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ROLES OF PULMONARY ANGIOTENSIN SYSTEM IN THE DEVELOPMENT OF PULMONARY FIBROSIS

presented by

XIAOPENG LI

has been accepted towards fulfillment
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B.D. Uhal
Major Professor's Signature

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Date

ROLES OF PULMONARY ANGIOTENSIN SYSTEM IN THE DEVELOPMENT OF PULMONARY FIBROSIS

By

Xiaopeng Li

A DISSERTATION

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ABSTRACT

ROLES OF PULMONARY ANGIOTENSIN SYSTEM IN THE DEVELOPMENT OF PULMONARY FIBROSIS

By

Xiaopeng Li

Treatment for IPF targeting the suppression of inflammation has not been successful, suggesting that inflammation is not the sole mechanism underlying lung fibrogenesis. Mortality of IPF patients is not dependent on severity of inflammation, but correlates well with the presence of "fibroblastic foci" and adjacent failure of reepithelization. Loss of alveolar epithelial cells (AECs) and failure of reepithelization characterized in pulmonary fibrosis can be considered as profibrotic and are believed to initiate the fibrotic lesion.

The loss of AECs could result from necrosis and/ or apoptosis. Increased level of apoptosis was found in AECs in experimental and human pulmonary fibrosis. One indication that the angiotensin system is involved in fibrogenesis is findings that blockade of angiotensin systems blocked Pulmonary Fibrosis (PF) at least in several animal models. Our lab showed that angiotensin II (ANG II) induces apoptosis of the primary AECs through ANG II type I (AT1) receptor in vitro. Apoptosis of AECs induced by Fas Ligand, TNF-alpha, and amiodarone requires angiotensin synthesis de novo as AECs apoptosis can be blocked by the antisense oligonucleotide against the mRNA of the angiotensinogen (ANGEN), angiotensin converting enzyme (ACE) inhibitors, and ANG II receptor (AT receptor) antagonists in vitro. Taken together, those data suggest that ANG II, the processed product of ANGEN, is the key to regulate apoptosis of AECs and subsequent lung fibrosis.

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The existence of "local angiotensin system" in the lung, which is independent of the endocrine RAS, is supported by studies demonstrating the expression of angiotensin system components in cultured primary rat AECs in response to Fas Ligand, TNF-alpha, and amiodarone and myofibroblasts from human fibrotic lungs. Bleomycin-induced rat and mouse pulmonary fibrosis model is a well-studied model for fibrogenesis. But there is much still unknown about the components and roles of pulmonary angiotensin system in bleomycin-induced pulmonary fibrosis. The overall objective of my thesis project was to determine the roles of pulmonary angiotensisn system in the development of pulmonary fibrosis. We tested the overall hypothesis: the pulmonary angiotensin system including its components angiotensinogen, cathepsin D and AT1 receptor, plays an essential role in the development of bleomycin-induced pulmonary fibrosis at least in part through ANG II-AT1 receptor pathway mediated apoptosis of AECs. Our data showed that: 1) AEC apoptosis in response to bleomycin (BLEO) requires ANG synthesis and is inhibited by ANG system antagonists; 2) Cat D is required for AEC apoptosis in response to bleomycin at least in part by converting angiotensinogen to ANGI; 3) Administration of the AT1-selective receptor antagonist and deletion of the AT1a receptor gene block BLEO-induced AEC apoptosis and lung fibrosis; 4) The apoptotic type II alveolar epithelial cells and myofibroblasts are the major cellular sources of lung-derived ANGEN in vivo; 5) Administration of the antisense against ANGEN messenger RNA attenuates bleomycin-induced AEC apoptosis and lung fibrosis.

To my lovely wife,Min Luo, you make this possible.

To my parents, Han-Fu Li and Bao-Yu Chen, for your uttermost love and support

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Acknowledgments

This dissertation project was the outcome of my hard work and the input, advice, and the assistance of many other people.

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Then, I thank Dr. Jump and Dr. Olson's lab for letting me use their space and equipments for part of my experiments. In addition, I would to thank all those individuals who have helped me along the way. They are my colleagues in Dr. Uhal lab including Dr. Jiaju Zhuang, Dr. Huiying Zhang, Ruijie Shu, Jong-kyong Kim, Heather Rayford, Valerie Conrad.

I also would like to thank my American Friendship family: Hank Hanson and Chris Boesen. I appreciate their friendship, support and endeavors to enrich my experience of living in this country for the past years.

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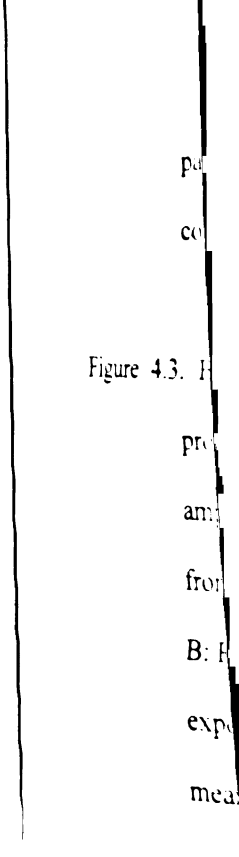

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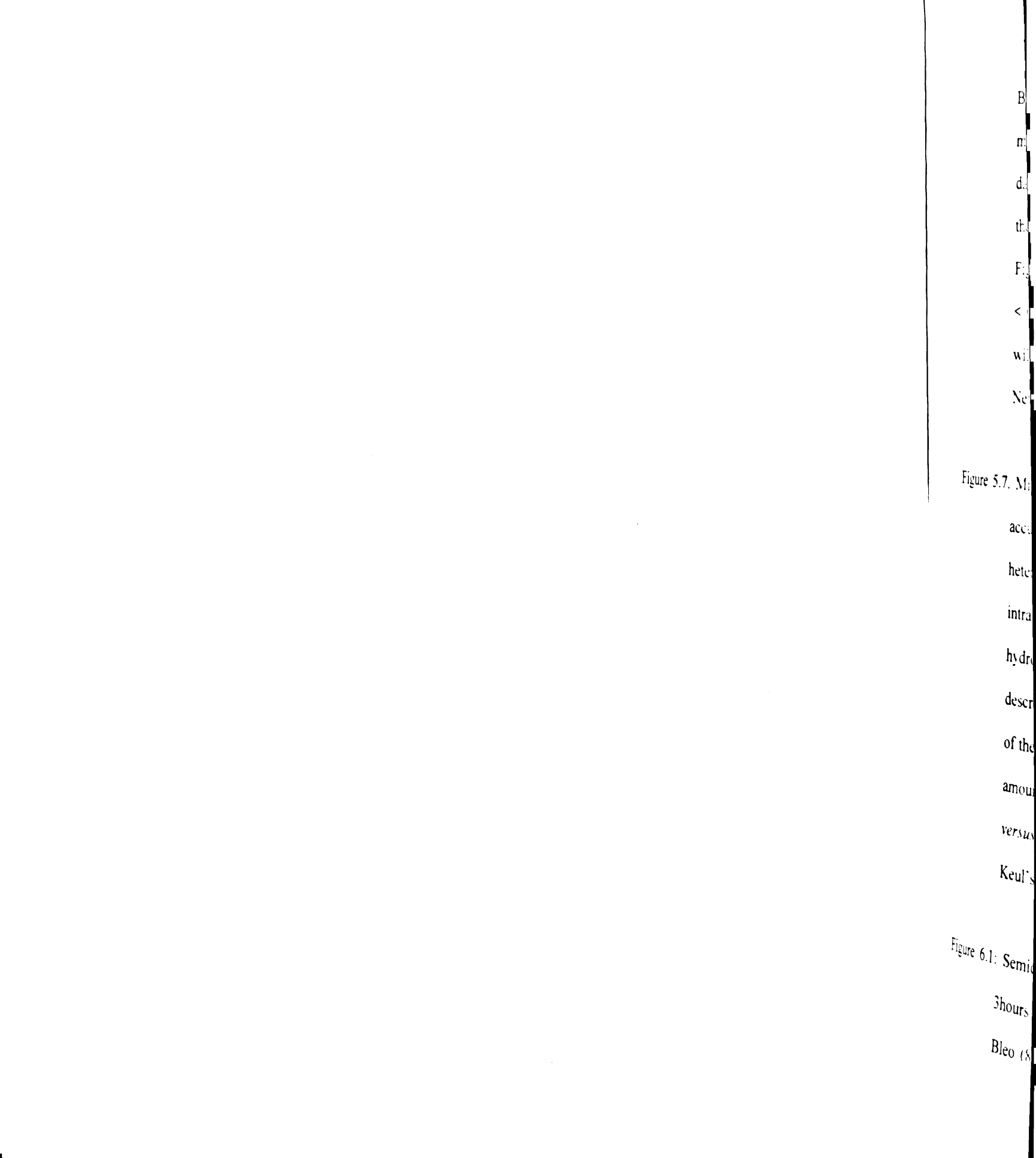

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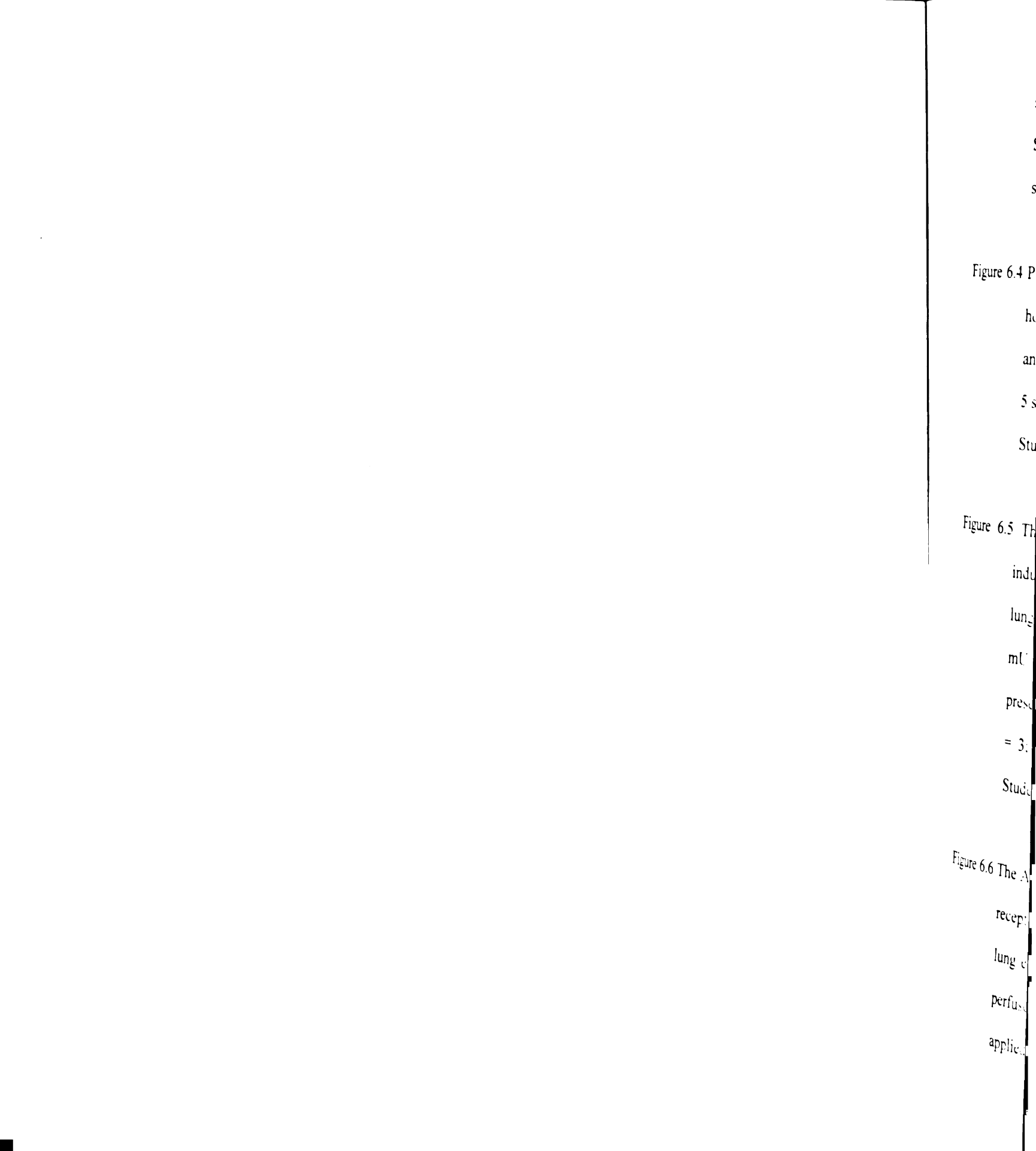

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ACE

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AECs

ANGEN

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ANG I

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APR

α-SMA

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ATA

AT I cell

AT II cell

AT1 Receptor

ATF

BALF

bio-dUTP

BLEO

BPAEC

cAMP

CAPT

LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACEis	Inhibitors of ANG converting enzyme
AECs	Alveolar Epithelial Cells
ANGEN	Angiotensinogen
ANG	Angiotensin
ANG I	angiotensin I
ANG II	angiotensin II
APR	Acute Phase Response Element
α-SMA	Alpha-Smooth Muscle Actin
AS	antisense oligonucleotides
ATA	aurintricarboxylic acid
AT I cell	Alveolar type I cell
AT II cell	Alveolar type II cell
AT1 Receptor	ANG II type I receptor
ATF	Activating transcription factor
BALF	bronchoalveolar lavage fluid
bio-dUTP	biotinylated deoxyuridine trisphosphate
BLEO	bleomycin
BPAEC	Bovine pulmonary artery endothelial cells
cAMP	Cyclic AMP
CAPT	captopril

CatD

CMV

CTD-PF

DAG

dig-dUTP

DL_{CO}

EBV

ECL

ECM

ELISA

Fas L

FBS

F1-14

HGF

HP

HRCT

IHC

IL-6

ILD

IP3

IPF

ISEL

ISH

CatD	cathepsin D
CMV	cytomegalovirus
CTD-PF	Connective tissue disease-pulmonary fibrosis
DAG	diacylglycerol
dig-dUTP	digoxigenin-labeled deoxyuridine trisphosphate
DL_{CO}	Diffusion capacity of carbon monoxide
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
ECM	extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
Fas L	Fas ligand
FBS	Fetal bovine serum
F1-14	angiotensinogen fragment 1-14
HGF	Hepatocyte growth factor
HP	hydroxyproline
HRCT	High-resolution CT
IHC	Immunohistochemistry
IL-6	Interleukin-6
ILD	Interstitial lung disease
IP3	Inositol-1, 4, 5-trisphosphate
IPF	Idiopathic Pulmonary Fibrosis
ISEL	In situ end labeling
ISH	In Situ Hybridization

I.T.

ITS

KGF

LIPO

LOS

LTα

Km

β -MG

PAIs

PBS

PepA

PDGF

PF

PGE_2

PI

PIP2

PKA

PKC

PLA2

PLC β

PLD

ProSP-C

RAS

SARAL

SCR

I.T.	intratracheal
ITS	Insulin-transferrin-selenium
KGF	Keratinocyte growth factor
LIPO	lipofectin
LOS	losartan
LTα	lymphotoxin-α
Km	Michaelis Constant
β -MG	β -Microglobulin
PAIs	plasminogen activator inhibitors
PBS	Phosphate-buffered saline
PepA	pepstatin A
PDGF	Platelet-derived growth factor
PF	Pulmonary Fibrosis
PGE_2	Prostaglandin E2
P I	propidium iodide
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	phospholipase A2
PLC β	phospholipase C-beta
PLD	phospholipase D
proSP-C	pro-surfactant protein-C
RAS	renin-angiotensin system
SARAL	saralasin
SCR	Scrambled-sequence control oligonucleotides

SNPs

SP-A

TBS

TGF-β

TNF-α

TNF-RII

tPA

TUNEL

UPA

w.t.

ZVADfmk

SNPs	Single nucleotide polymorphisms
SP-A	Surfactant protein A
TBS	Tris-buffered saline
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor alpha
TNF-RII	TNF-α/LTα receptor 2
tPA	tissue-type plasminogen activator
TUNEL	Terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine End Labeling
UPA	urokinase-type plasminogen activator
w.t.	wild type
ZVADfmk	*N*-benzylcarboxy-Val-Ala-Asp-fluoromethylketone

GENERAL

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Chapter 1

GENERAL INTRODUCTION

1. Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive, usually fatal, form of interstitial lung disease resulting from injury to the lung and an ensuing fibrotic response. Fibrosis leads to thickening of the alveolar walls and the obliteration of the alveolar space with unknown etiology (Fonseca et al., 2000). The characteristics of IPF are failure of alveolar reepithelialization, persistence of fibroblasts/myofibroblasts, deposition of extracellular matrix, and distortion of lung architecture, which ultimately results in respiratory failure.

1.1 Epidemiology of IPF

IPF is a relatively rare disease and more common in males than in females. The estimated annual incidence is 7 cases per 100,000 women and 10 cases per 100,000 men (Coultas et al., 1994). The incidence, prevalence, and death rate increase with age (Coultas et al., 1994; Mannino et al., 1996; Schwartz et al., 1994). For the age group between 35 to 44 years there are 2.7 cases of IPF in a population of 100,000. For the age group older than 75 years there are about 175 cases in the same population (Coultas et al., 1994). Two thirds of patients diagnosed with IPF are with an age greater than 60 years (Johnston et al., 1997). The mean age at diagnosis of IPF is 66 years (Johnston et al., 1997; Carrington et al., 1978).

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1.2 Predisposing factors for IPF

Some studies indicate that genetic factors and environmental exposure to dusts, organic solvents, or urban pollution increases the risk of developing IPF (Johnston et al., 1997; Hubbard et al., 1996; Iwai et al., 1994).

1.2.1 Genetic factors

Genetic factors may play an important role in the pathogenesis of IPF and about 3% of the IPF patients aggregated in families (White et al., 2003). Studies seeking the abnormal genes in those patients demonstrated a mutation in the pro-surfactant protein-C (proSP-C) gene, which lead to the substitution of leucine by glutamine in the C-terminus of that protein (Thomas et al., 2000). Electron microscopic studies demonstrated that this mutant protein is aberrantly located in the cell and lamellar bodies containing surfactants is abnormal (Thomas et al., 2000). These results suggest that the mutation of the gene produces protein that could not be properly processed to the correct subcellular compartment to be secreted out of type II alveolar epithelial cells. The mutation results in abnormal surfactant production in alveolar type II cells. These findings suggest that in this familial IPF, improper cellular processing of proSP-C may contribute to pulmonary fibrosis.

Genetic polymorphisms of several other enzymes and cytokines have been associated with either the incidence or progression of IPF (Whyte et al., 2000; Pantelidis et al., 2001). Angiotensin converting enzyme (ACE) gene polymorphisms were studied in pulmonary fibrosis. The D allele of the insertion/ deletion (I/D allele) polymorphism of ACE confers a higher level of ACE gene expression compared to I allele. The incidence

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of D allele and D/D genotype of ACE gene in 24 patients with interstitial pneumonia and moderate to severe pulmonary fibrosis was examined (Morrison et al., 2001). The incidence of the D allele was approximately 15% higher and the D/D genotype was approximately 11% higher in the patients group than in the general population (Morrison et al., 2001). That study suggests that gene polymorphisms that confer higher levels of angiotensin converting enzyme (ACE) gene expression predispose patients to lung fibrosis (Morrison et al., 2001).

Single nucleotide polymorphisms (SNPs) of some pro-inflammatory genes was evaluated in 74 IPF patients with confirmed diagnosis by clinical or biopsy data (Pantelidis et al., 2001). Four candidate genes were proposed including tumour necrosis factor-α (TNF-α), lymphotoxin-α (LTα), high affinity TNF-α/LTα receptor 2 (TNF-RII), and interleukin-6 (IL-6). No difference in genotype, allele, or haplotype frequencies of those genes was found between patients inflicted with IPF and control population. However, the frequency of carriage of the IL-6 allele (intron 4G) and the TNF-RII allele (1690C) increased in patients, which was not observed in controls. In addition, a strong association was found between progression of IPF and IL-6 genotype since diffusion capacity for carbon monoxide (DL_{CO}) was decreased, suggesting that the rapidity of disease progression is higher in patients with IL-6 genotype, this may account for decreased production of IL-6 (Pantelidis et al., 2001).

1.2.2 Environmental factors:

Environmental factors have also been considered to contribute to the development of pulmonary fibrosis.

