that there is a difference in the quantity or quality of endothelial cell and endothelial BM injury in these diseases related to the different regeneration potential of endothelial cells. It is also possible that there are anastomosing capillaries from bronchial circulation in BOOP (Turner-Warwick 1963, Bignon *et al.* 1974).

6.1.1 Expression of VEGF, bFGF and receptors of VEGF in newly formed intraluminal connective tissue in BOOP and UIP

VEGF and bFGF were expressed both in BOOP and in UIP, which is in agreement with earlier studies upon acute lung injury in humans and pulmonary fibrosis in animal models (Henke *et al.* 1991, Liebler *et al.* 1997, Fehrenbach *et al.* 1999a). The present study shows that VEGF is probably an important growth factor involved in the pathogenesis of idiopathic interstitial pneumonias. VEGF and bFGF were both more widely expressed in the intraluminal connective tissue lesions in BOOP than in UIP. This is in line with the results of the extent of capillarization in the newly formed connective tissue in BOOP and in UIP, and strongly suggests that both of these growth factors are important stimulators of angiogenesis in the newly formed intraluminal connective tissue in BOOP. The results can be compared to the phase of capillary proliferation of granulation tissue in cutaneous wound healing, in which both bFGF and VEGF are needed for the angiogenic stimulus (Nissen *et al.* 1998). On the other hand, in UIP the delayed capillarization at the initial site of the lung injury is followed by expression of VEGF and bFGF and capillarization in the areas of mural incorporating fibrosis. This may correspond to the development of irreversible fibrosis, providing sufficient vasculature to the remodeled interstitium.

In recent studies there are interesting observations concerning the role of VEGF in lung injury and fibroproliferative response. Preterm babies who developed bronchopulmonary dysplasia (BPD) had lower VEGF concentrations in tracheal aspirate samples than did those surviving without BPD (Lassus *et al.* 1999). Also, patients with UIP had lower VEGF concentrations in BALF compared with normal volunteers or patients with cystic fibrosis or sarcoidosis (Meyer *et al.* 2000, Koyama *et al.* 2002). Moreover, VEGF was decreased in BALF from patients with ARDS, which is a disease also characterized by a fibroproliferative response (Maitre *et al.* 2001). When summarized, it seems that VEGF has a most important role in pulmonary repair after lung injury, and is needed for the normal regeneration of alveoli. The exact role of VEGF in the pathogenesis of intraluminal connective tissue remains to be determined, however. According to our study it seems possible that VEGF is needed for the quick capillary response and angiogenesis in order to devoid an exaggerated fibroproliferative response. It is also possible that VEGF levels correlate with the extent of alveolar wall injury, and thus sufficient capillarization is one manifestation of good overall regeneration potential, paralleling controlled regulation of mesenchymal cells.

The cell-specific localization and immunohistochemical staining pattern of VEGF and bFGF correspond well to previous studies (Cordon-Cardo *et al.* 1990, Henke *et al.* 1991, Uchida *et al.* 1994, Iijima *et al.* 1996, Sannes *et al.* 1996, Jackson *et al.* 1997, Liebler *et al.* 1997, Decaussin *et al.* 1999, Fehrenbach *et al.* 1999a,b). The results suggest that the strong positive staining pattern of epithelial cells and fibroblasts/myofibroblasts is a sign of synthesis or storage of VEGF and bFGF in these cells. The bioactive forms of VEGF and bFGF are found both in ECM and inside cells (Li & Keller 2000, reviewed by Robinson & Stringer 2001, and Powers *et al.* 2000), but no extracellular staining was found in the present study. This may be because no proteolytic enzymes were used for cleavage of the growth factors from ECM. Also, the concentrations of the growth factors in ECM may be too small to be detected by immunohistochemistry, or the small extracellular concentrations may be lost during tissue processing.