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Several viruses including Epstein-Barr virus (EBV), cytomegalovirus (CMV) have been implicated in pathogenesis of IPF as those viruses and latent viral infections have been detected in patients with IPF. However, there exists no convincing evidence to demonstrate certain viruses directly cause IPF (Yonemaru et al, 1997; Stewart et al., 1999; Kelly et al., 2002).

Investigators have also made some extent of associations between unidentified occupational and dust exposures and IPF (Baumgartner et al., 2000; Mullen et al., 1998).

Cigarette smoking is the most extensively studied environmental risk factor for IPF. Howerve, instead of showing smoking as a risk factor for IPF, some clinical studies indicate that current smokers have improved mortality in established cases of IPF (King et al., 2001; Flaherty et al., 2002). In agreement with those studies, those IPF patients who currently smoke also had lower granulation/connective tissue (FF) scores, another independent predictor of disease survival, indicating better prognosis (Flaherty et al., 2003; King et al., 2001). These observations thus suggest that some factors produced in the process of cigarette smoking may be helpful for treating IPF (Nobukuni et al., 2002).

Genetic susceptibility and environmental factors may contribute to the development of IPF, yet evidence of predisposing or etiologic factors is not strong since most patients do not demonstrate any obvious risk factors (Selman et al., 2001).

1.3 Clinical features

The clinical manifestations include dyspnea on exertion, nonproductive cough, and inspiratory crackles; at the late stage of this disease patients also present shortness of

breath at rest (Crystal et al., 1976). Bi-basilar and end-expiratory rales are demonstrated through physical examination in more than 80% of patients with IPF (ATS, 2000). Digital clubbing is noted in up to half of all patients (Johnston et al., 1997). Cyanosis and signs of pulmonary hypertension may be seen in patients at the late stage of IPF (Panos et al, 1999).

There is no laboratory test specific for the diagnosis of IPF. Laboratory evaluation of patients with suspected IPF is primarily to rule out alternative causes of interstitial lung disease such as sarcoidosis or connective tissue disease-pulmonary fibrosis (CTD-PF). Pulmonary function tests reveal restrictive impairment, reduced diffusing capacity for carbon monoxide, and arterial hypoxemia exaggerated or elicited by exercise (Selman et al., 2001).

More than 90% of patients with IPF at the time of diagnosis had abnormal chest radiographs (Johnston et al., 1997). The characteristic pattern of abnormal chest radiography is diffuse bilateral interstitial or reticulonodular infiltrates, predominantly in the basilar and subpleural regions of the lower lung (Guerry-Force et al., 1987). Diagnosis for patients with suspected IPF can be improved by using the high-resolution CT (HRCT) scanning since HRCT can produce reconstructed images of thin scan sections (1-2 mm slices) in three-dimensions to visualize the enhanced spatial structure of the lung parenchyma (White et al., 2003). Patterns of HRCT images typically seen in IPF patients include coarse reticular or linear opacities indicating intralobular and interlobular septal thickening, a predilection for the periphery and lower lobes of the lungs, honeycomb cysts, and traction bronchiectasis (Kazerooni et al., 1997). Ground glass opacities and ill-defined hazy zones that represent active alveolitis or fibrosis of the

intralobular and alveolar septae can also be present (Kazerooni et al., 1997; Wells et al., 1993). Extensive honeycombing, septal thickening, and a lack of ground glass opacities often predict a poor prognosis.

The definite diagnosis of IPF must meet the following criteria: 1) a compatible clinical history; 2) the exclusion of other known causes of interstitial lung disease such as drug injuries, environmental exposures, or collagen vascular disease; 3) a surgical lung biopsy showing usual interstitial pneumonia histologic pattern (ATS, 2000). The histologic hallmark of usual interstitial pneumonia is a heterogeneous appearance with alternating areas of dense fibrosis, fibroblastic foci, interstitial inflammation, and honeycomb change and normal lung at low magnification.

1.4 Histopathology

In IPF the normal architecture and functional integrity of the lung are destroyed. The main histological features of the fibrotic lung are persistent and unrepaired epithelial damage, proliferation and accumulation of fibroblast/myofibroblast cells, and increased collagen deposition (Selman et al., 2001).

Temporal heterogeneity is a histopathologic hallmark of IPF. At low magnification, a typical heterogeneous appearance can be observed with normal areas alternating with areas with peripheral fibrosis, interstitial inflammation, and honeycomb changes (ATS, 2000). The inflammatory component is typically not intensive and consists primarily of lymphocytes and plasma cells. Other inflammatory cells such as neutrophils and eosinophils may be present with low abundance. Under high-power magnification, fibroblastic foci can be found at the border between fibrotic and normal lung where

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fibroblasts/myofibroblasts accumulate with collection of dense, relatively acellular collagen bundles. The presence of fibroblastic foci may be an important prognostic factor for IPF as it represents the active lesion of IPF (Selman et al., 2001). The number of fibroblastic foci is correlated with the morality of patients with IPF (Nicholson et al., 2002; King et al., 2001).

Another important characteristic of IPF is unrepaired alveolar epithelial damage. Alveolar epithelial injury with abnormal type II pneumocytes is often seen at areas of active fibrosis (Kuhn et al., 1991). Honeycomb changes are formed by enlarged, cystic airspaces lined by abnormal type II pneumocytes.

1.5 Therapeutic options for pulmonary fibrosis

The traditional view considers pulmonary fibrosis is an inflammatory disorder, which provides the basis for using potent anti-inflammatory agents such as corticosteroids to treat IPF (Flaherty et al., 2001). Anti-inflammatory agents were often supplemented with immunosuppressive and cytotoxic agents such as cyclophosphamide (Zisman et al., 2000), azathioprine (Raghu et al., 1991) and colchicines (Douglas et al., 1998). Unfortunately, clinical studies indicate that those traditional therapeutic approaches can only offer a marginal benefit at best (King et al., 2000). In contrast, new approaches employing anti-fibrotic agents such as angiotensin system antagonists (captopril), pirfenidone, interferon-Ɣ and statin have been shown to have antifibrotic effects in vitro and/or in vivo in experimental pulmonary fibrosis models (Selman et al., 2001). However, prospective, randomized, placebo-controlled multi-center clinical trials are needed to evaluate their efficacy in the treatment of IPF.

2. Bleomycin-Induced Experimental Pulmonary Fibrosis Model:

A number of animal models of pulmonary fibrosis have been developed to address the pathogenesis of pulmonary fibrosis. One of them is the bleomycin-induced experimental pulmonary fibrosis model. [see review (Thrall and Scalise, 1995)].

Bleomycin is an antineoplastic antibiotic, which was isolated from a strain of *Streptomyces verticillus* (Umezawa et al., 1966). Bleomycin is not a single peptide. Instead, it consists of a family of complex glycopeptides with different amine groups (Umezawa et al., 1966, 1967; Umezawa, 1973, 1974). It is used in the treatment of squamous cell carcinomas and various lymphomas (Ichikawa et al., 1967, 1969; Yagoda et al., 1972; Blum et al., 1973). The mechanisms of antineoplastic action of bleomycin are complex and cell cycle-dependent. Briefly, bleomycin intercalates between DNA base pairs, causing DNA to unwind and impairing protein synthesis. Bleomycin increases radical oxygen species by reducing molecular oxygen to superoxide and hydroxyl radicals. Those free radicals then attack DNA and cause strand cleavage (Sausville et al., 1978).

Lack of side effects on bone marrow (Kimura et al., 1972; Boggs et al, 1974) and immunocompetence (Dlugi et al., 1974; Lahane et al., 1975) are the major advantages of using bleomycin over other chemotherapeutic agents. However, the lung and skin are the major organ systems affected by the toxic side effects of bleomycin (Thrall and Scalise, 1995). The fact that these organ systems are susceptible to bleomycin can be explained in part, but not entirely, by the pharmacokinetics of bleomycin. Bleomycin reaches the highest concentration in the lung and skin after parenteral administration. This is likely

due to the lack of a hydrolase, which inactivates bleomycin, in the lung and skin as bleomycin-induced cytotoxicity is higher with low cellular levels of hydrolase activity (Umezawa et al., 1972). Currently the bleomycin induced pulmonary fibrosis animal model is the most commonly used model for studying the pathogenesis of fibrotic lung disease.

The bleomycin-induced pulmonary fibrosis is dose and administration route dependent. Two most common administration routes are parenteral and intratracheal. Cumulative doses of 100-200 mg (units)/kg were used in the parenteral injection model whereas the intratracheal single–injection model uses doses in the range of 2.5-7.5 mg/kg (Thrall and Scalise, 1995). The parenteral injection produces lesions with a more diffuse pattern in perivascular and subpleural locations whereas the intratracheal injection produces patchy focal lesions in peribronchial locations. Various animals, including mice and rats, have been used for models of bleomycin induced pulmonary fibrosis (Thrall and Scalise, 1995).

Generally speaking, the development of lung injury in various bleomycin animal models is quite similar regardless of species and route of administration. The lung injury progresses through three stages (Thrall and Scalise, 1995): (1) The acute inflammatory stage (1-3 days post-intratracheal instillation of bleomycin and later in the parenteral models). This stage features the activation of inflammatory mediator systems and emerging of pulmonary edema. (2) The subacute stage (4-21 days post intratracheal instillation of bleomycin). This stage is characterized by the synthesis of collagen and elevation of the levels of net lung collagen as well as other connective tissue components.

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(3) The chronic stage (21 days to termination). This stage is dominated by the metabolism of connective tissue towards reepithelialization.

In summary, as an effective antineoplastic agent used in the treatment of various carcinomas and lymphomas, bleomycin has toxic side effects on lung tissues, which confers the major limitation to its therapeutic use. The bleomycin animal models, however, provide a valuable tool to investigate the pathogenesis of fibrotic lung disease with direct clinical relevance.

3. Mechanisms of Fibrogenesis

IPF is the most common interstitial lung disease (ILD) encountered by respiratory physicians (Mapel et al., 1998). Unfortunately, despite years of research on its pathogenesis, the treatment of PF has not been successful. The traditional therapeutic approaches using potent anti-inflammatory agents supplemented with an immunosuppressive agent could only offer a marginal benefit at best (King et al., 2000). Recent NHLBI Workshops have concluded that our current understanding of the pathogenesis of the IPF is incomplete (Mason et al., 1999; Crystal et al., 2002).

Current opinions regarding the pathogenesis of PF are controversial. The traditional view considers pulmonary fibrosis as an inflammatory disorder, in which inflammation in the lower respiratory tract (alveolitis) is responsible for derangements of the alveoli and results in scaring of the lung parenchyma (Gallin et al., 1992). The consequence of the inflammation is the loss of functional alveolar–capillary unit, the accumulation of collagen, and the formation of honeycomb lung. The limited success of

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anti-inflammatory/ immunosuppressive therapy for IPF has stimulated the generation of alternative hypothesis regarding the pathogenesis of IPF (Crystal et al., 2002).

Current evolving hypothesis is that IPF results from the epithelial microfoci injury and a failure of reepithelization (Selman et al., 2001). Haschek and Witschi first proposed this hypothesis more than 20 years ago, speculating that epithelial damage drives fibrogenesis and efficient epithelialization would prevent fibrogenesis (Haschek et al., 1979; Witschi, 1990). There is some evidence from both animal model and human patients that supports this hypothesis. In the animal models, experimental delay of epithelial repair after injury facilitates subsequent fibrogenesis (Haschek et al., 1979). In human lung biopsies, nascent fibrotic foci were colocalized with unrepaired or abnormal epithelia (Uhal et al., 1998).

3.1 The roles of alveolar epithelial cells (AECs)

The lung is the respiratory organ that functions to maintain the constant internal environment by inspiring oxygen into blood circulation and removing carbon dioxide from blood circulation. To fulfill this function, the lung has special architecture. The basic functional unit of the lung is alveoli where gas exchange occurs between the air and the circulating blood. Alveoli consists of several components: 1) the alveolar epithelium that covers the air space; 2) the endothelium lining the capillaries; 3) thin interstitium separating the epithelium and the endothelium, which includes the connective tissue and extracellular matrix components with a few resident fibroblasts (Fonseca et al., 2000).

The intact alveolar epithelium is composed of two cell types, alveolar type I cells (AT I cell) and type II cells (AT II cell) (Uhal et al., 1997; Sutherland et al., 2001). They

are morphologically and functionally different although the cell numbers of these two types are similar (Mason and Williams, 1991). AT I cells are large elongated cells and are terminally differentiated. They cover over 90% of the alveolar surface. The thin and attenuated cytoplasm of the AT I cell facilitates the gas exchange by minimizing the diffusion distance between alveolar gas and blood. These cells are metabolically active and harbor cell surface receptors for a variety of substances, including extracellular matrix (ECM) proteins, growth factors, and cytokines. AT II cells are cuboidal in shape with rounded nuclei. They are predominantly located in the corners of the alveoli and cover about 10% of the alveolar surface. Type II cells have distinctive features such as apical microvilli and cytoplasmic lamellar bodies that contain the alveolar lining material surfactant. Type II cells have abundant intracellular organelles including extensive endoplasmic reticula, Golgi complexes, peroxisomes, and mitochondria. AT II cells are not terminally differentiated and have a number of unique functions (Sutherland et al., 2001). For example, AT II cells can synthesize and secrete surfactant that is involved in regulating the surface tension in the alveolar epithelium. AT II cells are also involved in xenobiotic metabolism by regulating the activities of the cytochrome P-450 monooxygenase, transepithelial ion and H_2O movement. In addition, AT II cells have immuno-modulatory functions and regulate the lung extracellular matrix (ECM) metabolism (Pardo et al., 1997). Another important function of the AT II cells is the maintenance of the alveolar epithelium. It can function as stem cells to proliferate to give rise to new AT II cells or differentiate into AT I cells. As such, AT II cells play important role in the repairing of the alveolar epithelium by replacing the lost cells and restoring normal tissue architecture and lung function (Uhal, 1997).

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3.2 Consequence of alveolar epithelial cells damage/ injury:

Loss of AECs during IPF has a number of adverse effects, which likely contribute to the pathophysiology of IPF [see review (Simon, 1995)].

3.2.1 Loss of permeability barrier

The major function of type I alveolar cells is gas exchange. Diffusion distances between alveolar air and capillary blood are very short due to the thin shape of the type I cells, which facilitates gas exchange. Intercellular junctions between adjacent capillary endothelial cells are not tight. Therefore, plasma proteins can leak into the interstitial space. In order to maintain the functional integrity of the lung, the plasma has to be prevented from leaking into the airspaces. The alveolar wall provides a barrier that fulfills this function. This barrier of the alveolar wall consists of the capillary endothelium and alveolar epithelium (Gorin and Stewart, 1979). Compared to the intercellular junctions between adjacent capillary endothelial cells, the intercellular junctions between alveolar epithelial cells are much tighter which prevent air space from flooding. The pulmonary lymphatic system also helps to prevent air space from flooding by draining the normal efflux of plasma components back to the circulation. However, in patients with IPF, plasma proteins, e.g., fibrinogen, leak into the alveolar space (Basset et al., 1986; Kuhn et al., 1989; Crouch, 1990).

3.2.2 Loss of the barrier limiting fibroblast migration into the alveolar airspace

Migration of fibroblasts into the airspaces would lead to collagen deposition and obliteration of alveoli. In addition to providing a barrier for plasma proteins leakage, the

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alveolar epithelium also prevents the migration of fibroblasts into the alveolar airspaces. Rat tracheal grafts with or without denuded epithelial cells were used to demonstrate that epithelial cells suppress migration of fibroblasts (Terzaghi et al., 1978). When the grafts without epithelial cells were transplanted into another rat, the tracheal lumen rapidly becomes obliterated by the inward migration of fibroblasts and formation of connective tissue. When the tracheal grafts were repopulated with epithelial cells, intralumenal fibrosis did not occur. Therefore, an intact epithelia layer appears to be crucial for preventing fibrosis. This is likely due to the fact that AECs produce inhibitory factors for fibrosis. For example, prostaglandin E2 (PGE_2) that is secreted by alveolar epithelial cells is a potent inhibitor of fibroblast collagen synthesis and proliferation (Goldstein et al., 1982; Lama et al., 2002). Furthermore, the PGE_2 levels are lower in broncho-alveolar lavage (BAL) fluid from patients with IPF compared to those from control patients (Borok et al., 1991). Therefore, loss of AECs or damaged AECs with deceased ability to synthesize PGE2 may contribute to pathogenesis of IPF.

3.2.3 Loss of cell surface that prevents collapse and fusion of alveolar walls

Alveolar collapse and fusion of adjacent walls can occur in severely damaged areas of lung parenchyma in IPF patients due to loss of the epithelial cell surface. Fibrotic connective tissue replaces the collapsed alveolar space and nuked basement membrane. Those processes lead to the decrease of alveolar surface area and contribute to the restrictive lung volumes (Simon, 1995).

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Surfactant is a lipid-rich material that is synthesized exclusively by type II alveolar cells. Its function is to lower the surface tension of the air-fluid interface so that alveolar geometry can be maintained to protect smaller alveoli from collapsing.

Bronchoalveolar lavage fluid obtained from patients with IPF has abnormalities in its surfactant components. Particularly, the composition of phospholipids is out of ratio (Low, 1989; McCormack et al., 1991). The protein component of surfactant in IPF is also abnormal. For example, surfactant protein A (SP-A), the major protein species within surfactant, was shown to decrease in bronchoalveolar lavage fluid (McCormack et al., 1991). It was also revealed that patients with better-preserved levels of SP-A had better prognosis showed by lung functions test (McCormack et al., 1991).

The deficiencies in surfactant have several impacts on the pathophysiology of IPF. First, abnormal surfactant causes higher surface tension on the alveoli that can lead to the collapse of smaller alveoli. Permanent collapse will occur where those alveoli have areas of naked basement membrane. Second, the abnormally high surface tension will decrease the hydrostatic pressure within the alveolar lining fluid below the air-fluid interface (Guyton and Moffatt, 1981). Pressure gradients between the vascular and interstitial compartments and the alveolar space will increase causing water movement into alveolar airspace and leading to alveolar flooding. Third, the deficiency of SP-A can affect surfactant turnover as SP-A serves as a ligand for the uptake and reprocessing of previously secreted surfactant by alveolar epithelial cells (Wright et al., 1987).

3.2.5 Loss of the Alveolar Progenitor Cells

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Type II cells are known to serve as the progenitor cells for the alveolar epithelium whereas Type I cells are terminally differentiated and do not proliferate (Uhal, 1997). In damaged areas of the alveolar surface where type I cells are lost, type II cells proliferate and differentiate into type I cells. If the injury to the alveolar surface is too severe for type II cells to survive, the potential for reconstituting a normal alveolar surface is lost. The regeneration and reepithelialization of the normal alveolar wall are critical for normal healing without the consequence of fibrosis. Hence, the current presiding view regarding the mechanism of fibrogenesis holds that IPF results from epithelial injury and failure of reepithelization (Selman et al., 2001).

3.2.6 Loss of Active Transport Properties

Type II cells, and probably type I cells, help to maintain a relatively dry alveolar compartment by clearing fluid within the compartment via active transporter coupled to the sodium-potassium ATPase. The sodium-potassium ATPase located on the basolateral surface of the cells pumps sodium ions out of the cell into the interstitial space. This process was first noted through tissue culture studies which demonstrasted domes formed by monolayers of type II cells (Mason et al., 1982; Goodman and Crandall, 1982). These domes are caused by accumulation of the fluid from the media under the monolayer as a result of import of sodium molecules and accompanying water. Consequently, the trapped fluid lifts up the monolayer and form domes (Goodman et al., 1983; Cott et al., 1986). Studies have also been performed on intact lungs and confirmed that solute molecules can be removed from the alveolar space against concentration gradients (Bassett et al., 1987; Matthay and Wiener-Kronish, 1990). Therefore, damage to or loss

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3.2.7 Loss of the ability to clear intra-alveolar fibrin and regulate the plasminogen activation system:

Alveolar epithelial cells are in part responsible for both the formation and clearance of intra-alveolar fibrin. When alveolar epitheliums are damaged, plasma components such as fibrinogen leak into the alveolar space. Tissue factors expressed on the surface of type II alveolar cells (Gross et al., 1991) and alveolar macrophages (McGee and Rothberger, 1985) convert fibrinogen to fibrin, which can act as scaffolds for fibroblast migration. The clearance of this intra-alveolar fibrin is critical to the outcome of the lung injury (Sitrin et al., 1987; Brown et al., 1989). If extravascular fibrin is cleared in an orderly fashion, it is possible to reconstitute the normal alveolar space. Otherwise, fibroblasts will attach to and migrate along the remaining fibrin leading to the deposition of interstitial collagens and obliteration of the alveolar space, which are the characteristics of pulmonary fibrosis.

The fate of the newly formed fibrin in alveolar space depends on whether the profibrinolytic and antifibrinolytic processes within the alveolar compartment is balanced. The fibrinolytic process is mediated by the plasminogen activation system, which tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) can activate (Martin et al., 2002). Both tPA and uPA can transform the plasminogen to plasmin, the primary fibrinolytic enzyme responsible for degrading fibrin. uPA is considered to be the major contributor for the fibrinolytic activity within

bronchoalveolar lavage fluid from normal lungs (Chapman et al, 1986; Hasday et al., 1988; Idell et al., 1989). In IPF the plasminogen activation system has been implicated to regulate fibrin turnover and ECM degradation (Simon et al., 1995). The antifibrinolytic process is mediated by plasminogen activator inhibitors (PAIs), which negatively regulate the activity of plasminogen and thus plasmin. Studies using animal models suggest that overexpression of PAIs (inhibiting plasmin activity) promotes fibrosis, whereas lack of PAIs (allowing greater plasmin activity) prevents the formation of significant fibrosis (Eitzman et al, 1996). Epithelial cells control the balance between fibrolytic and antifibrolytic process by synthesizing both urokinase (Gross et al., 1990) and plasminogen activator inhibitor-l (Gross et al., 1990, 1991; Simon et al., 1992; Hasegawa 1997). Epithelial cells express urokinase that activates the plasminogen present within the plasma clot. Alveolar epithelial cells also express cell surface receptors for urokinase, which contain the fibrinolytic activity on the cell surface.

Normal alveolar epithelium is efficient in clearing intra-alveolar fibrin and profibrinolytic. However, damaged alveolar epithelial cells often show impaired ability to remove intra-alveolar fibrin. For example, the fibrinolytic activity of bronchoalveolar lavage fluid from IPF patients was deficient (Chapman et al., 1986). The compromised fibrinolysis was due to a decreased level of urokinase protein and an increased level of the inhibitors for urokinase and plasmin, an imbalance between profibrinolytic and antifibrinolytic processes (Chapman et al., 1986). Kotani *et al* showed that BAL fluid from patients with IPF contains significantly greater amounts of tissue factor and PAIs than normals whereas uPA levels were similar between the two groups (Kotani et al., 1995). Combined together, those studies suggest that the alveolar microenvironment in

IPF favors a pro-coagulant, anti-fibrinolytic state promoting ECM accumulation and retarding alveolar re-epithelialization.

3.2.8 Abnormal AECs as a primary source of profibrotic cytokines

Dying or abnormal AECs in IPF synthesize numerous growth factors and cytokines that activate fibroblasts and mesenchymal cells. AECs are the primary source for transforming growth factor-beta (TGF-β) which transdifferentiate normal fibroblasts into the myofibroblast producing most of the ECM in the fibrotic lungs (Khalil et al., 1996). Additionally, AECs produce ANG II, platelet-derived growth factor (PDGF) (Antoniades et al., 1990), tumour necrosis factor alpha (TNF-α) (Kapanci et al., 1995), and endothelin-1 (Giaid et al., 1993). PDGF is a potent mitogen and chemoattractant for fibroblasts. The mRNA and protein level of PDGF have been shown to be upregulated in epithelial cells of patients with IPF (Antoniades et al., 1990). TNF-α promotes DNA synthesis and proliferation of fibroblasts (Battegay et al., 1995) and was secreted by hyperplastic type II AECs in pulmonary fibrosis (Kapanci et al., 1995; Nash et al., 1993). Endothelin-1 has also been shown to stimulate fibroblast DNA synthesis and proliferation as well as to induce transdifferentiation of fibroblasts to myofibroblasts (Shahar et al., 1999). The various fibrosis-promoting effects of those factors produced by damaged AECs support the view that death of the AECs within alveolar epithelium can be regarded as profibrotic and could initiate a fibrotic lesion.

4. Apoptosis of AECs is involved in pulmonary fibrogenesis

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There are two kinds of cell deaths in alveolar epithelium: necrosis and programmed cell death also known as apoptosis. The morphological and biochemical characteristics of necrosis and apoptosis are different. Necrosis is considered as a passive and un-regulated process associated with inflammation which results in disintegration of membrane and cellular organelles (Saraste and Pulkki, 2000). In contrast to necrosis or "accidental" cell death, apoptosis is an active form of cell death that requires the activation of specific enzymes such as caspases, endonucleases and other components of signaling pathways (Uhal 2002). Apoptotic cells often are characterized with intact cell membrane, shrunk cytoplasm and fragmented DNA. These hallmarks are commonly used to identify apoptotic cells (Allen et al., 1997). Apoptosis results in single cell death and deletion from tissues with minimum inflammation. By doing so, apoptosis leads to the removal of unwanted or damaged cells. As apoptosis is a highly regulated physiological process of cell removal, it plays a fundamental role in the homeostatic control of cell population (Sutherland et al., 2001).

4.2 Epithelial cell apoptosis in fibrotic lungs

A relatively small increase in the incidence of apoptosis within a given cell population can result in considerable cell loss over time. Therefore, a seemingly minor upregulation of apoptosis is theoretically capable of accounting for the excessive loss of AECs and the failure of the reepithelization characteristic of pulmonary fibrosis. Recent studies strongly suggested a role for epithelial apoptosis as a key profibrotic event in lung fibrogenesis. Evidence in support of this viewpoint is summarized below.

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First, apoptosis of AECs is found both in the lungs of patients with IPF and in animal models of the disease. Fragmented DNA, a hallmark of apoptosis, was found in bronchiolar cells and AECs within lung biopsies from patients with IPF (Kuwano et al., 1996) and in the lungs of mice and rats with bleomycin-induced lung fibrosis (Hagimoto et al., 1997; Wang et al., 2000). This finding was confirmed by simultaneous double labeling of fragmented DNA and α-smooth muscle actin, a marker for myofibroblasts, in biopsies from patients with IPF (Uhal et al., 1998). Fragmented DNA in the alveolar epithelium was found frequently and immediately adjacent to α-smooth muscle actin-positive interstitial cells and foci of collagen. Thus epithelial apoptosis colocalizes with myofibroblasts where collagen deposition is severe, at least in patients with IPF. Very recently, apoptosis within AECs of fibrotic human lungs was reconfirmed by Barbas-Filho et al. (Barbas-Filho et al., 2001), also through the detection of fragmented DNA.

Consistent with those findings, the "death receptor" Fas was found to be expressed in AECs within the lungs of IPF patients by several independent research groups (Kazufumi et al., 1997; Domagala-Kulawik et al., 2000). Thereafter, increased circulating levels of soluble FasL were shown to correlate with disease activity in patients with IPF (Kuwano et al, 2002). In animal models, similar observations were obtained; Hagimoto et al. (1997) showed epithelial apoptosis and upregulation of Fas on the epithelium in bleomycin-induced pulmonary fibrosis in mice. Another study demonstrated that Fas is expressed on the luminal surface of a subset of alveolar type II cells in the mouse model (Fine et al., 1997).

Second, induction of apoptosis in the epithelium is sufficient to initiate a fibrotic response in animal models. Hagimoto et al. (1997) showed that intratracheal instillation of an

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antibody that activates Fas-induced apoptosis of bronchial and AECs (both of which express Fas constitutively) initiated a fibrotic response detectable 1 wk later. Moreover, knockout mice deficient in the receptor Fas were found to be resistant to the profibrotic effect of bleomycin (Kuwano et al., 1999). However, another study found that the development of bronchiolar and alveolar epithelial apoptosis and fibrosis after bleomycin instillation in the lungs in Fas-null *lpr* mice and *gld* mice was similar to development shown in wild-type mice (Aoshiba et al., 2000). Thus the role of Fas-induced apoptosis in the development of the pulmonary fibrotic response is still controversial; in addition, pathways other than Fas can initiate epithelial apoptosis and facilitate fibrogenesis.

Third, pharmacological blockade of apoptosis can prevent the fibrotic response. Wang et al. (Wang et al, 2000) first found that bleomycin-induced accumulation of lung collagens could be blocked by the angiotensin-converting enzyme (ACE) inhibitor captopril or by daily intraperitoneal injections of *N*-benzylcarboxy-Val-Ala-Asp-fluoromethylketone (ZVADfmk), a broad-spectrum inhibitor of caspases (cysteine proteases) required for the induction of apoptosis. Soon thereafter, Kuwano et al. (2001) confirmed the blockade by using the same caspase inhibitor (ZVADfmk) administered by aerosol to mice. Another strategy to interrupt AEC apoptosis proved effective in blocking bleomycin-induced pulmonary fibrosis; Inoshima et al (2004) showed that the forced expression of p21, predominantly in lung epithelial cells, exerted both antiapoptotic and antifibrotic effects. Thus the blockade of collagen deposition in vivo by inhibitors of apoptosis suggests that the fibrotic response is secondary to the apoptotic death of certain lung cell types. This premise in turn is consistent with the theories put forth by Witschi (1990) and Adamson and Bowden (1976) that alveolar reepithelialization is necessary to prevent subsequent

fibrogenesis after lung injury. Other data consistent with this theory include studies of keratinocyte growth factor (KGF), a potent proliferation and differentiation factor for alveolar type II cells known to promote alveolar epithelial repair (Stern et al., 2003). Both KGF and the related hepatocyte growth factor (HGF) prevented bleomycin-induced lung fibrosis in rats and mice (Yi et al., 1996; Deterding et al., 1997; Sugahara et al, 1998; Yi et al., 1998; Dohi et al., 2000).

4.3 Mechanism and signaling of epithelial apoptosis in fibrotic lung

Alterations in the expression of various antiapoptotic and proapoptotic factors likely regulate apoptosis of AECs. The proapoptotic factors p53 and p21 are upregulated in bronchiolar and AECs within lung biopsy specimens from patients with IPF and in bleomycin-induced pulmonary fibrosis animal models (Kuwano et al, 1996; Kuwano et al., 2000). Upregulation of the proapoptotic factor BAX in patients with diffuse alveolar damage and in bleomycin-induced pulmonary fibrosis may enhance the susceptibility of AECs to apoptosis (Guinee et al., 1997; Kuwano et al., 2000).

In vitro studies of epithelial cell lines or primary cells showed that JNK, a member of the MAPK family is involved in stress-induced apoptosis. Activation of JNK (Adler et al., 1995: Janssen et al., 1999; Lander et al., 1996) leads to phosphorylation of its targets, c-Jun and activating transcription factor (ATF)-2 (Ip et al., 1998; Schaeffer et al., 1999), and activation of downstream gene expression when epithelial cells are exposed to oxidative stress, which is a very important contributor to pulmonary injury and fibrosis. An in vivo study of p38 MAPK in bleomycin-induced pulmonary fibrosis showed that p38 MAPK and its substrate, ATF-2, were phosphorylated in BAL fluid cells after

intratracheal instillation of bleomycin. The phosphorylation of ATF-2 was inhibited by subcutaneous administration of a specific inhibitor of p38 MAPK, FR-167653. FR-167653 also prevented the apoptosis of lung cells and fibrosis induced by bleomycin administration (Matsuoka et al, 2002). Signaling pathways that can contribute to the apoptosis of AECs in IPF patients were investigated by Yoshida et al. (2002), who found that active ERK was decreased and active JNK was increased in epithelial cells and was accompanied by the progression of fibrosis. Activated p38 MAPK in epithelial cells was increased at the intermediate stage of fibrosis, in which the TUNEL-positive cells were predominantly detected (Yoshida et al., 2002).

5. Renin-Angiotensin System

5.1 General information

The conventional renin-angiotensin system (RAS) is an endocrine system known to regulate fluid homeostasis, electrolyte metabolism and blood pressure etc. Renin, an aspartyl protease, is secreted by the granular cells of the juxtaglomerular apparatus of the kidney. Synthesis and release of renin into the circulation is considered as the rate-limiting step of the RAS and is activated by decreased renal perfusion and plasma sodium concentration. Angiotensinogen (ANGEN), the precursor for angiotensin II (Ang II), is secreted by the liver and cleaved by circulating renin to form the decapeptide: angiotensin I (Ang I). Angiotensin converting enzyme primarily located at the endothelial surface of pulmonary capillaries converts Ang I to an octapeptide Ang II. Ang II is considered the primary effector molecule of the RAS (Bader et al., 1994; Griendling and Alexander, 1994).

5.2 Effects of Angiotensin II

ANG II exerts its effects through at least two subtypes of Ang II receptors, AT1 and AT2. These receptors are G-protein coupled receptors that have seven transmembrane domains (Murphy et al., 1991; Sasaki et al., 1991). The majority of physiological and pathophysiologic functions of ANG II are mediated by AT1 receptors. Those effects include, but are not limited to: 1) Vasoconstriction in arterial smooth muscle and concomitant increase of blood pressure; 2) Secretion of aldosterone from the adrenal gland, which increases renal sodium reabsorption; 3) Facilitated release of catecholamines in the sympathetic nerve, which increases cardiac output and total peripheral resistance and blood pressure; 4) Elevated sympathetic nerve activity and vasopressin secretion in the brain; 5) Enhanced cardiac contractility and ventricular hypertrophy; 6) Remodeling of the vascular wall resulting in increased total peripheral resistance (Geisterfer et al., 1988; Robertson and Nicholls, 1993); and 7) Direct action on the renal tubular cells to promote sodium and water reabsorption in the kidney (Deshmukh et al., 1998). ANG II is also proposed to play an important role in the pathogenesis of lung injury that will be discussed later in this chapter.

5.3 Angiotensin II signal pathway

There exist several signaling pathways to transduct the effects of ANG II (Timmermans et al., 1993; Lee and Severson, 1994). AT1 receptor-mediated signaling pathways have been more extensively studied than that AT2 mediated. AT1 receptors are guanine

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nucleotide-binding regulatory proteins (G proteins)-coupled. The signaling pathways mediated by AT1 receptors are as follows:

1) Increases intracellular [Ca^{2+}]:

The binding of ANG II to AT1 receptors activates the Gq protein and consequently phospholipase C-beta (PLCβ). PLC β hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP2) to generate inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to receptors on IP3-sensitive organelles with intracellular Ca^{2+} stores and stimulates the release of intracellular Ca^{2+}. In addition, binding of ANG II to AT1 receptor directly activates the cytoplasmic membrane-bound Ca^{2+} channels to allow the influx of Ca^{2+} into the cytoplasm. Both pathways lead to the elevation of intracellular Ca^{2+} concentration, which produce a variety of effects. Then Ca^{2+} binds to calmodulin, and the Ca^{2+}/ calmodulin complex activates a number of intracellular enzymes, such as ATPase and kinases that contribute to the cellular response. The binding of ANG II to AT1 receptors also activates phospholipase D (PLD), which hydrolyzes phosphatidylcholine to generate phosphatidic acid. Phosphatidic acid is then transformed into DAG by phosphatidate phosphohydrolase. DAG derived from phosphatidylcholine through PLD and phosphatidylinositol-4, 5-bisphosphate through PLC activates protein kinase C (PKC) leading to the phosphorylation of downstream proteins and cascade events to produce physiological response.

2) Increases Arachidonic Acid metabolites:

AT1 receptor activation also stimulates activation of phospholipase A2 (PLA2), which converts phosphatidylcholine to arachidonic acid. Arachidonic acid is metabolized to prostaglandins and thromboxane A2 by cyclooxygenase and to hydroxyeicosatetraenoic

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3) Decrease intracellular [cAMP]:

In some cells AT1 receptor activation leads to the activation of the inhibitory G-protein (Gi) and subsequently inhibition of adenylyl cyclase activity and thus the decreased level of intracellular cyclic AMP (cAMP). Decreased intracellular [cAMP] level leads to the inhibited activity of protein kinase A (PKA) and thereby supressed phosphorylation state of substrates for PKA causing corresponding biological effect.

4) Increases growth factors production:

Activation of AT1 receptors by ANG II stimulates cell growth by regulating gene expression of transcriptional factors of MAP kinase and JAK tyrosine kinase(s) pathway such as AP1 (Fos and Jun) and STAT(s) (Bhat et al., 1994). AP1 (Fos and Jun) and STAT(s) activate the transcription of a number of growth factors and extracellular matrix proteins such as TGF β and collagen.

In addition, internalized ANG II together with intracellular ANG II can directly bind to DNA associated AT1 like receptors and regulate gene transcriptions of renin, ANGEN, PDGF (Re, 2004).

6. Pulmonary angiotensin system in idiopathic pulmonary fibrosis

6.1 Local pulmonary angiotensin system:

Recently a few studies suggested the existence of "local" angiotensin systems in various organs and tissues. For example, the ANG II concentrations in the interstitial compartment of heart and eye were found to be 5-100 fold higher (about 50 –500pM)

than that in plasma (~5-10pM) (van Kats et al., 1998; Danser et al., 1994). The higher interstitial levels of ANG II compared to the circulating level could not be explained by diffusion and/or receptor-mediated uptake of circulating angiotensin II. These results thereby suggest that tissue angiotensin II is largely, if not completely, synthesized locally. Furthermore, cultured cells from various organs including heart (Lindpaintner et al., 1988), vascular endothelium (Li et al., 1999), brain (Campbell et al., 1986; Dzau et al., 1982; Ohkubo et al., 1986) and lung (Filippatos et al., 2001) were shown to express the RAS components such as ANGEN, ANG II and their corresponding converting enzymes and angiotensin receptors. In contrast to the classical endocrine system of RAS in which angiotensin II is delivered to tissues via circulating blood, local angiotensin systems can be from either "intrinsic "(independent of the endocrine RAS) or "extrinsic" (relying on the endocrine RAS as its components sources) sources.

6.2 Pulmonary angiotensin system components:

The local pulmonary angiotensin system appears to consist of ANGEN, ANG I, ANG II and their corresponding converting enzymes and angiotensin receptors.

6.2.1 ANGEN:

It was shown that two types of cells in the lung could produce ANGEN in vitro under certain conditions: AECs and myofibroblasts. For example, primary AECs could synthesize and secrete ANGEN, which was converted to ANG II when undergoing apoptosis induced by Fas ligand (Wang et al., 1999), TNF-alpha (Wang et al., 2000), amiodarone (Bargout et al., 2000) and bleomycin (Li et al., 2003). Primary myofibroblasts isolated from fibrotic human lungs (IPF biopsies) expressed the ANG II

precursor ANGEN mRNA and protein (Wang et al., 1999), suggesting that human lung myofibroblast can synthesize ANGEN in culture. Furthermore, preincubation of the culture medium of myofibroblast with purified renin and ACE increased the Enzyme-Linked Immunosorbent Assay (ELISA)- detectable ANG II concentration eight folds, indicating that there are abundant ANGEN synthesized constitutively waiting to be converted to ANG II (Wang et al., 1999). Despite of evidence from in vitro studies, there is no in vivo study demonstrating the existence of lung-derived ANGEN.

6.2.2 Renin and Renin- like enzymes:

Production of the local angiotensin systems can use elements that are "extrinsic" such as relying on the renin from the endocrine RAS. In many organs and tissues such as the heart and vessel wall, the synthesis of local ANG II was shown to depend on the uptake of circulating renin and/ or prorenin either via diffusion into the interstitial space or through binding to prorenin receptors (Re, 2004). No renin mRNA has been detected in the lungs and it is unclear whether there exists samilar mechanism in the lung to locally produce ANG II. Nevertheless, there exist non-renin proteases capable of generating angiotensins in the lung such as cathepsin D. Cathepsin D might be responsible for the synthesis of generating angiotensin I from ANGEN and subsequent synthesis of ANG II in some cells like vascular interstitial cells (Weber et al., 1995). Other proteases, such as the enzyme tonin, can also generate angiotensin I from angiotensinogen (Re, 2004).

6.2.3 ACE and ACE-like enzymes:

There exist two forms of ACE. One is membrane-bound and the other is soluble. The membrane-bound ACE is attached to the plasma membrane of endothelial cells via a short hydrophobic sequence at the C-terminus and is found in most organs. The soluble ACE, detached form of the membrane-bound ACE, is found in most of the body fluid including lymph, plasma, cerebrospinal fluid and amniotic fluid (Erdos, 1990). The soluble form lacks the hydrophobic terminus and is derived from the membrane-bound form via post-translational cleavage by an ACE-secretase. Membrane-bound ACE is abundantly expressed by the vascular endothelium of the pulmonary circulation and is primarily responsible for the conversion of ANG I to ANG II in the circulation (Oparil et al., 1971). The extent of the conversion of ANGI to ANG II is a function of the vascular surface area and the transit time of blood circulating through the lung (Oparil et al., 1971). The contribution of soluble ACE to ANG II generation is negligible (Ng and Vane 1968; Admiraal et al., 1993).