A difference in the expression of VEGF compared with earlier studies was observed in the immunostaining of endothelial and smooth muscle cells. In pulmonary artery of the human fetus and in arterioles and bronchioles of normal adult human lung, VEGF expression has been localized in smooth muscle cells without any reaction in vascular endothelial cells (Shifren *et al.* 1994, Fehrenbach *et al.* 1999a). In the present study, there was VEGF immunoreactivity in endothelial cells, whereas there was only occasional positivity in the vascular or bronchiolar smooth muscle cells. Hypoxia stimulates expression of VEGF in epithelial cells (Christou *et al.* 1998) and endothelial cells (Liu *et al.* 1995, Namiki *et al.* 1995), and it is possible that the widely upregulated expression of VEGF in epithelial and endothelial cells has caused a negative feedback to nearby smooth muscle cells of blood vessels and bronchioles. It is also possible that the concentrations of VEGF in smooth muscle cells, as also in normal pneumocytes, are too small to be detected by our immunohistochemical procedures.

Changes in the vascular permeability mediated by VEGF may be of great significance in the pathogenesis of fibrous pulmonary disorders. The physiological role of VEGF is related to the regulation of vascular permeability (Senger *et al.* 1983). It has been shown that normal human ELF contains high concentrations of VEGF (Kaner & Crystal 2001). In an animal model, overexpression of VEGF induced pulmonary edema (Kaner *et al.* 2000). In chronic inflammation the development of edema may also be mediated by local migration of VEGF-positive plasma cells (Ito *et al.* 1995). Thus, it seems that both an injury of the alveolar epithelial barrier and migration of inflammatory cells can contribute to the changes in vascular permeability and leakage of plasma into the alveolar space in acute lung injury. Increased vascular permeability is needed for transfer of macromolecules and formation of fibrin gel matrix for proliferating capillaries (Nehls & Herrmann 1995). Prolonged or uncontrolled increase in permeability of capillaries can, however, lead to pulmonary edema inhibiting physiological gas exchange and repair process.

Besides induction of angiogenesis and granulation tissue, bFGF may also have other functions in BOOP and UIP. In fact, bFGF participates in many processes relevant to the pathogenesis of lung fibrosis, such as stimulation of fibroblast proliferation (Henke *et al.* 1993), collagen synthesis and collagenolytic activity (McGee *et al.* 1988, Kennedy *et al.* 1995), and proliferation of alveolar type II cells (Leslie *et al.* 1990, Li *et al.* 2001). Interestingly, bFGF-stimulated fibroblasts are more sensitive to the inhibitory effect of captopril than are unstimulated cells (Nguyen *et al.* 1994), which also indicates that bFGF

modulates fibroblast proliferation and phenotype. Whether the net effect of bFGF to the fibroproliferative response is promoting or degrading probably depends on temporal and spatial signals from other growth factors, epithelial cells, BMs and ECM components.

Both Flt-1 and Flk-1 were expressed in BOOP and in UIP. Originally, Flt-1 and Flk-1 were thought to be endothelial cell-specific. Subsequently, however, trophoblast cells, monocytes, renal mesangial and tubular cells, hematopoietic stem cells, megakaryocytes, retinal progenitor cells, pancreatic duct cells, non-small cell lung carcinoma cells, lung fibroblasts and muscle cells have also been shown to express one or both of these receptors (reviewed by Neufeld *et al.* 1999, Öberg *et al.* 1994, Decaussin *et al.* 1999, Kanellis *et al.* 2000, Ishida *et al.* 2001, Rissanen *et al.* 2002). In the present study, both receptors were expressed in the vascular endothelium and in addition, positivity was seen in the normal and metaplastic epithelium and in fibroblasts/myofibroblasts. The results support our theory of the importance of VEGF for alveolar regeneration after lung injury.