Primary cultures of AECs were shown to express the ACE mRNA (Wang et al., 1999a, b). Other non-ACE enzymes that also convert angiotensin I to angiotensin II include chymase found in human cardiac tissue, cathepsin G and ACE2, a recently described enzyme (Re, 2004).

6.2.4 Angiotensin II receptors:

Bullock et al. demonstrated that in the human lung AT1 receptor mRNA and protein were localized on vascular smooth muscle cells, macrophages and in the stroma underlying the airway epithelium, possibily relating to underlying fibroblasts (Bullock et al., 2001). However, the AT1 receptor protein was not detected in the epithelium although there was

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a low level of mRNA. In contrast, AT2 receptor RNA and protein was strongly stained in the epithelium, particularly on the bronchial epithelial cell brush border and the underlying mucous glands as well as some endothelial cells (Bullock et al., 2001). This study, however, did not exam the AT1 and AT2 receptors in the alveolar epithelial cells (Bullock et al., 2001). Consistently, another study on rat lung reported that AT1 was localized on alveolar macrophages, alveolar type II cells, vascular smooth muscle cells, endothelial cells and fibroblasts (Otsuka et al., 2004). AT1 expression in the lung increased markedly after intratracheal administration of BLM, which is known to generate fibrosis, suggesting that angiotensin II as a ligand of AT1 is involved in pulmonary fibrosis (Otsuka et al., 2004).

6.3 Potential roles of pulmonary ANG II in pulmonary fibrosis:

6.3.1 Pulmonary angiotensin system is linked to lung epithelial apoptosis:

Purified ANG II was shown to induce apoptosis of AECs in culture (Wang et al., 1999). This effect was mediated by AT1 receptors (Papp et al., 2002). It was also shown that apoptosis of AECs induced by Fas Ligand (Wang et al., 1999), TNF-alpha (Wang et al 2000), amiodarone (Bargout et al., 2000) and bleomycin (Li et al., 2003) required angiotensin synthesis de novo as AECs apoptosis could be blocked by the antisense oligonucleotides against mRNAs of the ANGEN, ACE inhibitors, and ANG II receptor (AT receptor) antagonists in vitro. Furthermore, human lung myofibroblast–derived inducers of alveolar epithelial apoptosis were identified as angiotensin peptides (Wang et al., 1999). These data suggest that ANG II is the "Master Switch" for apoptosis of AECs induced by Fas Ligand, TNF-alpha, amiodarone and bleomycin. This "Master Switch"

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function of ANG II for AECs apoptosis could account for the role of angiotensin system in fibrogenesis since apotosis of AECs is essential for the development of pulmonary fibrosis.

6.3.2 Mitogenic for fibroblasts and Activate TGF beta expression:

ANG II stimulates fetal and adult human lung fibroblast proliferation *in vitro* via the AT1 receptor and the autocrine action of transforming growth factor beta (TGF β) (Marshall et al., 2000). ANG II also increased procollagen synthesis by human lung fibroblasts *in vitro* via AT1 receptors (Marshall et al., 2000).

6.3.3 Inhibition of the fibrinolytic pathway:

Mice with deficient fibrinolytic system developed pulmonary fibrosis in response to bleomycin, suggesting that IPF could result from compromised fibrolytic capability (Swaisgood et al., 2000). Plasminogen activator inhibitor 1 (PAI-1) inhibits plasmin and subsequently decreases the fibrinolytic capability promoting the deposition of collagen and other extracellular matrixs in bleomycin-induced lung injury in animal models (Eitzman et al., 1996; Swiderski et al., 1998). ANG II has been shown in rat microvessel endothelial cells to control thrombosis by inducing, PAI-1, the inhibitor for extracellular matrix turnover and fibrinolysis (Nishimura et al., 1997). In addition, in a rat model used to investigate the cardiac vasculopathy mediated by nuclear factor-κB and activator protein-1, ANG II was shown to regulate the thrombogenic pathway by increasing the expression of tissue factor which can convert fibrinogen into fibrin (Muller et al., 2000).

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Hence, ANG II can break the balance between the fibrolytic and antifibrolytic process and induce an environment favoring the deposition of collagen.

6.3.4 Downregulation of hepatocyte growth factor (HGF) expression:

HGF has been shown to be important in suppressing bleomycin-induced fibrosis in mice, suggesting the antifibrotic function of HGF (Yaekashiwa et al., 1997). Furthermore, Taniyama et al. (2000) using a cardiomyopathic hamster model showed that HGF prevented myocardial fibrosis and that both ANG II and TGF-beta are strong inhibitors for local HGF production. Therefore, ANG II may have profibrotic role by downregulation of the hepatocyte growth factor (HGF) expression in the lung.

6.4 Angiotensin system is involved in pulmonary fibrosis:

The angiotensin system consists of ANGEN, ANGI, ANG II, corresponding converting enzymes and angiotensin II receptors (Proudn 1991; Filippatos et al., 2001). There is much evidence suggesting that angiotensin system is involved in pulmonary fibrosis.

6.4.1. Increased levels of converting enzymes and AT1 receptors in IPF

A number of studies show that enzymes required for the production of local angiotensin peptide were upregulated in pulmonary fibrosis. ACE which converts ANGI to ANG II is upregulated in both human and animal fibrotic lungs. ACE activity was shown to be increased in the bronchoalveolar lavage fluid (BALF) of animals with bleomycin-induced pulmonary fibrosis (Venkatesan, et al.1997) and human patient with IPF (Specks et al., 1990). Furthermore, bleomycin upregulated gene expression and enzyme activity of ACE

in bovine pulmonary artery endothelial cells (BPAEC) (Day, et al., 2001). Those data suggest ACE may play a critical role in development of pulmonary fibrosis. In addition, the study to examine the incidence of D allele of the insertion/ deletion (I/D) polymorphism of ACE in patients with interstitial pneumonia and moderate to severe pulmonary fibrosis showed that the incidence of the D allele which confers a higher ACE expression was approximately 15% higher in the study population than in the general population (Morrison et al., 2001). Although the sample size is limited, these data indicate that polymorphisms that confer higher levels of ACE predispose patients to lung fibrosis and thus support the hypothesis that ACE and its product ANG II are involved in the pathogenesis of human pulmonary fibrosis.

Cathepsin D (Cat D) is one of the enzymes capable of cleaving ANGEN to ANG I. Cat D was upregulated in both animal and human fibrotic lung. Our lab showed that Cat D immunolabeling in alveolar wall cells morphologically consistent with type I and type II cells increased following intratracheal administration of bleomycin. Meanwhile, soluble Cat D enzymatic activity was elevated in cell-free bronchoalveolar lavage fluid (BALF) from the same lungs. Upregulation of Cat D was also been shown in human lungs from PF patients (Kasper et al., 1996) and was induced in L132 lung cells during apoptosis (Kasper et al., 1999).

AT1 receptors were also upregulated in animal fibrotic lung (Otsuka et al., 2004). AT1 expression in the lung increased markedly after intratracheal administration of bleomycin, suggesting that angiotensin II as a ligand of AT1 is involved in pulmonary fibrosis (Otsuka et al., 2004).

6.4.2. Blockade of experimental lung fibrosis by angiotensin system antagonists

Application of ACE inhibitor, which presumably inhibits ANG II production, has been shown to attenuate experimental pulmonary fibrosis in animal models induced by various agents. For example, ACE inhibitor captopril exerted inhibitory effects on monocrotaline (Molteni et al, 1985) as well as γ irradiation-induced lung fibrosis in rats (Ward et al, 1990). Captopril also inhibited the proliferation of human lung fibroblast in vitro (Nguyen et al., 1994). More recently, the AT1 receptor-selective antagonists L158809 as well as the nonthiol ACE inhibitor enalapril were shown to have similar effects on radiation-induced pulmonary fibrosis in rats (Molteni et al., 2000). Uhal et al demonstrated that captopril prevented collagen deposition in bleomycin-treated rats (Wang et al., 2000). It was later shown that captopril and AT1 selective antagonist losartan blocked the amiodarone induced pulmonary fibrosis in rats. (Uhal et al., 2002) These results combined together indicate that ANG II plays an important role in lung fibrogenesis via AT1-receptor.

6.4.3. Blockade of apoptosis by ACE inhibitor captopril or caspases inhibitors blocks pulmonary fibrosis

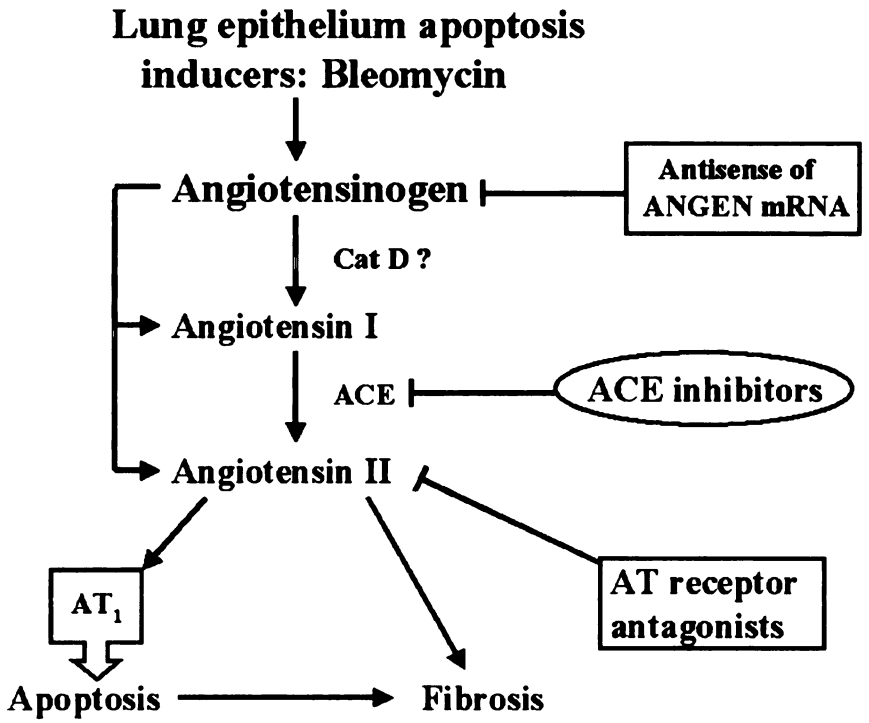
Caspase is one of the key enzymes mediating apoptosis. Collagen deposition and epithelial apoptosis were blocked by both captopril and ZVADfmk, a broad-spectrum inhibitor of caspases in intratracheally administered bleomycin-induced pulmonary fibrosis in rats (Wang et al., 2000). Another group confirmed this result by showing that the same caspase inhibitor delivered by inhalation attenuated bleomycin-induced

pulmonary fibrosis in mice (Kuwano et al., 2001). Those results suggest that both ANG II and epithelial apoptosis are required for lung fibrogenesis.

7. Working hypothesis:

The pulmonary angiotensin system including its components angiotensinogen, cathepsin D and AT1 receptor plays an essential role in the development of bleomycin-induced pulmonary fibrosis at least in part through ANG II-AT1 receptor pathway mediated apoptosis of alveolar epithelial cells.

The role of pulmonary angiotensin system during PF can be summarized in the following figure (Fig. 1.1):



Bleomycin induces apoptosis of lung AECs by upregulation of ANGEN gene expression. Synthesized ANGEN can be converted to ANG I by cathepsin D (Cat D). ANG I can be further converted to ANG II by ACE. ANG II can induce apoptosis of the AECs through

AT1 receptor, which will cause excessive loss of AECs and insufficient epithelial repair. ANG II produced by apoptotic epithelial cells can also directly contribute to the fibroblast activation. Both ways will lead to lung fibrosis. Synthesis of ANGEN is the key upstream event in this process, which can be blocked by the antisense against ANGEN mRNA.

8. Significance:

Our study is one of the first studies to determine the existence of intrinsic pulmonary angiotensin system and its role in the development of bleomycin induced pulmonary fibrosis. This study helps us to better understand the mechanism of lung fibrogenesis with regard to the pulmonary angiotensin system, which will likely lead us to the finding of effective therapeutic or preventive approaches for IPF.

Chapter 2

HYPOTHESIS AND SPECIFIC AIMS

Treatment for IPF targeting the suppression of inflammation has not been successful, suggesting that inflammation is not the sole mechanism underlying lung fibrogenesis. Mortality of IPF patients is not dependent on severity of inflammation, but correlates well with the presence of "fibroblastic foci" and adjacent failure of reepithelization (Selman et al., 2001). The normal alveolar epithelium has "anti-fibrotic" functions including inhibiting fibroblast proliferation. Loss of alveolar epithelial cells (AECs) and failure of reepithelization characterized in pulmonary fibrosis can be considered as profibrotic and are believed to initiate the fibrotic lesion.

The loss of AECs could result from necrosis and/ or apoptosis. Increased level of apoptosis was found in AECs in experimental and human pulmonary fibrosis. One indication that the angiotensin system is involved in fibrogenesis is findings that blockade of angiotensin systems blocked Pulmonary Fibrosis (PF) at least in several animal models. Our lab showed that angiotensin II (ANG II) induces apoptosis of the primary AECs through ANG II type I (AT1) receptor in vitro. Apoptosis of AECs induced by Fas Ligand, TNF-alpha, and amiodarone requires angiotensin synthesis de novo as AECs apoptosis can be blocked by the antisense oligonucleotide against the mRNA of the angiotensinogen (ANGEN), angiotensin converting enzyme (ACE) inhibitors, and ANG II receptor (AT receptor) antagonists in vitro. Taken together, those data suggest that ANG II, the processed product of ANGEN, is the key to regulate apoptosis of AECs and subsequent lung fibrosis.

The existence of "local angiotensin system" in the lung, which is independent of the endocrine RAS, is supported by studies demonstrating the expression of angiotensin system components in cultured primary rat AECs in response to Fas Ligand, TNF-alpha, and amiodarone and myofibroblasts from human fibrotic lungs. Bleomycin-induced rat and mouse pulmonary fibrosis model is a well-studied model for fibrogenesis. But there is much still unknown about the components and roles of pulmonary angiotensin system in bleomycin-induced pulmonary fibrosis.

Overall Hypothesis:

The pulmonary angiotensin system including its components angiotensinogen, cathepsin D and AT1 receptor plays an essential role in the development of bleomycin-induced pulmonary fibrosis at least in part through ANG II-AT1 receptor pathway mediated apoptosis of alveolar epithelial cells.

Specific Aims:

1) To examine if bleomycin-induced apoptosis of alveolar epithelial cells requires angiotensin synthesis de novo.

Specific hypothesis: AEC apoptosis in response to bleomycin (BLEO) requires ANG synthesis and might be inhibited by ANG system antagonists.

2) To identify the primary aspartyl protease that could convert angiotensinogen to ANGI and contribute to pulmonary angiotensin system in vitro.

Specific hypothesis: Cat D is required for AEC apoptosis in response to bleomycin at least in part by converting angiotensinogen to ANGI.

3) To examine if angiotensin receptor AT1 is essential for AEC apoptosis and lung fibrosis *in vivo*.

Specific hypothesis: Administration of the AT1-selective receptor antagonist and deletion of the AT1a receptor gene block BLEO-induced AEC apoptosis and lung fibrosis.

4) To identify the cellular sources of lung-derived ANGEN in situ:

Specific hypothesis: The apoptotic type II alveolar epithelial cells and myofibroblasts are the major cellular sources of lung-derived ANGEN.

5) To examine the effect of blockade of lung-derived ANGEN on pulmonary fibrosis.

Specific hypothesis: Administration of the antisense against ANGEN messenger RNA attenuates bleomycin-induced AEC apoptosis and lung fibrosis.

Chapter 3

BLEOMYCIN-INDUCED APOPTOSIS OF ALVEOLAR EPITHELIAL CELLS REQUIRES ANGIOTENSIN SYNTHESIS *DE NOVO*

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ABSTRACT

Primary cultures of rat type II alveolar epithelial cells (AECs) or human AEC-derived A549 cells, when exposed to bleomycin (BLEO), exhibited dose-dependent apoptosis detected by altered nuclear morphology, fragmentation of DNA, activation of Caspase 3 and net cell loss over time. In both cell culture models, exposure to BLEO caused time-dependent increases in angiotensinogen (ANGEN) mRNA. Antisense oligonucleotides against ANGEN mRNA inhibited BLEO-induced apoptosis of rat AEC or A549 cells by 83% and 84%, respectively ($p<0.01$ and $p<0.05$) and prevented BLEO-induced net cell loss. Apoptosis of rat AECs or A549 cells in response to BLEO was inhibited 91% by the ACE inhibitor captopril or by 82%, respectively, by neutralizing antibodies specific for ANGII (both $p<0.01$). Antagonists of ANG receptor AT1 (losartan, L158809 or saralasin), but not an AT2-selective blocker (PD123319), inhibited BLEO-induced apoptosis of either rat AECs (79%, $p<0.01$) or A549 cells (83%, $p<0.01$) and also reduced the activity of Caspase 3 by 52% ($p<0.05$). These data indicate that BLEO, like FasL or TNF-α, induces transactivation of ANG synthesis *de novo* that is required for AEC apoptosis. They also support the theory that ANG system antagonists have potential for the blockade of AEC apoptosis *in situ*.

INTRODUCTION

Alveolar epithelial cells (AECs) have many important roles that are critical to normal lung function (Mason and Williams, 1991). The death of AECs by apoptosis is now believed to be an important event in the pathogenesis of lung fibrosis (Haschek and Witschi, 1979) and in more acute lung injury (Matute-Bello et al., 2001; Uhal, 2001). A variety of investigations have implicated important roles for key molecules such as tumor necrosis factor alpha (TNF-α) and Fas ligand (Fas_L), both known inducers of apoptosis in a variety of cell types, in the events that lead to fibrogenesis in the lung (Hagimoto et al., 1997a; Hagimoto et al., 1997b; Ortiz et al., 1998).

In earlier work (Wang et al., 2000; Wang et al., 1999), we showed that exposure of either primary cultures of rat AECs or the human AEC-derived A549 cell line to Fas_L or TNF-α increases angiotensinogen (ANGEN) mRNA and protein, and evokes its subsequent conversion to angiotensin II (ANGII). Moreover, we found that transactivation of ANGII synthesis is required for AEC apoptosis in response to TNF-α or Fas ligand. Thus, AEC death in response to these could agents could be blocked by ANG receptor antagonists or inhibitors of ANG converting enzyme (ACEis), at least *in vitro*. Studies of another inducer of AEC apoptosis, the antiarrythmic agent amiodarone, also showed that antagonists of ANG production or receptor interaction could prevent apoptosis of AECs in response to this benzofuran compound (Bargout et al., 2000).

For these reasons we hypothesized that AEC apoptosis in response to bleomycin (BLEO) might also require ANG synthesis and might therefore be inhibitable by ANG system antagonists. We report here that bleomycin, if applied to rat or human AECs *in vitro*, induces the expression of angiotensinogen mRNA and subsequent apoptosis that can be

blocked by ANGEN antisense oligonucleotides, by ACEis or by ANG receptor antagonists of the AT1-selective subtype.

MATERIALS AND METHODS

Reagents and materials: The AT1-selective antagonists L158809 and losartan were obtained from Merck and Co., West Point, PA. The AT2-selective antagonist PD123319 was obtained from Parke Davis Research Division, Ann Arbor, MI. The caspase inhibitor ZVAD-fmk (N-benzylcarboxy-Val-Ala-Asp- [O-Me]-CH2F) was obtained from Kamiya Biomedical, Seattle, WA. DEVDfmk (Asp-Glu-Val-Asp- [O-Me]-CH2F) was obtained from Pharmingen, San Diego, CA. Alkaline phosphatase-conjugated streptavidin, digoxigenin-labeled deoxyuridine trisphosphate (dig-dUTP) and biotinylated deoxyuridine trisphosphate (bio-dUTP) were obtained from Boerhinger Mannhiem, Indianapolis, IN. Bleomycin (BLEO), anti-angiotensin antibodies, ATA, captopril and saralasin were obtained from Sigma Chemical Co., Saint Louis, MO. Reagents for detection of alkaline phosphatase and other secondary reagents for in situ end labeling of DNA or western blotting were from sources described earlier (Wang et al., 2000). All other materials were of reagent grade and were obtained from Sigma Chemical Co., Saint Louis, MO.

Cell culture: The human lung adenocarcinoma cell line A549 was obtained from the American Type Cell Culture Collection and cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). Primary alveolar epithelial cells isolated from adult male Wistar rats as described earlier (Wang et al., 1999). The primary cells were studied at day two of culture, a time at which they are type II cell-like by accepted morphologic and biochemical criteria (Paine and Simon, 1996). Primary cell preparations were of better than 90% purity assessed by acridine orange staining as described previously (Wang et al., 2000; Wang et al., 1999). All cells were grown in 24-

or 6-well chambers and were analyzed at subconfluent densities of 80-90%. All subsequent incubations with BLEO and/or other test agents were performed in serum-free medium. The cells were exposed to caspase inhibitors or antagonists of the angiotensin system 30 minutes before exposure to BLEO for 1-20 hours as indicated.

Quantitation of apoptosis and cell loss: Detection of apoptotic cells with propidium iodide (PI) was conducted as described earlier (Wang et al., 2000; Wang et al., 1999) following digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5ug/ml PI. In these assays, detached cells were retained by centrifugation of the 24-well culture vessels during fixation with 70% ethanol. Cells with discrete nuclear fragments containing condensed chromatin were scored as apoptotic. As in earlier publications, the induction of apoptosis was verified by in situ end labeling (ISEL) of fragmented DNA (Uhal et al., 1998; Wang et al., 2000; see Figure 3.1). Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group.

Cell loss over 20 hours of culture was quantitated by cell counts of the adherent plus detached cell populations. These were obtained following centrifugation of the culture vessels as described in the preceding paragraph, without prior washing. Thus, detached cells (routinely less than 10% of the total cell number) were included in the cell loss data. Total cell counts (attached plus detached) were scored over a minimum of 200 nuclei per field, 4 fields per well with a minimum of 6 culture wells, or 4,800 nuclei, per treatment group. Data from each treatment group were compiled and analyzed by ANOVA followed by Student-Newman-Keul's post hoc analysis.

Detection and quantitation of caspase 3 activity: Activation of Caspase 3 was detected through: a) immunolabeling of fixed cells adherent to plastic culture surfaces with an antibody that recognizes only the active form of the enzyme (Biovision, Mountain View, CA). The primary antibody was detected with an alkaline phosphatase-conjugated secondary antibody followed by nitro-blue tetrazolium. The enzymatic activity of Caspase 3 was measured in adherent cells incubated for 20 hours with the membrane permeable substrate Ac-DEVD-AMC (Upstate Biotech, Saranac Lake, NY) at 50uM. Quantitation of the fluorescent product was achieved with a Biotek FL600 fluorescence plate reader. Fluorescence values were normalized to cell number determined on the same culture well after cell fixing and staining of DNA with propidium iodide (Uhal and Rannels, 1991).

RTPCR and antisense experiments: Semiquantitative reverse transcriptase polymerase chain reaction was performed as described earlier (Wang et al., 2000; Wang et al., 1999). The annealing temperatures for PCR reactions were optimized for each primer by preliminary trials. All PCR amplifications were terminated at or near the center of the linear range for each gene product analyzed, as determined by sequential withdrawal of sample at 5-cycle intervals between 20 and 40 cycles (not shown). The identity of expressed genes was determined by expected size of the PCR product in 1.6% agarose gels.

For RTPCR of rat-specific gene products, the following primers were used: for angiotensinogen, coding = 5'-CCTCGCTCTCTGGACTTATC-3', and uncoding = 5'-CAGACACTGAGGTGCTGTTG-3', which yields a PCR product of 226bp by single-step RTPCR (Pierzchalski et al., 1997). For □-microglobulin, the primers used were:

coding = 5' -CTCCCCAAA-TTCAAGTGTACTCTCG-3', and uncoding = 5'-GAGTGACGTGTTTAACTCTGCA-AGC-3', which yields a product of 249bp (Katwa, et al., 1995). For RT-PCR from human A549 cells, the following primers were used: for angiotensinogen, coding = 5'GCTTTC-AACACCTACGTCCA3', and uncoding = 5'AGCTGTTGGGTAGACTCTGT3'. These primers yield a final PCR product of 509bp (Lai et al., 1998). For □-actin, single-step RTPCR was used with the primers: coding = 5'AGG-CCAACCGCGAGAAGATGACC3', and uncoding = 5'GAAGTCCAGGGCGACGT-AGC3', which produces a PCR product of 332bp (Ponte et al., 1984).

For antisense studies, phosphorothioated control and antisense oligonucleotides against angiotensinogen (18-mers) were synthesized and transfected into A549 cells or primary rat AEC (both at 40nM final concentration) using the lipofectin reagent OligofectAMINE (Invitrogen Life Technologies, Grand Island, NY) at 4ul/ml as the vehicle, diluted in the OPTIMEM medium accompanying the lipofectin. The control nucleotides were of the same length and base composition as the antisense, but with scrambled sequence. The oligonucleotide: lipofectin ratio was optimized (over a 4hr tranfection) to yield transfection efficiencies of 50-75% with no apparent cell loss or detachment. Transfection efficiency was monitored with FITC-labeled 25-mer oligonucleotide for luciferase (not shown). Transfections were conducted for 4 hours followed by 5 times washing with serum-free cell culture medium; immediately thereafter, BLEO or vehicle was applied as described above for 20 hours. The transfection protocol itself had no significant effect on basal or BLEO-induced apoptosis (see Results). Phosphorothioated oligonucleotides used for transfection were: (ANGEN antisense) 5-

CCGTGGGAGTCATCACGG-3', and (ANGEN scramble) 5'-CAGGGATCTCTGGCGGAC-3' as described by Phillips et al. (Phillips et al., 1994).

Attention: Images in this dissertation are presented in color.

RESULTS

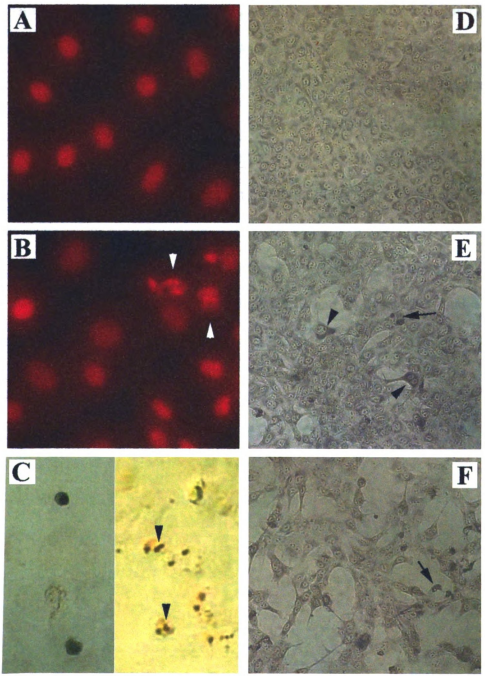
Exposure of primary cultures of rat AECs or A549 cells to BLEO for 20 hours caused apoptosis detectable by nuclear fragmentation, DNA fragmentation and by immunolabeling of the active form of Caspase 3 (Figure 3.1). Although these markers were detected in a minor fraction of the cells, the apoptosis induced was sufficient to reduce the total cell number significantly over time (see Figure 3.1D-F and quantitation in Figure 3.7B). Scoring of fragmented nuclei revealed dose-dependent apoptosis that reached statistical significance beginning at 0.5mU/ml in primary AECs (Figure 3.2) and at 1mU/ml in A549 cells (not shown). The nuclear fragmentation was blocked by the broad-spectrum caspase inhibitor ZVAD-fmk (60uM) or by the endonuclease inhibitor aurintricarboxylic acid (ATA, 10uM), confirming the specificity of the assay for apoptosis. Apoptosis of the rat AECs also was blocked by the ACE inhibitor captopril (CAPTO, 500ng/ml) and by the nonselective ANG receptor antagonist saralasin (SARAL, 50ug/ml), in agreement with earlier studies of Fas L and TNFα-induced AEC apoptosis (Wang et al., 2000; Wang et al., 1999).

In a separate experiment (Figure 3.3), BLEO-induced apoptosis of primary AECs was blocked by the Caspase 3-selective blocker DEVD-fmk (60uM) and by the ANG receptor subtype AT1-selective blocker losartan (10^{-6}M, $p<0.01$), suggesting that subtype AT1 mediates BLEO-induced apoptosis as it does AEC apoptosis in response to ANGII (Papp et al., 2002). This was found to be the case in human AECs as well (Figure 3.4); BLEO-induced apoptosis of A549 cells was inhibited 83% by the AT1-selective blocker L158809, but was not reduced by the AT2-selective antagonist PD123319. Moreover, BLEO-induced apoptosis was also prevented by a neutralizing antibody specific for

ANGII (anti-ANGII), but not by an isotype-matched nonimmune immunoglobulin (N.S.IgG). Further, the total enzymatic activity of Caspase 3 was elevated by exposure of A549 cells to BLEO (Figure 3.5) but the increase was inhibited 52% by saralasin.

These data suggested that BLEO induces ANGII synthesis *de novo* in primary AECs and A549 cells. Consistent with this theory, semiquantitative RTPCR for angiotensinogen (ANGEN) revealed more abundant ANGEN mRNA in primary AECs at 3 hours and especially at 20 hours after challenge with 25mU/ml BLEO (Figure 3.6A). In A549 cells (Figure 3.6B), 25mU/ml BLEO stimulated a significant increase in ANGEN mRNA that was detectable at 1 hour after addition of BLEO and increased by 7 hours.

To determine if functional ANGEN mRNA is required for the apoptotic response to BLEO, phosphorothioated antisense or scrambled-sequence control oligonucleotides against ANGEN mRNA were transfected into rat AECs and A549 cells immediately before challenge with BLEO for an additional 20 hours. As shown in Figure 3.7, BLEO-induced apoptosis of primary AECs (Panel A) was inhibited by 83% by the ANGEN antisense but not by the scrambled control oligonucleotides (+scram). In A549 cells (B), the antisense also reduced BLEO-induced apoptosis by 84% but the scrambled oligonucleotide had no significant effect. Moreover, exposure to BLEO for 20 hours reduced the total cell number of A549 cells (attached plus detached, bottom panel) by 43%, but the ANGEN antisense prevented the BLEO-induced cell loss.


A
D
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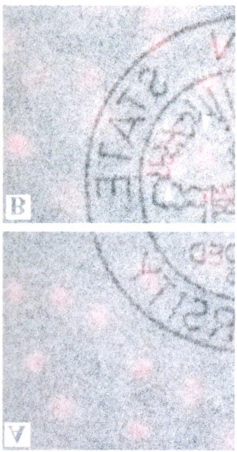

B
A

Figure 3.1: Detection of apoptosis in primary AECs and A549 cells. A, B and C: Primary cultures of AECs were exposed to vehicle (A) or BLEO (B and C) at 25mU/ml for 20hr and were then fixed in 70% ethanol without washing (see Methods). Cells exhibiting chromatin condensation and nuclear fragmentation with propidium iodide (arrowheads, B) were scored as described earlier (27 and Methods). C: BLEO-exposed cells were fixed and prepared for in situ end labeling (ISEL, left) or TUNEL (right) of fragmented DNA (25); note colocalization of label (blue or brown, respectively) in nuclear fragments (arrowheads) identified by propidium in B. D, E and F: A549 cells exposed to vehicle (D) or BLEO at 25mU/ml (E) or 100mU/ml (F) for 20hrs were fixed and prepared for immunolabeling for the active form of Caspase 3 (see Methods). Note labeling of active Caspase 3 (purple) in cells with either normal morphology (arrowheads, E) or in cells with condensed cytoplasm and nucleus (arrows, E and F). Note also reduced total cell number with increasing BLEO doses (E and F).

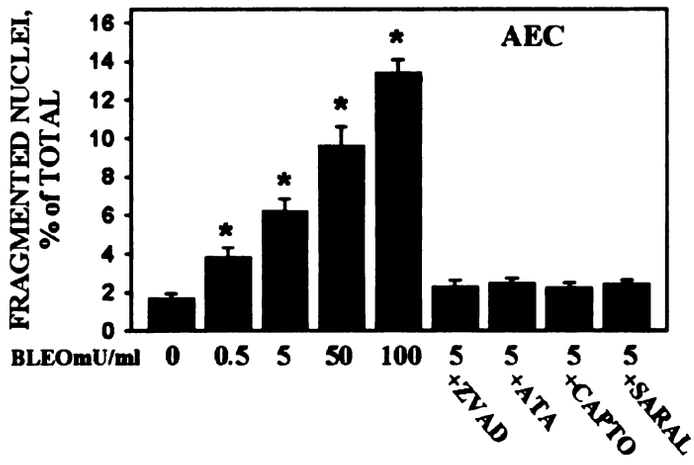


Figure 3.2: Dose-dependent induction of nuclear fragmentation by bleomycin (BLEO) in primary rat AECs and blockade by inhibitors of caspases, endonucleases, ANG converting enzyme (ACE) and ANG-receptor interaction. Rat AECs were isolated and challenged with the indicated concentrations of BLEO on Day 2 of primary culture (see Methods). Putative inhibitors were added 30 minutes prior to addition of BLEO; nuclear fragmentation was scored as described in Fig.3.1B and Methods. ZVAD = ZVAD-fmk (N-benzylcarboxy-Val-Ala-Asp-[O-Me]-CH_2F, 60uM); ATA = aurintricarboxylic acid 10uM); CAPTO = captopril (500ng/ml); SARAL = saralasin (50ug/ml). Bars are the mean $\pm$ S.E.M. of at least 4 observations; * = $p<0.05$ versus control (0.0 BLEO).

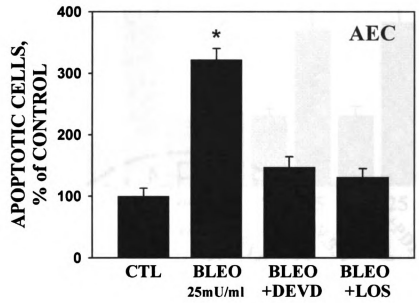


Figure 3.3: Blockade of bleomycin-induced apoptosis in primary AECs by selective caspase or ANG receptor blockers. Rat AECs were isolated and challenged with 25mU/ml BLEO alone or in the presence of the Caspase 3-selective inhibitor DEVD-fmk (60uM) or the ANG receptor AT1-selective antagonist losartan (LOS, 10^{-6}M). Control cultures (CTL) received BLEO and blocker vehicles only. Nuclear fragmentation was scored as described in Fig.1 and Methods. Bars are the mean $\pm$ S.E.M. of at least 4 observations; * = $p<0.05$ versus control.

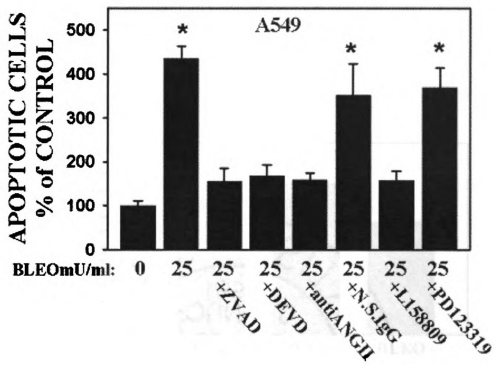


Figure 3.4: Inhibition of bleomycin-induced apoptosis of A549 cells by inhibitors of caspases or ANGII-receptor interaction. A549 cells were cultured to 8% confluence as described in Methods and were challenged with 25mU/ml BLEO in the presence or absence of the indicated compounds. Anti-ANGII = neutralizing antibody to ANGII (1ug/ml); N.S.IgG = isotype matched nonimmune immunoglobulin (1ug/ml); L158809 = ANG receptor AT1-selective antagonist (10^{-6} M); PD123319 = ANG receptor AT2-selective antagonist (10^{-6}M). Other abbreviations and concentrations are the same as in Figs. 2 and 3. Nuclear fragmentation was scored as described in Fig.3.1 and Methods. Bars are the mean $\pm$ S.E.M. of at least 4 observations; * = $p<0.05$ versus control (0 dose).

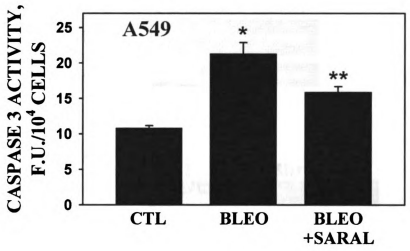


Figure 3.5. Induction of caspase 3 activity by bleomycin and inhibition by an ANG receptor antagonist. A549 cells were challenged with BLEO (25mU/ml) for 20 hours in the presence and absence of the nonselective ANG receptor antagonist saralasin (SARAL, 50ug/ml). Assay of Caspase 3 was conducted on adherent cells as described in Methods. * = $p<0.05$ versus control (CTL) and ** = $p<0.05$ versus BLEO.

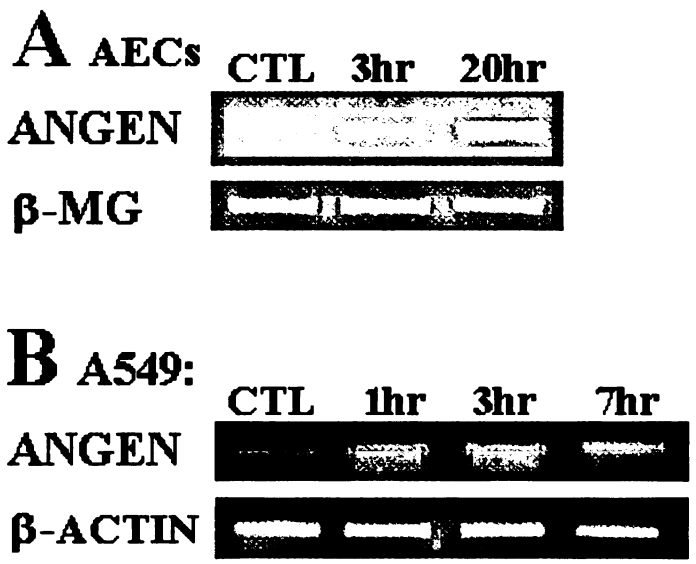
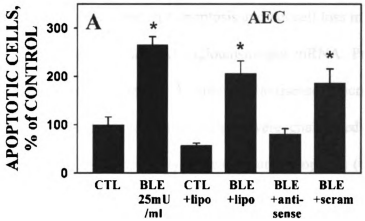


Figure 3.6. Semiquantitative RTPCR of angiotensinogen (ANGEN) mRNA in AECs after bleomycin exposure. Primary cultures of rat AECs (A) and A549 cells (B) were exposed to BLEO (25mU/ml) for the indicated times and total RNA was isolated. RTPCR was performed as described before with primers specific for rat or human angiotensinogen (ANGEN), β-microglobulin (β-MG) or α-actin as control mRNAs.


A
AEC
APOPTOTIC CELLS, % of CONTROL
300
200
100
0
*
*
*
CTL
BLE 25mU /ml
CTL +lipo
BLE +lipo
BLE +anti-sense
BLE +scram

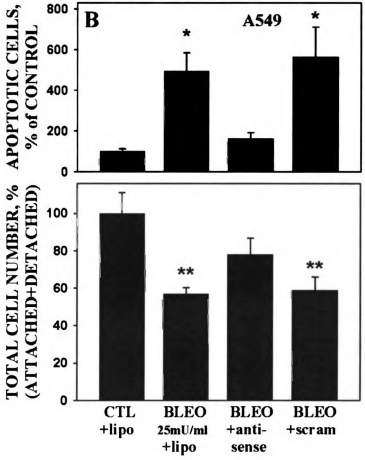

B
A549
APOPTOTIC CELLS, % of CONTROL
800
600
400
200
0
*
*
TOTAL CELL NUMBER, % (ATTACHED+DETACHED)
100
80
60
40
20
0
**
**
CTL +lipo
BLEO 25mU/ml +lipo
BLEO +anti-sense
BLEO +scram

Figure 3.7. Blockade of bleomycin induced apoptosis and net cell loss in AECs and A549 cells by antisense oligonucleotides against angiotensinogen mRNA. Primary rat AECs (A) or A549 cells (B) were transfected for 4 hours with antisense or scrambled sequence (scram) oligonucleotides as described earlier. Cells were challenged with bleomycin (BLE, 25mU/ml) for 20 hours immediately thereafter, and apoptosis (upper panel) was scored as detailed in Figs.1-3; net cell loss (bottom panel of B) was measured as detailed in Methods. lipo = lipofectamine; see Methods for details. Bars are the mean $\pm$ S.E.M. of at least 4 observations; * = $p<0.05$ and ** = $p<0.01$ versus corresponding control (CTL).