The co-expression of VEGF and its receptors in the same cell types suggests an autocrine growth factor effect of VEGF on these cells. The possibility of an autocrine regulatory mechanism for endothelial cells has been suggested in a previous study (Namiki *et al.* 1995). An interesting finding was the co-expression of VEGF and Flt-1 and Flk-1 in fibroblasts/myofibroblasts, a finding also observed in fibroblasts in non-small cell lung carcinomas (Decaussin *et al.* 1999). It remains to be determined whether this is linked to regulation of angiogenesis or fibrogenesis. In an animal model with bleomycin induced fibrosis, both VEGF and Flt-1 and Flk-1 were upregulated in fibrous areas without any sign of capillarization, suggesting that VEGF promotes fibrosis (Fehrenbach *et al.* 1999a,b). Indeed, VEGF can induce expression of connective tissue growth factor (CTGF) via Flt-1 and Flk-1 (Suzuma *et al.* 2000), and CTGF is known to promote fibrosis in IPF (Allen *et al.* 1999). Moreover, CTGF coding genes are upregulated in fibroblasts in systemic sclerosis (Shi-wen *et al.* 2000). However, it is also possible that the co-expression of VEGF and Flt-1 and Flk-1 in fibroblasts/myofibroblasts is a sign of an attempt to enhance synthesis of VEGF for angiogenesis. In this case there are evidently other regulating factors prohibiting formation of new capillaries in UIP. In chronic wounds with impaired healing response, expression of VEGF is elevated, but increased proteolytic activity results in its degradation (Lauer *et al.* 2000). This may also contribute to the prevention of capillarization in UIP.

6.2 Apoptotic activity in newly formed intraluminal connective tissue in BOOP and UIP

The present results demonstrate that apoptotic activity is higher among the cells of the newly formed connective tissue in BOOP when compared to those in UIP. All kind of cell types present in newly formed connective tissue lesions (i.e. fibroblasts/myofibroblasts, endothelial cells, inflammatory cells and epithelial cells) showed increased apoptotic activity. The results are in agreement with Polunovsky *et al.* (1993), and thus suggest that apoptosis plays an important role in the resolving process of the intraluminal connective

tissue in BOOP. In this sense the newly formed connective tissue in BOOP resembles granulation tissue of skin wound in which apoptosis mediates the decrease in cellularity (Desmoulière *et al.* 1995).

Apoptotic activity was also seen in the newly formed connective tissue in UIP, but to a remarkably lesser extent than in BOOP. In a former study the apoptotic activity of interstitial cells of normal lung (n=6) was reported to be very low, the apoptotic index being $0.11 \pm 0.03\%$ (Törmänen *et al.* 1995). Against this background, in UIP, the apoptotic activity of the interstitial cells of the newly formed connective tissue is about of the same level as that of normal lung interstitial cells, or can even be decreased, whereas the apoptotic activity of the newly formed connective tissue is clearly increased in BOOP. The persistent fibrosis in UIP can be partly due to a lowered apoptotic activity of cells of newly formed connective tissue, leading to a decreased resolution of the intraluminal connective tissue lesions. However, in order to better clarify the role of apoptosis in pulmonary fibrosis, studies combining apoptotic activity with proliferation activity of fibroblasts/myofibroblasts are needed.

There are studies pointing out that human lung fibroblasts from patients with an active and early stage of pulmonary fibrosis proliferate significantly faster than those obtained from normal lungs (Jordana *et al.* 1988, Raghu *et al.* 1988). The weakness of these studies is the heterogeneity of the diseases and thus also of the cell lines selected to the studies. However, these results and the results of the present study contrast with the observation of Ramos *et al.* (2001), who found that fibroblasts from UIP lungs in culture have a lower growth rate than fibroblasts from normal lungs, and exhibit spontaneous apoptosis. The present results of low apoptotic activity of cells of fibroblast foci are supported by the fact that transforming growth factor β_1 , which is one of the major profibrotic growth factors in UIP (Sime *et al.* 1997, Broekelmann *et al.* 1991, Coker *et al.* 2001), also inhibits myofibroblast apoptosis (Zhan & Phan 1999). The present study also focused on fibroblast foci, which are sites of active collagen synthesis (Kuhn *et al.* 1989) and thus presumably also proliferation of fibroblasts/myofibroblasts. On the other hand, the number of myofibroblasts is diminished in the areas of end stage fibrosis (Kapanci *et al.* 1995), which means that the apoptotic activity of myofibroblasts must increase at certain time point during the progression of fibrosis. So the opposite findings of different studies on proliferation rates and apoptotic activity of fibroblasts/myofibroblasts may be due to differences in sampled material and cell behavior *in vivo* and *in vitro*.