DISCUSSION

The results described here agree with previous studies from this laboratory that showed a requirement for autocrine ANGII production and receptor interaction for AEC apoptosis in response to Fas_L or TNF-α (Wang et al., 2000, Wang et al., 1999).

Other authors also have reported DNA damage and death of AECs in response to bleomycin (Hagimoto et al., 1997; He et al., 2001). Although a previous report of bleomycin action on A549 cells described no influence of the drug on cell viability *in vitro* (Sato et al., 1999), the levels of apoptosis described here (apoptotic index about 10-20%, see Figures 3.1 and 3.2) suggest that cell death at the relatively low doses used in that study (0.1-2mU/ml) may have gone undetected. Nonetheless, the levels of apoptosis reported here are more than sufficient to result in significant net cell loss in a relatively short period of time (see Figure 3.7). More importantly, both the cell loss and markers of apoptosis could be blocked by ANG system antagonists, consistent with the theory that these agents can prevent apoptosis and thus cell loss in response to bleomycin.

The findings that bleomycin-induced apoptosis of AECs could be blocked by the AT1-selective antagonists losartan (Figure 3.3) and L158809 (Figure 3.4) but not by the AT2-selective antagonist PD123319 are in agreement with our recent demonstration that the AT1 receptor subtype mediates AEC apoptosis in response to purified ANGII (Papp et al., 2002). They also support the contention that autocrine production of ANGII and binding to its receptor(s) are required for the apoptotic response. This contention also is supported by the ability of ANGEN antisense oligonucleotides or a neutralizing antibody that recognizes ANGII, but not ANGI or ANGEN, to essentially abrogate apoptosis and prevent net cell loss in response to bleomycin (Figures 3.4 and 3.7).

In an earlier report we described the induction of apoptosis in AECs by exposure to purified ANG II (Wang et al., 1999), which also occurred in a concentration-dependent manner with an EC_{50} of 10 and 50 nM for primary AECs and A549 cells, respectively. Those data indicated that exposure of AECs to exogenous ANG II is sufficient, in the absence of other stimuli, for the induction of apoptosis. Measurements of the ANG II concentration in the cell culture media at a single sampling time (20 h) suggest that Bleo increases ANG II in the medium by at least two- to threefold, to a level close to that required for induction of apoptosis by purified ANG II (2-3 nM, data not shown). However, intracellular receptors for ANG II have been demonstrated in other cell types (Eggena et al., 1993; Haller et al., 1996), and ANG II administered intracellularly by microinjection (Haller et al., 1996) has been shown to invoke a variety of signaling pathways. Thus, in the present study, intracellular generation of ANG II by Bleo may be sufficient to stimulate apoptosis independently of extracellular ANG II. In both serum and interstitial fluid, the half-life of ANG II is short (15 s-15 min), and receptor-bound ANG II is known to be internalized in many cell types (Filippatos et al., 2001); for all these reasons, the interpretation of extracellular ANG II levels is difficult at best.

The RT-PCR data of Fig.3.7 and measurements of ANGEN protein by Western blotting (not shown) suggest that there is a basal level of ANGEN expression by AECs, even in the absence of other proapoptotic stimuli. In experiments not reported here, the ANG receptor antagonist saralasin, applied alone, decreased the basal rate of spontaneous AEC apoptosis (normally 1-2% of total cells at steady state); this finding supports the notion that basal ANGEN expression is involved in the basal rate of AEC death. On the other hand, the rate at which ANGEN protein is proteolytically cleaved, both inside and outside

the cell, likely constitutes another point of regulation, but at present we have little data to indicate which point of control is most critical. Moreover, it seems reasonable to suspect that Bleo and other inducers of AEC apoptosis alter the expression of additional components of the local renin-angiotensin system in AECs, such as angiotensin-converting enzymes and receptors as well as ANGEN expression. Consistent with this theory, recent work by Day et al. (Day et al., 2001) demonstrated upregulation of ACE in cultured endothelial cells by Bleo. Alveolar epithelial cells also express ACE mRNA, and ACE inhibitors block AEC apoptosis in response to Fas_L (Wang et al., 1999), TNF-α (Wang et al., 2000), and Bleo (Figure 3.2). Investigations to define the possible regulation of ACE and other peptidases in AECs are currently underway.

Although the mechanism by which bleomycin upregulates ANGEN mRNA was not addressed in this study, the findings that BLEO, Fas_L and TNF-α all increased ANGEN mRNA (Wang et al., 1999; Wang et al., 2000) imply the involvement of a pathway common to these inducers. Regulation of ANGEN expression is best studied in the hepatocyte, in which the stimulatory effects of TNF-α and Interleukin-6 have been shown to be mediated through the interaction of transcription factors NF-κB and STAT-3 with the Acute Phase Response Element (APR) of the ANGEN promoter (Brasier et al., 1994; Sherman and Brasier, 2001). In contrast, studies of the regulation of ANGEN expression by the cardiac myocyte have shown that p53 is a key regulator of its expression in response to a variety of stimuli (Leri et al., 1998; Pierzchalski et al., 1997). Whether this difference reflects the distinct developmental lineages of these cell types or

other factors is unknown. Viewed in this context, future investigations of the regulation of ANGEN expression by cells of the lung will be most interesting, particularly in light of the fact that the lung contains many different cell types that are in very close proximity, but which arose from distinct embryologic origins. As an example, the results reported here for AECs compliment our earlier report of ANGEN expression by human lung myofibroblasts (Wang, et al., 1999), which reside immediately adjacent to AECs in the fibrotic lung *in situ*.

In summary, exposure of primary cultures of rat alveolar epithelial cells or the AEC-derived A549 cell line to bleomycin caused a time-dependent increase in angiotensinogen mRNA and caspase-dependent apoptosis. In either cell type, the apoptosis could be prevented by agents that prevent the synthesis of angiotensinogen protein, its conversion to the mature peptide angiotensin II, or the binding of the peptide to its receptors. These findings may have important implications toward possible therapeutic strategies to prevent lung injury and/or fibrogenesis. They also raise the possibility that previous clinical trials involving ACEis or ANG receptor antagonists may hold useful information related to the potential of these agents to affect pulmonary disease.

Chapter 4

ESSENTIAL ROLE FOR CATHEPSIN D IN BLEOMYCIN-INDUCED APOPTOSIS OF ALVEOLAR EPITHELIAL CELLS

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ABSTRACT

Earlier studies from this laboratory showed that bleomycin-induced apoptosis of type II alveolar epithelial cells (AECs) requires the autocrine synthesis and proteolytic processing of angiotensinogen into angiotensin II (ANGII), and that inhibitors of ANG converting enzyme (ACEis) block bleomycin-induced apoptosis (Li, X, H Zhang, V Soledad-Conrad, J Zhuang and BD Uhal. Bleomycin-induced apoptosis of alveolar epithelial cells requires angiotensin synthesis *de novo*. Am. J. Physiol. 284(3): L501-L507, 2003.). Given the documented role of cathepsin D (CatD) in apoptosis of other cell types, we hypothesized that CatD might be the AEC enzyme responsible for the conversion of angiotensinogen into ANGI, the substrate for ACE. Primary cultures of rat type II AECs challenged with bleomycin *in vitro* showed upregulation and secretion of CatD enzymatic activity and immunoreactive protein, but no increases in CatD mRNA. The aspartyl protease inhibitor pepstatin A, which completely blocked CatD enzymatic activity, inhibited bleomycin-induced nuclear fragmentation by 76% ($p<0.01$) and reduced bleomycin-induced caspase 3 activation by 47% ($p<0.05$). Antisense oligonucleotides against CatD mRNA reduced CatD immunoreactive protein and inhibited bleomycin-induced nuclear fragmentation by 48% ($p<0.01$). A purified fragment of angiotensinogen (F1-14) containing the CatD and ACE cleavage sites, when applied to unchallenged AEC *in vitro*, yielded mature ANGII peptide and induced apoptosis. The apoptosis induced by F1-14 was inhibited 96% by pepstatin A and 77% by neutralizing antibodies specific for CatD (both $p<0.001$). These data indicate a critical role for CatD in bleomycin-induced apoptosis of cultured AEC, and suggest that the

role(s) of CatD in AEC apoptosis include the conversion of newly synthesized angiotensinogen to ANGII.

Key Words:

aspartyl protease lung injury programmed cell death

lung fibrosis type II pneumocyte

INTRODUCTION

Angiotensin II (ANGII) is a potent inducer of apoptosis in alveolar epithelial cells (AEC) and is synthesized and released from AEC undergoing apoptosis in response to other stimuli (Filippatos and Uhal, 2003). Work from this laboratory has shown that ANGII is secreted by AEC challenged in vitro with Fas ligand (Wang et al., 1999) or TNF-alpha (Wang et al., 2000), and demonstrated that the production of ANGII is required for the signaling of apoptosis by these inducers. More recent investigations showed that AEC apoptosis in response to the fibrogenic agent bleomycin requires the autocrine synthesis of angiotensin II (ANGII) and the subsequent binding of ANGII to receptor subtype AT1 (Li et al., 2003). Apoptosis of AEC in response to the fibrogenic antiarrhythmic agent amiodarone also is blocked by ANG receptor AT1-selective antagonists (Filippatos and Uhal, 2003;Uhal et al., 2003). Together, these findings have lead to the theory that autocrine production of ANGII by AEC *de novo*, i.e., from the precursor angiotensingen, is a common event required for AEC apoptosis regardless of the initiating stimulus (Uhal, 2002).

Primary cultures of rat AEC were shown to constitutively express low but functional levels of angiotensin converting enzyme (ACE) mRNA (Wang et al., 1999), and to respond to ACE inhibitors such as captopril or lisinopril (Uhal et al., 1998; Wang et al., 2000). However, the identity of the enzyme(s) in AEC which act upstream of the ACE reaction, i.e., performing the conversion of angiotensinogen to ANGI (the substrate for ACE), remains unknown. In the serum, the conversion of liver-derived circulating angiotensinogen into ANGI is accomplished by the kidney-derived enzyme renin; this system is now viewed as the classical "endocrine" renin-angiotensin system (Filippatos et

al., 2001). In contrast, tissue-specific "local" angiotensin systems exist in many tissues as either "extrinsic" systems (i.e., dependent on one or more components of the endocrine system) or as "intrinsic" ANG systems in which all the enzymes and substrates required for the production of ANGII are synthesized locally. In local intrinsic systems outside the lung, the primary aspartyl protease that converts newly synthesized angiotensinogen to ANGI is cathepsin D (CatD) (Weber et al., 1995), a ubiquitous lysosomal aspartyl protease expressed by virtually all cells (Isahara et al., 1999). The identity of this aspartyl protease in the pulmonary local ANG system is the subject of this study.

A critical role for CatD in the execution of apoptosis has been shown previously in a variety of cell types including kidney cell lines (Heinrich et al., 1999), PC12 cells and dorsal root ganglion-derived neurons (Isahara et al., 1999) and in ML1 leukemia or U1752 lung cancer cells exposed to etoposide or adriamycin (Wu et al., 1998). In those studies, apoptosis in response chemical stimuli or trophic withdrawal could be prevented by the aspartyl protease inhibitor pepstatin A or by antisense oligonucleotides against CatD mRNA. One investigation of CatD-dependent apoptosis in neuronal cells led to the hypothesis that activation of CatD by apoptosis inducers leads to the generation of an unidentified "bioactive molecule" that is required for the signaling of apoptosis, but is either degraded or expressed at low levels in viable cells under basal unstimulated conditions (Isahara et al., 1999).

In the light of previous demonstrations that AEC apoptosis requires the autocrine synthesis of ANGII and the documented ability of CatD to convert angiotensinogen to ANGI, we hypothesized that CatD might be required for AEC apoptosis. We also

theorized that the primary function of CatD in AEC apoptosis is the conversion of angiotensinogen to ANGI. We report here that AEC apoptosis in response to bleomycin is inhibited by CatD knockdown as a result of its blockage of ANGII synthesis.

MATERIALS AND METHODS

Reagents and materials: The ANG receptor AT1-selective antagonist L158809 was obtained from Merck and Co., West Point, PA. The CatD fluorescent substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH2 (Yasuda et al., 1999) was obtained from Peptides International, Louisville, KY. Bleomycin (BLEO), anti-angiotensin antibodies, ATA, captopril and saralasin were obtained from Sigma Chemical Co., Saint Louis, MO. A kit for ELISA quantitation of angiotensin II was obtained form Peninsula Laboratories (San Carlos, CA). All other materials were of reagent grade and were obtained from Sigma Chemical Co., Saint Louis, MO.

Cell culture: Primary alveolar epithelial cells isolated from adult male Wistar rats as described earlier (Wang et al., 1999). The primary cells were studied at day two of culture, a time at which they are type II cell-like by accepted morphologic and biochemical criteria (Papp et al., 2002). Primary cell preparations were of better than 90% purity assessed by acridine orange staining as described previously (Wang et al., 1999;Wang et al., 2000). All cells were grown in 24- or 6-well chambers and were analyzed at subconfluent densities of 80-90%. All subsequent incubations with BLEO and/or other test agents were performed in serum-free medium. The cells were exposed to caspase inhibitors or antagonists of the angiotensin system 30 minutes before exposure to BLEO for 1-20 hours as indicated.

Quantitation of nuclear fragmentation and caspase-3 activity: Detection of apoptotic cells with propidium iodide (PI) was conducted as described earlier (Wang et al., 1999; Wang et al., 2000) following digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5ug/ml PI. In these assays, detached cells were retained by centrifugation

of the 24-well culture vessels during fixation with 70% ethanol. Cells with discrete nuclear fragments containing condensed chromatin were scored as apoptotic. As in earlier publications, the induction of apoptosis was verified by in situ end labeling (ISEL) of fragmented DNA (Li et al., 2003). Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group.

The enzymatic activity of Caspase 3 was measured in adherent cells incubated for 20 hours with the membrane permeable substrate Ac-DEVD-AMC (Upstate Biotech, Saranac Lake, NY) at 50uM final concentration. Quantitation of the fluorescent product was achieved with a Biotek FL600 fluorescence plate reader. Fluorescence values were normalized to cell number determined on the same culture well after cell fixing and staining of DNA with PI (Wang et al., 1999).

Assay of CatD activity: The enzymatic activity of CatD was determined with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH2 as described by its inventors (Yasuda et al., 1999). Briefly, aliquots of AEC lysates or concentrated cell culture media were incubated in opaque 96-well culture plates (suitable for top reading in a fluorescence plate reader) in 1.0M sodium acetate buffer, pH 4.0 containing 50uM fluorogenic substrate. The total volume of reaction buffer, including sample, was 100ul. In the case of cell lysates, equal amounts of lysate protein were assayed per culture vessel in triplicate. For concentrated cell culture media, the volume of medium assayed was normalized to equivalent amounts of cells used for conditioning the media, as determined by the lysate protein concentration. Initial rates of fluorescent product formation were obtained from the slope of continuous readings taken over 30

minutes following the addition of substrate. Initial reaction rates were linear with both time and protein concentration (see Results).

RTPCR and antisense experiments: Quantitative realtime reverse transcriptase polymerase chain reaction was performed by standard protcols in the Genomics Technology Support Facility, Michigan State University. Primer sequences were designed on the basis of published sequence information and the public domain software Primer3 (MIT, Cambridge, MA). The annealing temperatures for PCR reactions were optimized for each primer by preliminary trials. The identity of expressed genes was determined by expected size of the PCR product in 1.6% agarose gels, followed by excision and sequencing of the PCR product.

For RTPCR of rat CatD, the primers used were: (primer set 1) coding = 5'-ACACTGTGTCGGTTCCATGT-3', and uncoding = 5'-TGCGATGAATACGACTCC-AG-3', which produces a PCR product of 101bp, and (primer set 2) coding = 5'-GCGTCTTGCTGCTCATTCTC-3', and uncoding = 5'-TGGGACCTTTAAGGATCA-GG-3', which produces a PCR product of 141bp.

For antisense studies, phosphorothioated control and antisense oligonucleotides against CatD (22-mers) were designed through published sequence information and public domain software, synthesized and transfected into primary rat AEC (both at 40nM final concentration) using the lipofectin reagent OligofectAMINE (Invitrogen Life Technologies, Grand Island, NY) at 4ul/ml as the vehicle, diluted in the OPTIMEM medium accompanying the lipofectin. The control nucleotides were of the same length and base composition as the antisense, but with scrambled sequence. The oligonucleotide: lipofectin ratio was optimized to yield transfection efficiencies of 50-

75% with minimal cell loss or detachment. Transfection efficiency was monitored with FITC-labeled 25-mer oligonucleotide for luciferase (not shown). Transfections were conducted for 4 hours followed by 5 times washing with serum-free cell culture medium, as described earlier (Wang et al., 1999; Wang et al., 2000). Immediately thereafter, BLEO or vehicle was applied as indicated for 20 hours. The transfection protocol itself had no significant effect on basal or BLEO-induced apoptosis (see Results). Phosphorothioated oligonucleotide sequences were: (CatD antisense) 5'-CATATAGTTTTGCTTCTGTCCT-3', and (CatD scramble) 5'-TGCCCTATATGTTAGTTC-TTTC-3'.

RESULTS

Measurements of cathepsin D (CatD) enzymatic activity with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH2 revealed CatD enzymatic activity in lysates of purified rat alveolar epithelial cells (AECs, Figure 4.1); the generation of fluorescent product was linear with time and lysate protein concentration. Incubation of primary AEC cultures with bleomycin for 20 hours, at a concentration previously shown to stimulate AEC apoptosis (25mU/ml)(Li et al., 2000), significantly increased the activity of CatD in both AEC lysates and in the serum-free cell culture medium (Figure 4.2). The aspartyl protease inhibitor pepstatin A (pepA) inhibited the CatD activity by over 90%.

Apoptosis inducers are known to increase CatD activity and mRNA in other cell types (Isahara et al., 1999;Wu et al., 1998). Analyses of CatD mRNA in primary AECs by RTPCR (Figure 4.3A) revealed PCR products of the correct size expected from two different primer sets, but no apparent change in response to bleomycin. Sequencing of both PCR products verified the specificity of the PCR for rat CatD (not shown). Quantitative analyses of CatD mRNA by realtime PCR was unable to detect significant changes in the mRNA in response to bleomycin challenge (Figure 4.3B). In contrast, western blotting of AEC lysates and culture media with CatD-specific antibodies and high resolution gels (Figure 4.3) revealed bleomycin-induced increases in immunoreactive CatD proteins in the cell culture medium, but apparently not in the cell lysates. Bleomycin increased isoforms of CatD of apparent MW 52, 48 and 44kdal in the culture medium, whereas a 44kdal protein was the major immunoreactive isoform of CatD present in AEC lysates.

To begin determining if CatD might play a role in apoptosis of AECs as it does in other cell types, AEC apoptosis was evaluated in the presence and absence of the aspartyl protease inhibitor pepstatin A. In Figure 4.5, pepstatin A (pepA) inhibited bleomycin-induced nuclear fragmentation of primary AEC by 76% ($p<0.01$) and reduced bleomycin-stimulated caspase 3 activity by 47% ($p<0.05$). The pepstatin A alone did not affect basal nuclear fragmentation or caspase 3 activity.

As an alternate test of the role of CatD in AEC apoptosis, phosphorothioated antisense oligonucleotides specific for CatD mRNA were designed and transfected transiently into primary rat AEC with lipofectin (LIPO, Figure 4.6). In Figure 6A, the antisense oligonucleotides (AS) significantly reduced the immunoreactive CatD released into AEC culture media, as determined by western blotting on low resolution gels. In contrast, scrambled-sequence control oligonucleotides (SCR), of the same length and base composition as the antisense, did not reduce CatD immunoreactivity. In Figure 4.6B, pretreatment of primary AECs with the same antisense oligonucleotides used in panel A (AS) reduced bleomycin-induced nuclear fragmentation by 48% ($p<0.05$).

Recent work from this laboratory showed that the induction of AEC apoptosis by bleomycin requires *de novo* synthesis of angiotensin II (ANGII) and its subsequent binding to ANG receptor subtype AT1 (Li et al., 2003). To begin addressing the theory that the primary role of CatD in AEC apoptosis is its ability to process angiotensinogen to the peptide angiotensin I, CatD knockdown strategies were evaluated for the ability to prevent AEC apoptosis in response to a synthetic angiotensinogen fragment consisting of amino acids 1-14. This domain of angiotensinogen contains the catalytic sites for both CatD and angiotensin converting enzyme (ACE), which together generate angiotensin II.

To confirm this premise with the reagents currently commercially available for this study, the purified angiotensinogen fragment 1-14 (F1-14) was treated in vitro (without cells) with purified ACE or purified CatD enzymes (Figure 4.7A). As expected, treatment of F1-14 with both ACE and CatD together resulted in significant production of the peptide ANGII (Figure 4.7A), as measured by an ELISA that detects ANGII but not ANGI or angiotensinogen. In contrast, neither purified ACE alone nor purified CatD alone could convert F1-14 to the peptide ANGII. In Figure 4.7B, incubation of primary rat AECs with fragment F1-14 alone in serum-free culture medium (but without added enzymes) yielded significant production of ANGII, confirming the constitutive expression of ANG converting enzymes by primary AEC (Wang et al., 1999).

In Figure 4.8, incubation of primary rat AEC with F1-14 induced apoptosis detected by nuclear fragmentation. The apoptosis was completely blocked by the nonspecific or AT1-selective ANG receptor antagonists saralasin (SARAL) or L158809, respectively. Moreover, the apoptosis was inhibited 96% by the CatD inhibitor pepstatin A (pepA), and was reduced 77% by neutralizing antibodies specific for CatD (CatD AB, both $p<0.001$).

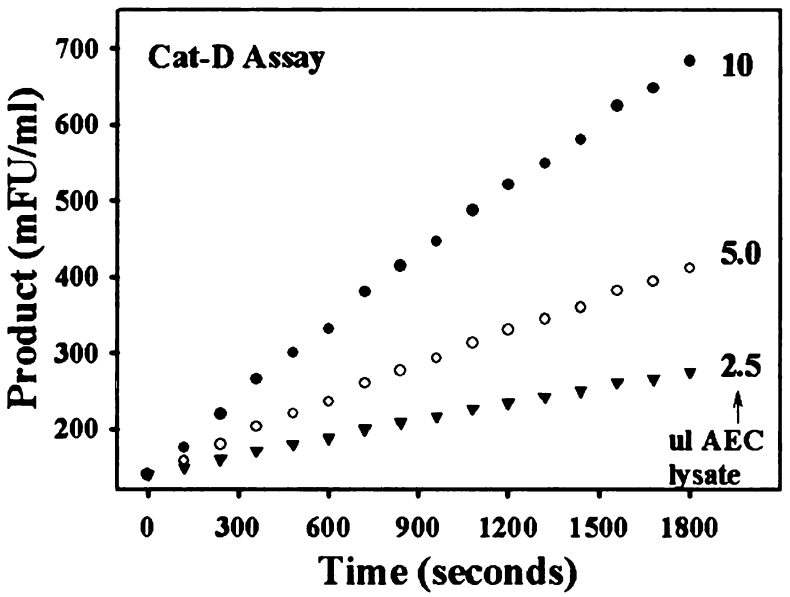

Figure 4.1. Cleavage of a fluorogenic substrate for Cathepsin D (CatD) is dependent on time and protein concentration. Lysates of primary alveolar epithelial cells (AECs) were incubated with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH2, and generation of fluorescent product was monitored continuously over 30 minutes (see Methods for details). Note linearity of product formation with time and amount of AEC lysate.

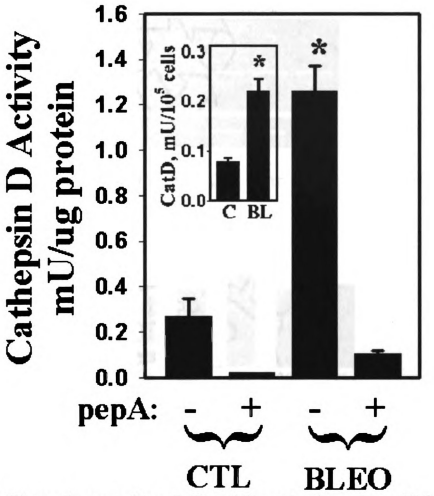

Figure 4.2. Bleomycin upregulates CatD activity and release from AECs. Primary cultures of rat alveolar epithelial cells (AECs) were exposed to bleomycin (BLEO) for 20 hours at a concentration known to induce AEC apoptosis (25mU/ml). CatD activity was measured in cell lysates as described in Figure 4.1, in the presence or absence of the aspartyl protease inhibitor pepstatin A (pepA).

Inset: CatD activity was measured in concentrated cell culture medium collected from BLEO-treated (BL) and untreated (C) cells studied in panel A. Bars are the mean ± S.E.M. of n = 6; * = $p<0.01$ versus untreated control (CTL) by ANOVA and Student-Newman-Keul's test.

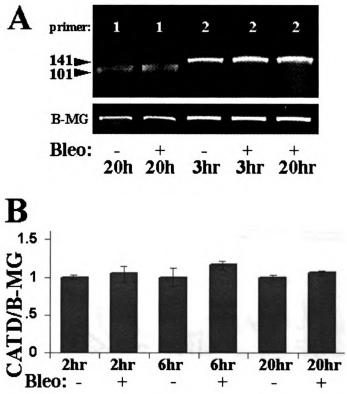

Figure 4.3. Bleomycin does not alter steady-state levels of CatD mRNA. A: PCR products from two different primer sets (1 and 2, see Methods) used to amplify CatD mRNA by RTPCR; starting material was total RNA isolated from primary rat AECs exposed to BLEO or vehicle for the indicated times. B: Realtime RTPCR of CatD mRNA (primer set 2) at the indicated times after exposure to BLEO (see Methods). β-MG = β-microglobulin; bars are the mean±S.E.M. of three separate AEC cultures.

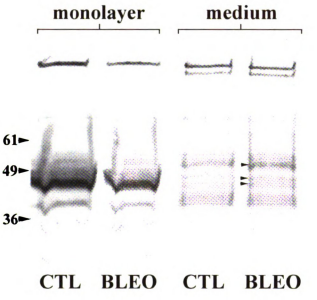

Figure 4.4. Bleomycin increases the release of immunoreactive CatD protein from cultured AECs. Primary cultures of AECs were exposed to bleomycin (BLEO) as in Figure 2; detergent lysates were harvested from the cells (monolayer), and the cell culture medium was collected and concentrated. Equal amounts of lysate protein (10ug/lane) or volume of culture medium (equivalent to 10^5 cells) were subjected to western blotting with Cat-D-specific antibodies (see Methods). Note increases in immunoreactive proteins of apparent MW~ 52, 48 and 44kdal (arrowheads) in medium from BLEO-treated AECs.

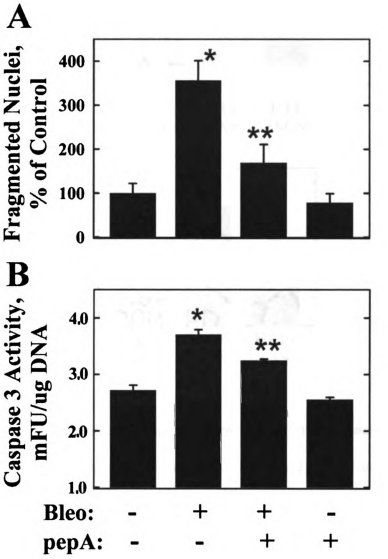

Figure 4.5. Pepstatin A inhibits bleomycin-induced apoptosis of AECs in vitro. Primary cultures of rat AECs were exposed to BLEO in the presence or absence of pepstatin A (pepA) at 100uM. Apoptosis was quantitated by scoring of nuclear fragmentation with propidium iodide (panel A) or by the enzymatic activity of Caspase 3 (B). See Methods for details. Bars are the mean ± S.E.M. of n = 6; * = $p<0.01$ versus untreated control and ** = $p<0.05$ versus BLEO by ANOVA and Student-Newman-Keul's test.

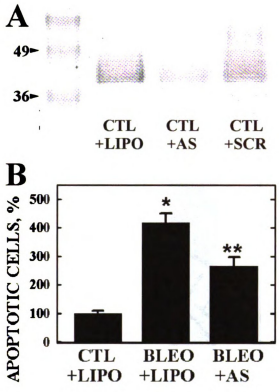

Figure 4.6. Antisense oligonucleotides reduce CatD immunoreactivity and inhibit bleomycin-induced apoptosis of AECs in vitro. A: Antisense (AS) or scrambled-sequence oligonucleotides (SCR) were transfected into primary cultures of rat AECs in the presence of lipofectin (LIPO, see Methods), without challenge with bleomycin (CTL). Western blotting of concentrated cell culture media was performed with CatD-specific antibodies; note decrease in immunoreactive CatD by AS but not SCR oligonucleotides. B: After antisense oligonucleotide transfection as in panel A, AECs were challenged with BLEO (25mU/ml) and harvested for detection of fragmented nuclei as in Figure 5. Bars are the mean ± S.E.M. of n = 3; * = $p<0.01$ versus untreated control (CTL) and ** = $p<0.05$ versus BLEO by ANOVA and Student-Newman-Keul's test.

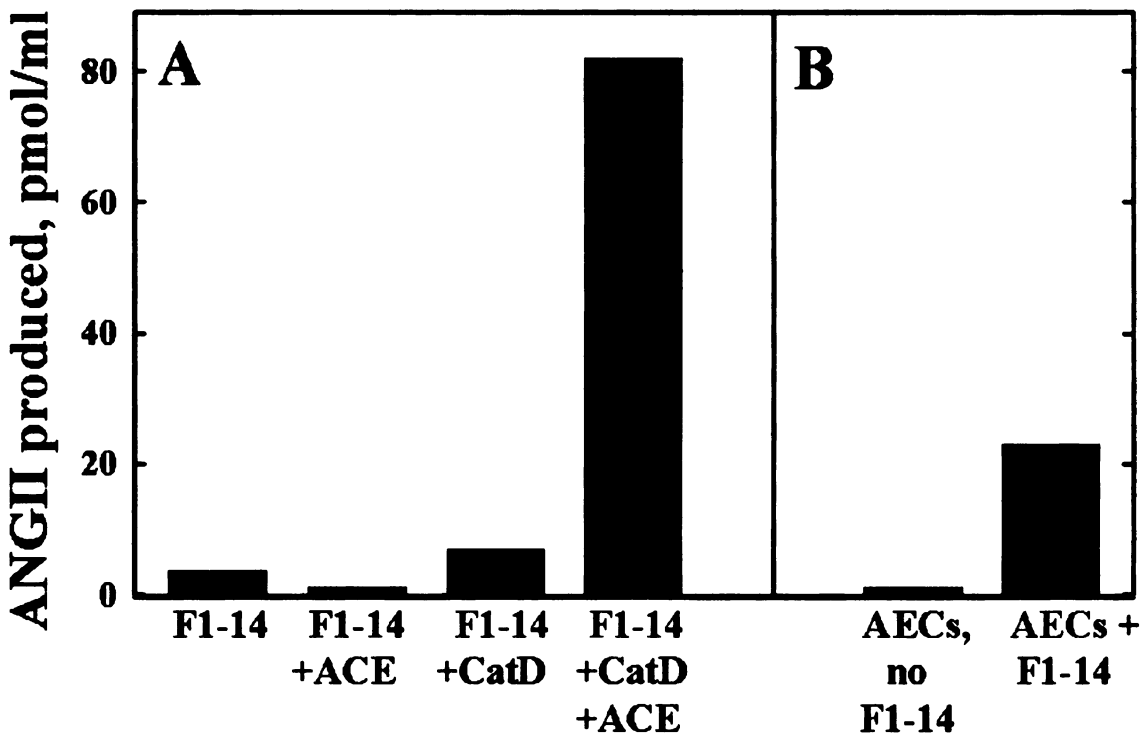

Figure 4.7. Production of ANGII from angiotensinogen fragment 1-14 in vitro.

A: Angiotensinogen fragment 1-14 (F1-14, 5uM) was incubated in vitro (without cells) with the indicated purified enzymes; ANGII was measured in the reaction buffer by specific ELISA (see Methods for details). Note production of ANGII by the combination of purified CatD + purified angiotensin converting enzyme (ACE), but not by either enzyme alone. B: Primary cultures of AECs were exposed to 5uM F1-14, and ANGII was measured in the serum-free cell culture medium; note production of ANGII by AECs challenged with F1-14, but not by untreated AECs.

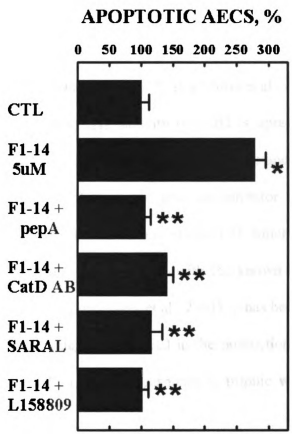

Figure 4.8. CatD-dependent induction of AEC apoptosis by angiotensinogen fragment 1-14. Primary cultures of AEC were incubated with F1-14 as in Figure 4.7, in the presence or absence of pepstatin A (pepA, 1uM); CatD-specific neutralizing antibodies (CatD AB, 1:100) and the ANG receptor antagonists saralasin (SARAL, 50ug/ml) or L158809 (10^{-6} M). See Methods for details. Bars are the mean ± S.E.M. of n = 3; * = $p<0.001$ versus untreated control (CTL) and ** = $p<0.001$ versus F1-14 by ANOVA and Student-Newman-Keul's test.

DISCUSSION

A role for the aspartyl protease CatD in apoptosis has been shown previously in HeLa cells exposed to interferon-γ, Fas ligand or TNF-α (Deiss et al., 1996) and in PA1 ovarian cancer cells (Wu et al., 1998). The activity of CatD is upregulated by the apoptosis inducer adriamycin in PA1 cells and in ML1 leukemia cells and U1752 lung cancer cells (Wu et al., 1998). Although the aspartyl protease inhibitor pepstatin A could block apoptosis in these cell types, the exact mechanism(s) by which CatD participates in the execution of apoptosis is unclear. In accord with the known ubiquitous expression of CatD as a lysosomal protease (Uchiyama et al., 2001), it has been suggested that this and other lysosomal proteases might be involved in the production of a bioactive molecule required for apoptosis of PC12 cells in response to trophic withdrawal (Isahara et al., 1999).

Cathepsin D also is known to be one of the enzymes capable of proteolytically processing the liver-derived and serum-borne protein angiotensinogen to the peptide angiotensin I, a function normally performed in the serum by the kidney-derived enzyme renin (Filippatos et al., 2001). On the other hand, evidence from several nonpulmonary cell types has established CatD as the primary enzyme that converts angiotensinogen to ANGI within local "intrinsic" angiotensin systems, independently of renin (Filippatos et al., 2001; Weber et al., 1995).

Recent studies from this laboratory have shown that bleomycin-induced apoptosis of alveolar epithelial cells requires the autocrine synthesis of angiotensinogen, angiotensin II and it's binding to ANG receptor AT1 (Li et al., 2003). Those data were consistent with related studies showing that purified ANGII itself was a potent inducer of apoptosis

in AEC (Wang et al., 1999), and implied that AEC express enzymes capable of converting angiotensinogen to ANGII. Although the same study showed constitutive expression of angiotensin converting enzyme (ACE) by alveolar epithelial cells, the aspartyl protease required for providing the substrate for ACE (ANGI) in AEC was unknown.

The data herein strongly suggest that CatD functions in this capacity in AEC; bleomycin-induced nuclear fragmentation and caspase 3 activity were significantly reduced by the aspartyl protease inhibitor pepstatin A (Figure 4.5) or by antisense oligonucleotides against CatD mRNA (Figure 4.6). In earlier investigations, bleomycin-induced apoptosis of AEC was competely blocked by specific angiotensin receptor antagonists or ANG-neutralizing antibodies (Li et al., 2003); this finding lead to the theory that autocrine generation of ANGII is required for AEC apoptosis regardless of the initiating stimulus (Uhal, 2002). In the light of those results, the finding that CatD antisense treatment did not completely block bleomycin-induced nuclear fragmentation (48%, Figure 4.6B) might indicate a potential role for additional protease(s) in angiotensinogen processing and subsequent AEC apoptosis. This interpretation is consistent with the finding that the protease inhibitor pepstatin A, which blocks all aspartyl proteases, also was incapable of complete blockade of nuclear fragmentation (76%, Figure 4.5) despite complete inhibition of CatD enzyme activity in AEC lysates (Figure 4.2). On the other hand, the antisense treatment, which is at least theoretically specific, did not completely eliminate immunoreactive CatD detected by western blotting (Figure 4.6A). Thus, it is difficult to know with certainty if the incomplete blockage of apoptosis is due to inefficient CatD knockdown or additional proteases activities.

Regardless, studies of angiotensinogen fragment 1-14 (Figures 4.7&8) are consistent with the theory that CatD is required for the conversion of angiotensinogen to ANGII by AEC, and with earlier work. For example, the finding that incubation of primary rat AECs with the fragment F1-14 alone in serum-free culture medium (but without added enzymes) yielded significant production of ANGII (Figure 4.7) is consistent with the earlier demonstration of constitutive, albeit low, expression of both ACE and an unidentified aspartyl protease by primary AECs (Wang et al., 1999). Moreover, the complete abrogation of AEC apoptosis in response to angiotensinogen fragment F1-14 by the nonselective and AT1-selective ANG receptor antagonists saralasin and losartan (Figure 4.8) confirmed that the induction of apoptosis was dependent on both the generation of ANGII from F1-14 and the binding of ANGII to receptor AT1. Those results also are consistent with our earlier demonstrations that AT1 receptor mediates AEC apoptosis in response to bleomycin (Li et al., 2003), amiodarone (Filippatos and Uhal, 2003; Uhal et al., 2003) or purified ANGII (Papp et al., 2002). Most important, the findings that AEC apoptosis in response to F1-14 was essentially abrogated by either pepstatin A or by CatD antibodies (Figure 4.8) strongly suggest that the conversion of angiotensinogen to ANGI, and subsequently ANGII to induce AEC apoptosis, is dependent on CatD activity.

The upregulation of CatD activity by bleomycin in this study is consistent with the earlier findings that CatD is upregulated in alveolar epithelial cells in fibrotic human lung (Kasper et al., 1996) and is induced in the L132 lung cell line during apoptosis in vitro (Kasper et al., 1999). In other cell types, apoptosis inducers upregulate both CatD protein and mRNA, which suggests control of activation at the level of RNA (Wu et al., 1998). In contrast, RTPCR studies of AEC transcripts after bleomycin treatment failed to detect

changes in CatD mRNA (Figure 4.3) despite significant increases in CatD activity (Figure 4.2) and immunoreactive protein by western blotting (Figure 4.4). It is possible that the relatively few sampling times chosen for realtime analyses of CatD mRNA may have missed a transient but shortlived increase in the mRNA that might be revealed by a more exhaustive timecourse study. On the other hand, CatD is known to undergo activation by proteolytic mechanisms as well; in human U937 cells, CatD was shown to undergo processing of the inactive prepro- isoform (52kdal) to the active proCatD (48kdal) and an active 32kdal isoform, in response to autocatalysis of the enzyme induced by the direct binding of the apoptosis mediator ceramide (Heinrich et al., 1999). Consistent with those findings, western blotting of rat AEC lysates did reveal bleomycin-induced increases in several isoforms of apparent MW 44-52kdal. However, two of the isoforms shown to be increased in AEC media (52 and 48kdal, see "medium" in Figure 4.4) are larger than the primary isoform detected intracellularly in AEC (44kdal, "monolayer" in Figure 4.4). This finding argues against proteolytic processing alone as a mechanism of CatD activation in AEC. Thus, the exact mechanism(s) by which bleomycin upregulates CatD in AEC is unknown, but will pose an interesting problem for future studies. Pepstatin A-inhibitable CatD activity also was upregulated by amiodarone (Uhal et al., 2003) and TNF-alpha (Wang et al., 2000), both of which induce apoptosis in AECs (Uhal et al., 2003; Wang et al., 2000), but a determination of whether the requirement for CatD is universal to all proapoptotic stimuli for AEC will require further investigation.