In BOOP, some of the apoptotic cells were characterized as lymphocytes. BOOP is a CD8-positive T-cell lymphocyte dominant disease (Nagai *et al.* 1992, Costabel *et al.* 1992b, Fukuda *et al.* 2001), and a pathogenetic role for cytotoxic T-lymphocytes expressing FasL is also suggested in BOOP (Kuwano *et al.* 2000). So the increased apoptotic activity of lymphocytes might be one disease-limiting mechanism in BOOP. Glucocorticoids are known to promote apoptosis of lymphocytes by activating endonuclease-like activity in lymphoid tissue (Compton & Cidlowski 1986), and the good response to corticosteroids in BOOP may also be partly explained by increased apoptosis of lymphocytes.

6.2.1 Expression of apoptosis regulating proteins *bcl-2*, *mcl-1* and *bax* in newly formed intraluminal connective tissue in BOOP and UIP

Compared with the current knowledge of the immunohistochemical expression of *bcl-2*, *mcl-1* and *bax* in normal lung (Krajewski *et al.* 1995, Guinee *et al.* 1997, Törmänen *et al.* 1999), all these proteins were upregulated in BOOP and in UIP. In a recent study, increase of *bcl-2* protein in BAL-fluid from patients with UIP was also observed (Mermigkis *et al.* 2001). However, in the present study, no correlation was found between the apoptotic events of the newly formed connective tissue and expression of apoptosis-regulating proteins, except the possible balance between *mcl-1* and *bax*. The expression of *bcl-2* family proteins did not explain the difference in the apoptotic index between the diseases, either.

The results of Guinee *et al.* on *bcl-2* and *bax* expression in diffuse alveolar damage (DAD) (1997) are very similar to the present results. They observed *bcl-2* positivity in about 10 % of the myofibroblasts while *bax* showed wider positivity, 20–100 % of the myofibroblasts staining positively. Considering the different clinical behavior of BOOP, DAD and UIP, it is possible that the expression of *bcl-2*, *mcl-1*, and *bax* is constant in the newly formed intraluminal connective tissue in lung injury despite the underlying disease, reflecting susceptibility of these cells to apoptosis. In this case there are other regulating factors determining whether the cell ends up in apoptosis or not. p21 and p53 are potent regulators of apoptosis of epithelial cells in pulmonary fibrosis (Kuwano *et al.* 1996, Guinee *et al.* 1996), and expression of p53 and p21 have also been observed in mesenchymal cells in diffuse alveolar damage, but to a lower extent than in epithelial cells (Guinee *et al.* 1996). Lovastatin has been shown to induce lung fibroblast apoptosis in cell cultures, but the mechanism remained unidentified (Tan *et al.* 1999). Soluble fibronectin peptides have also been shown to trigger lung fibroblast apoptosis in cell cultures by disruption of an integrin-mediated survival signaling pathway (Levrey Hadden & Henke 2000). There have been hopes that induction of lung fibroblast apoptosis might provide potential therapies for patients with UIP (Raghu *et al.* 1999, Ziesche *et al.* 1999). Modulation of activity of TGF- β 1 and CTGF might also provide tools for this (reviewed by Allen and Spiteri 2002).

The *bax* positivity of metaplastic type II pneumocytes is in line with the earlier observations of enhanced apoptosis of type II pneumocytes in acute alveolar damage and in UIP in humans and in experimental models (Uhal *et al.* 1995, Bardales *et al.* 1996, Guinee *et al.* 1997, Uhal *et al.* 1998, Barbas-Filho *et al.* 2001). The number of *bcl-2* positive type II pneumocytes was small and apparently of less importance compared to the wide and strong extent of *bax* positivity in epithelial cells. It is suspected that rapid re-epithelialization of newly formed connective tissue lesions is an important antifibrotic feed back mechanism in BOOP compared with the inefficient re-epithelialization in UIP (reviewed by Selman *et al.* 2001). On this basis the equal staining pattern of metaplastic epithelial cells in both BOOP and in UIP appears contradictory. In UIP, the strong *bax* positivity can lead to ongoing apoptosis of regenerated type II pneumocytes which, in turn, leads to ongoing fibroblast proliferation and collagen synthesis. However, in BOOP,

the strong bax positivity of regenerated type II pneumocytes can be seen as a sign of high turnover of the newly formed connective tissue and the epithelial cells covering it, leading to resolution of the intraluminal connective tissue lesion.

VEGF and bFGF inhibit apoptosis of endothelial cells and the antiapoptotic effect of both growth factors is at least partly mediated by increased expression of bcl-2 (Karsan *et al.* 1997, Gerber *et al.* 1998). There are studies suggesting that VEGF and bFGF could also have pro- or antiapoptotic effect on non-endothelial cells, some of which are mediated by bcl-2 (Wang *et al.* 1998, Bairey *et al.* 2001, Pidgeon *et al.* 2001). The present study does not clarify the possible roles of these growth factors in the context of apoptotic activity. Laminin-5 is also suspected to prevent apoptosis of epithelial cells (Esco *et al.* 2001). Loss of processed laminin-5 in the ECM may expose epithelial cells to increased apoptosis (Esco *et al.* 2001), which may be of great importance in the pathogenesis of UIP.

6.3 Re-epithelialization of newly formed intraluminal connective tissue with laminin-5 γ 2 chain in BOOP and UIP

The present study shows that the laminin-5 γ 2 chain is synthesized and widely expressed in regenerating pneumocytes in both BOOP and UIP. The proportional number of laminin-5 γ 2 chain-positive type II pneumocytes as well as the number of cells synthesizing laminin-5 γ 2 chain mRNA was equal in both diseases. This finding suggests that in both diseases there is an attempt to re-epithelialize the injured alveolar septa with laminin-5 γ 2 chain positive epithelial cells and thus stabilize the adhesions between the re-epithelializing cells and underlying stroma. The importance of laminin-5 for epithelial cell adhesion has been shown in several previous studies (Rousselle *et al.* 1991, Pulkkinen *et al.* 1994a,b, Kivirikko *et al.* 1995, Gagnoux-Palacios *et al.* 2001). Moreover, it has been shown that the short arm of the laminin-5 γ 2 chain plays a pivotal role in the incorporation of laminin-5 into the ECM and in cell adhesion (Gagnoux-Palacios *et al.* 2001).

The extent of the re-epithelialization of the newly formed connective tissue was statistically wider in BOOP when compared to that in UIP. In UIP the histological finding was heterogenous, poorly and well re-epithelialized intraluminal lesions occurring simultaneously. This gives rise to the question whether the low extent of re-epithelialization only reflects temporal heterogeneity of UIP. However, the clear morphological and structural abnormality of epithelial cells strongly favors the theory of disturbed or delayed re-epithelialization in UIP. In BOOP, the regenerating epithelium was morphologically uniform and usually showed appropriate layering above intraluminal connective tissue lesions. This regular histology contrasted with the histology of UIP, where there was disordered layering of regenerating epithelial cells, morphological variety of the cells, and exfoliation of laminin-5 γ 2 chain-positive epithelial cells into alveolar spaces. The morphological difference between the diseases suggests that despite the synthesis of laminin-5 γ 2 chain, the attachment of the cells to the underlying stroma is disturbed in UIP. This may be due to a plethora of different factors,

like altered extracellular processing of laminin-5 $\gamma 2$ chain or dysfunction of other laminin-5 chains, laminin receptors or other BM components. Loss of processed laminin-5 in the ECM may also expose epithelial cells to increased apoptosis (Esco *et al.* 2001) which may be of great importance in the pathogenesis of UIP (Uhal *et al.* 1998).

Laminin-5 $\gamma 2$ chain mRNA *in situ* hybridization correlated with the immunohistochemical results, showing that the laminin-5 $\gamma 2$ chain is synthesized in epithelial cells, with no detectable synthesis by stromal cells. This is well in line with the epithelial nature of laminin-5 $\gamma 2$ chain observed in earlier studies (Pyke *et al.* 1994, Virtanen *et al.* 1995, Pyke *et al.* 1995). The small amount of positive signals in *in situ* hybridization corresponds to the normal wound healing model, in which laminin-5 $\gamma 2$ mRNA has been observed only at the leading edge of the migrating epithelium (Pyke *et al.* 1994). At the same time, the small amount of laminin-5 $\gamma 2$ chain mRNA contrasts with the wide immunohistochemical expression of the protein. Obviously, in non-neoplastic cells the synthesis of laminin-5 $\gamma 2$ chain is strictly regulated and momentary.

In immunoelectron microscopy, positive labeling for laminin-5 $\gamma 2$ chain was seen at BMs of type I pneumocytes and regenerating type II pneumocytes in UIP, as well as in basal cells of a normal bronchus. BM stability is mediated by connections between laminin-5, and laminins-6 and -7 via integrin $\alpha 3\beta 1$ (Champlaud *et al.* 1996, DiPersio *et al.* 1997, Rousselle *et al.* 1997). In skin, laminin-5 also bridges hemidesmosomes with collagen type VII, which provides significant force to the connection between epithelial cells and the underlying stroma (Rousselle *et al.* 1997). In this study, no typical hemidesmosomes were seen in regenerating pneumocytes. In a previous ultrastructural study on BOOP, no hemidesmosomes were seen either (Myers & Katzenstein 1988). However, in a study on diverse human fibrotic lung disorders, hemidesmosomes were found in a subpopulation of cuboidal cells probably derived from bronchiolar cells and associating with advanced fibrosis (Kawanami *et al.* 1982). The presence of laminin-5 does not implicate that there are hemidesmosomes in epithelial cells (Leivo *et al.* 1996), but it is possible that in addition to the re-epithelialization after alveolar injury, the adhesive properties of laminin-5 also contribute to the interstitial remodeling by forming connections between subpopulation of epithelial and stromal cells via hemidesmosomes.

7 Conclusions

The present study addressed the role of angiogenesis, apoptosis and re-epithelialization in the pathogenesis of pulmonary fibrosis. In the newly formed intraluminal connective tissue of BOOP, there was wide and early capillarization often resembling granulation tissue formation. Capillarization paralleled with the immunohistochemical localization of VEGF, Flk-1, Flt-1 and bFGF. Apoptotic activity was increased within the newly formed connective tissue obviously contributing to the reduced cellularity of the intraluminal connective tissue in BOOP. There was also regular re-epithelialization of the newly formed intraluminal connective tissue with orderly layered and morphologically uniform regenerating pneumocytes, synthesizing laminin-5 γ 2 chain.

In UIP the capillarization was sparse within the newly formed intraluminal connective tissue, but pronounced in the areas of mural incorporating fibrosis, showing delayed capillarization at the primary site of the injury. Also, the apoptotic activity of the newly formed intraluminal connective tissue was lower in UIP than in BOOP, and the apoptotic activity can even be lower than in normal lung interstitium. Despite the synthesis of laminin-5 γ 2 chain, the re-epithelialization seems to be disturbed or delayed in UIP. The extent of re-epithelialization of the newly formed connective tissue was lower in UIP when compared to BOOP, and there was both morphological and structural abnormality of regenerating epithelial cells in UIP.

Based on these findings it seems evident that in BOOP, an efficient repair process takes place after lung injury, while in UIP, the repair process is disturbed or delayed. Thus, the results of the present study support the new concept of abnormal healing of lung injury as an important part of the pathogenesis of UIP. However, the pathogenesis of pulmonary fibrosis is most complex with multiple interactions between different cell types and signaling molecules, and more knowledge is needed about pathogenetic factors leading to the exaggerated fibroproliferative response in UIP. Understanding the pathogenesis of pulmonary fibrosis is essential for the development of new therapeutic strategies against UIP.

8 References

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