In summary, bleomycin upregulated CatD enzymatic activity and immunoreactive protein in primary cultures of rat alveolar epithelial cells (AEC). Apoptosis of cultured AEC in

response to bleomycin was significantly inhibited by the aspartyl protease inhibitor pepstatin A or by antisense oligonucleotides against CatD mRNA. The same inhibitors also prevented the enzymatic processing of a synthetic fragment of angiotensinogen (amino acids 1-14), and completely blocked AEC apoptosis in response to the same peptide. These data are consistent with earlier studies showing that apoptosis of AEC in response to bleomycin requires the autocrine synthesis and proteolytic processing of angiotensinogen to angiotensin II, and suggest that the proteolytic processing requires CatD. The data herein also suggest that blockade of CatD and other aspartyl proteases might provide a potential strategy for preventing AEC apoptosis and lung injuries that involve this mode of cell death.

Chapter 5

ESSENTIAL ROLES FOR ANGIOTENSIN RECEPTOR AT1a in BLEOMYCIN-INDUCED APOPTOSIS AND LUNG FIBROSIS IN MICE

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ABSTRACT:

Apoptosis of alveolar epithelial cells (AECs) has been implicated as a key event in the pathogenesis of lung fibrosis. Recent studies demonstrated a role for the synthesis and binding of angiotensin II to receptor AT1 in the induction of AEC apoptosis by bleomycin (BLEO) and other proapoptotic stimuli. On this basis we hypothesized that BLEO-induced apoptosis and lung fibrosis in mice would be inhibited by the AT1 antagonist losartan (LOS) or by targeted deletion of the AT1 gene. Lung fibrosis was induced by intratracheal administration of BLEO (1 U/kg) to wild-type C57BL/6J mice. Co-administration of LOS abrogated BLEO-induced increases in total lung caspase 3 activity detected 6 hours after in vivo administration and reduced by 57% BLEO-induced caspase 3 activity in blood-depleted lung explants exposed to BLEO ex vivo (both $P < 0.05$). Co-administration of LOS in vivo reduced DNA fragmentation and immunoreactive caspase 3 (active form) in AECs, measured at 14 days after intratracheal BLEO, by 66% and 74%, respectively (both $P < 0.05$). LOS also inhibited the accumulation of lung hydroxyproline by 45%. The same three measures of apoptosis and lung fibrosis were reduced by 89%, 85%, and 75%, respectively (all $P < 0.01$), in mice with a targeted disruption of the AT1a receptor gene (C57BL/6J-Agtr1a^{tm1Unc}). These data indicate an essential role for angiotensin receptor AT1a in the pathogenesis of BLEO-induced lung fibrosis in mice and suggest that AT1 receptor signaling is required for BLEO-induced apoptosis of AECs in mice as it is in rat and human AECs.

INTRODUCTION:

Idiopathic pulmonary fibrosis is a progressive and often fatal human disease characterized by infiltration of inflammatory cells into interstitial and alveolar spaces, ongoing damage to the lung parenchyma, fibroblast proliferation, and accumulation of interstitial collagens (Selman et al., 2001). Ongoing evaluations of both older and newer data have lead to the recent characterization of idiopathic pulmonary fibrosis as a disease of abnormal wound repair, in which abnormalities in epithelial-mesenchymal interactions are of key importance (Selman et al., 2001; Gauldie et al., 2002). This evolving theory about the pathogenesis of idiopathic pulmonary fibrosis is, in some respects, a revival of the hypothesis first put forth by Haschek and Witschi (Haschek and Witschi, 1979) and Adamson and colleagues (Adamson et al., 1988) that the severity of the fibrogenic response in the lung is directly related to the severity of epithelial injury. A growing body of evidence suggests that alveolar epithelial cell (AEC) death by apoptosis is a key event in the initiation and progression of lung fibrosis. In mice exposed to bleomycin (BLEO) by intratracheal instillation, up-regulation of the receptor Fas on lung epithelial cells and Fas ligand on infiltrating lymphocytes were associated with DNA fragmentation in epithelia and subsequent accumulation of collagens (Hagimoto et al., 1997a). Intratracheal instillation of Fas-activating antibodies caused epithelial cell apoptosis and subsequent collagen accumulation, the severity of which was proportional to the amount of Fas-activating antibody instilled (Hagimoto et al., 1997b). Other investigators have shown that BLEO itself induces apoptosis of AECs, which precedes the deposition of collagens (Wang et al., 2000). More importantly, several groups have found that blockage of epithelial apoptosis with caspase inhibitors administered *in vivo* can prevent

BLEO-induced lung cell apoptosis and the subsequent accumulation of lung collagens (Wang et al., 2000; Kuwano et al., 2001)

Recent work from this laboratory has shown that exposure of cultured AECs to Fas ligand (Wang et al., 1999), tumor necrosis factor-α (Wang et al. R, 2000), or BLEO (Li et al., 2003) all induce expression of angiotensinogen mRNA and protein, and its cleavage to the peptide angiotensin II (ANGII). Moreover, apoptosis of cultured AECs in response to these apoptosis inducers was abrogated by antagonists of ANG receptor AT1, such as losartan (LOS) or L158809 (Li et al., 2003; Uhal et al., 2003; Filippatos and Uhal, 2003) For all these reasons, it was hypothesized that angiotensin receptor AT1 is essential for AEC apoptosis and lung fibrosis *in vivo*. To test this theory, normal mice and mice deficient in ANG receptor AT1a, the AT1 subtype expressed in lung (Burson et al., 1994), were subjected to intratracheal BLEO administration and quantitation of apoptosis and lung collagens. We report here the prevention of both BLEO-induced AEC apoptosis and lung collagen accumulation in mice by administration of the AT1-selective receptor antagonist LOS or by targeted deletion of the AT1a receptor gene.

MATERIALS AND METHODS

Reagents and Materials

The AT1-selective antagonist LOS was obtained from Merck and Co., West Point, PA. Alkaline phosphataseconjugated streptavidin, digoxigenin-labeled deoxyuridine trisphosphate (dig-dUTP), and biotinylated deoxyuridine trisphosphate (bio-dUTP) were obtained from Boehringer Mannheim, Indianapolis, IN. BLEO was obtained from Sigma Chemical Co., Saint Louis, MO. Reagents for detection of alkaline phosphatase and other secondary reagents for *in situ* end labeling (ISEL) of DNA or Western blotting were from sources described earlier (Wang et al., 2000). All other materials were of reagent grade and were obtained from Sigma Chemical Co.

Animals, Induction of Pulmonary Fibrosis, and Surgical Procedures

All mice were obtained from The Jackson Laboratories, Bar Harbor, ME, and were housed in a satellite facility of University Laboratory Animal Resources, Michigan State University. Control animals were wild-type C57BL/6J mice used at 7 to 8 weeks of age. Some experiments also used mice of the same genetic background but with a targeted disruption in the ANG receptor AT1a gene (C57BL/6J-*Agtr1a*^{tm1Unc}) that removes a portion of the coding region sufficient to eliminate specific binding of AT1-selective agonists in all organs tested (Ito et al., 1995). Heterozygous animals were used on the basis of availability at the same age and body weight as wild types.

Induction of Lung Injury and Fibrosis

Animals under pentobarbital anesthesia received a single intratracheal instillation of bleomycin sulfate (BLEO) at 1 U/ kg body weight, in 50 μl of sterile saline. The 50-μl dose was instilled at end-expiration, and the liquid was followed immediately by 300 μl of air to ensure delivery to the distal airways. Control animals were instilled with an equal volume of sterile saline. In some studies the AT1 receptor antagonist LOS was added to the intratracheal instillate at 20 μmol/L; the same animals also received daily intraperitoneal injections of LOS at 10 mg/kg in sterile saline throughout the test interval. Other treatment groups received daily intraperitoneal sham injections of the saline alone. LOS or sham injections were continued for 14 days after instillation of BLEO, at which point all animals were sacrificed for histology, detection of collagen or DNA fragmentation, and caspase-3 activation in epithelial cells.

Surgical Procedures

Immediately before sacrifice, animals were given intraperitoneal injections of sodium pentobarbital and the trachea was cannulated. The left lung was ligated at the hilus, excised distal to the ligation, and immediately frozen in liquid N2 for hydroxyproline assay of total collagen (see below). The remaining lung tissues were carefully removed and were instilled with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 20 cm of H_2O constant pressure, then immersed in the same fixative for 30 minutes followed by storage in 70% ethanol. The fixed tissues were washed with PBS three times for 15 minutes and were then embedded in paraffin. Five μm sections of lung were deparaffinized by passing through xylene, xylene: alcohol 1:1, 100% alcohol, and 70% alcohol for 10 minutes each. Ethanol was removing by rinsing with distilled water.

Lung Explant Culture

Explants of 1 mm^2 were prepared by mincing of blood depleted (PBS-perfused) mouse lung, and were cultured in Transwell polycarbonate inserts (3.0μm pore; Costar, Corning, NY) under a thin layer (1 mm) of Dulbecco's modified Eagle's medium cell culture medium to facilitate gas exchange (Taylor et al., 2000). All explants were obtained from normal mouse lung that was PBS-perfused *in situ* before excision of the lungs. After excision of the lungs, treatment with BLEO or LOS was initiated *ex vivo* by intratracheal instillation of BLEO at 25 mU/ml in 300 μl of sterile Dulbecco's modified Eagle's medium (+/- LOS at 10^{-6} mol/L). The culture medium for explants also contained BLEO at 25 mU/ml, +/- LOS at 10^{-6} mol/L. Explants were harvested by transfer into liquid N2 and storage at -80°C until assay.

Identification and Quantitation of Apoptotic Cells and Total Lung Caspase 3 Activity

Localization of DNA Fragmentation

ISEL of fragmented DNA was conducted by a modification of the method of Mundle and colleagues (Mundle et al., 1994). Briefly, ethanol was removed from deparaffinized lung sections by rinsing in distilled water for at least 10 minutes. The slides were then placed in 3% hydrogen peroxide (Sigma Chemical Co.) for 30 minutes at 20°C, rinsed with PBS, and incubated with Proteinase K (Sigma) in standard saline citrate for 15 minutes at 37°C. Samples were rinsed once in water, three times in 0.15 mol/L PBS for 4 minutes each, and were then incubated in standard saline citrate (0.3 mol/L NaCl and 30 mmol/L

sodium citrate in water, pH 7.0) at 80°C for 20 minutes. After four rinses in PBS and four rinses in buffer A (50 mmol/L Tris/HCl, 5 mmol/L MgCl, 10 mmol/L B-mercaptoethanol, and 0.005% bovine serum albumin in water, pH 7.5), the sections were incubated at 18°C for 2 hours with ISEL solution (0.001mmol/L digoxigenin-dUTP; 20 U/ml DNA Polymerase I; and 0.01 mmol/L each of dATP, dCTP, and dGTP in buffer A). Afterward the sections were rinsed thoroughly five times with buffer A and three additional times in PBS. Detection of incorporated dUTP was achieved with by incubation for 2 hours at 37°C with AP-conjugated antidigoxigenin (Boehringer Mannheim) at 1/400 dilution. Bound AP-antibody was then detected with the Fast Blue chromogen system and the sections were mounted with Fluoromount solution (Southern Biotechnology, Birmingham, AL).

Immunohistochemistry (IHC) for Activated Caspase 3

IHC was performed with an antibody that recognizes only the active form of the enzyme (BioVision, MountainView, CA). Deparaffinized lung sections were blocked with a solution of 3% bovine serum albumin in PBS for 1 hour; the primary antibody was then applied overnight at 4°C in 3% bovine serum albumin/PBS. After washing in PBS, the antibody was detected with a biotin-conjugated secondary antibody and avidin-linked chromogen system. Type II pneumocytes were identified with the anticytokeratin antibody MNF116, an established marker of type II cells (Fehrenbach et al., 2000). Detection of mouse lung antigens with this mouse monoclonal antibody was achieved with the Mouse-on-Mouse Iso-IHC kit (InnoGenex, San Ramon, CA) according to the manufacturer's instructions.

For quantitation of ISEL- or caspase 3-positive epithelial cells, the number of positive cells within the surfaces of the alveolar walls was counted in a minimum of six randomly selected x 400 microscopic fields per lung section. Positive cells within the alveolar airspaces, or otherwise clearly not within the surface of the alveolar wall, were not scored. The counts of positive nuclei per field were expressed as a percentage of the total number of nuclei in the same microscopic field. Sections from each of at least five mice per treatment group were analyzed by an investigator blinded to sample identity.

Caspase 3 Enzyme Activity

Assay of total lung caspase-3 enzyme activity was conducted with a commercially available kit (Molecular Probes, Eugene, OR). Fast-frozen lung was homogenized in assay kit buffer and was analyzed according to the manufacturer's instructions on a Biotek FL600 fluorescence plate reader. In all samples, specificity of the reaction for caspase 3 was verified by abrogation of the signal with a caspase 3-selective irreversible inhibitor (data not shown).

Quantitation of Lung Collagen

For quantitation of total lung collagen, tissues frozen in liquid N2 were dried to constant weight in preweighed tubes at 80°C. The weighed dry tissue was hydrolyzed in 6 N HCl and was subjected to determination of hydroxyproline as described earlier by Woessner (Woessner, 1961) The efficiency of the hydrolysis was verified with rat-tail collagen by comparison to standard hydroxyproline (Sigma Chemical Co.).

Attention: Images in this dissertation are presented in color.

RESULTS

Quantitation of Apoptosis and Lung Injury

On the basis of earlier work with rat models (Wang et al., 2000; Li et al., 2003), we hypothesized that BLEO would induce apoptosis of mouse lung AECs that might be detected *in situ* by end labeling of fragmented DNA or by IHC for the active form of caspase 3. Consistent with this expectation, intratracheal instillation of BLEO caused increased ISEL and positive caspase 3 IHC within cells in the surfaces of alveolar corners, the expected locations of type II pneumocytes (Figure 5.1; B, F, and G, arrows; see subsequent figures for quantitation). Many ISEL- or caspase 3-positive cells colocalized with positive immunoreactivity to monoclonal antibody MNF116 (Figure 5.1C), an established marker of type II pneumocytes (Fehrenbach et al., 2000). Interestingly, MNF116 immunoreactivity was observed in relatively normal regions of BLEO-exposed lung (Figure 5.1D, right) but not in more severely affected regions (Figure 5.1D, left), consistent with the elimination of type II cells in these areas. Co-administration of the AT1 receptor antagonist LOS with the BLEO (Figure 5.1H) significantly reduced caspase 3 IHC (see below for quantitation).

The instillation of BLEO also resulted in histological changes typical of BLEO-induced lung fibrosis by day 14 after instillation (Figure 5.2). These include the infiltration of inflammatory cells, thickening of alveolar walls, and collagen accumulation (Figure 5.2B); for quantitation, see Figures 5.5 and 5.7 below. Again, the co-administration of LOS with the BLEO (Figure 5.2C) significantly reduced the alterations in lung morphology and collagen deposition (see below).

Inhibition of Apoptosis and Collagen Deposition by an AT1 Antagonist

Apoptosis also was detected as an increase in the total activity of caspase 3 in lung tissue, measured by enzyme assay of lung homogenates. As early as 6 hours after instillation of BLEO intratracheally (Figure 5.3A), lung caspase-3 activity was increased 100%, but the increase was prevented by co-administration of the AT1 receptor antagonist LOS (see Materials and Methods). In Figure 5.3B, BLEO also increased caspase-3 activity when applied *in vitro* at 25 mU/ml to mouse lung explants that were depleted of blood before explant culture. Application of BLEO *in vitro* increased caspase-3 activity in the explants by nearly 100% in 24 hours (P <0.05), but LOS (10^{-6} mol/L) inhibited the increase by 57%. The ability of LOS to inhibit lung epithelial apoptosis was also observed *in vivo* at 14 days after BLEO administration. In Figure 5.4A, intratracheal BLEO increased the abundance of ISEL-positive cells by 11-fold ($P < 0.01$), but LOS inhibited the increase by 66% ($P < 0.05$). Similarly, intratracheal BLEO increased the number of caspase 3-positive cells by 25-fold (Figure 5.4B, $P < 0.01$), but LOS blocked the increase by 74% ($P < 0.05$). Measurement of lung collagen accumulation in the same animals by hydroxyproline assay (Figure 5.5) revealed an increase in total lung collagen of 56% by 14 days after intratracheal BLEO, but LOS reduced the increase by 45%, to a value not significantly different from the control (CTL).

Inhibition of Apoptosis and Collagen Deposition by AT1 Gene Deletion

Receptor subtype AT1a is the AT1 isoform expressed in the lungs of mice (Burson et al., 1994). To test the hypothesis that angiotensin receptor AT1 is essential for BLEO-induced epithelial apoptosis and lung fibrogenesis, heterozygous AT1a-null mice were

exposed to intratracheal BLEO in the same manner as wild-type mice of the same genetic background. In Figure 5.6, the deletion of one allele of the AT1a gene (+/-) reduced BLEO-induced ISEL by 89% (Figure 5.6A) and inhibited BLEO-induced caspase 3 IHC by 85% (Figure 5.6B), both relative to the response in wild-type mice (**, $P < 0.01$).

When the susceptibility of the same mice to BLEOinduced fibrosis was measured, heterozygous AT1a-null mice did not exhibit a statistically significant increase in lung hydroxyproline at 14 days after intratracheal BLEO (Figure 5.7A), in contrast to wild-type mice. Expression of the hydroxyproline data as the absolute amount of collagen per left lung (Figure 5.7B) suggested that unchallenged AT1a +/- mice, of the same age and body weight as the wild types, have more total collagen per left lung at baseline relative to wild-type mice, but the difference was not statistically significant.

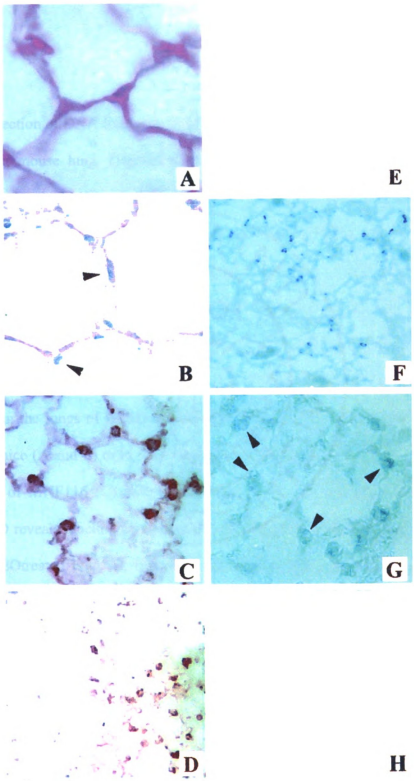
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Figure 5.1. Detection of DNA fragmentation, activation of caspase 3, and alveolar type II pneumocytes in mouse lung. Deparaffinized lung sections were prepared from mice instilled intratracheally 14 days earlier with sterile saline (A, C, and E) or BLEO (B, D, F, and G). The sections were subjected to ISEL of fragmented DNA (A and B) or IHC with antibodies against the active form of caspase 3 (E–H) or with the type II cell-specific antibodyMNF116 (C and D). G: Higher magnification of active caspase 3 labeling in F. H: Active caspase 3 labeling in mice treated with BLEO and LOS, an AT1 receptor antagonist. Note ISEL and active caspase 3 labeling in cells in the corners of alveolar walls in the lungs of BLEO-treated mice (B, F, and G, arrowheads) but not in saline-treated mice (A and E) or in mice treated with BLEO and LOS (H). Note also the co-localization of MNF116 (C) with anti-caspase 3 IHC (G) or ISEL (B) in BLEO-treated lungs. D reveals labeling of the type II cell marker MNF116 in relatively normal regions of BLEOtreated lung (D, right) but not in more severely affected regions (D, left). See text for details. Original magnifications: X400 (A, B, C, G); X200 (D); X100 (E, F, H).

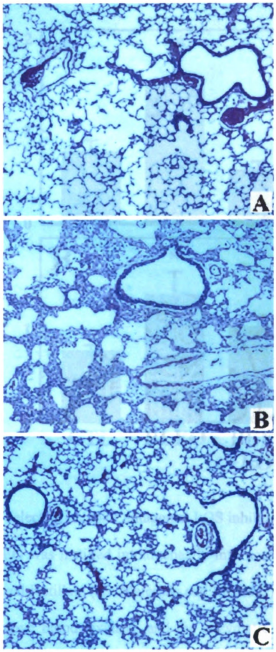

Figure 5.2. Histology of mouse lungs at 14 days after instillation of BLEO. A–C: Hematoxylin and eosin preparations of mouse lung instilled intratracheally14 days earlier with sterile saline (A), BLEO (B), or BLEO and LOS (C). See text and Materials and Methods section for details. Original magnifications X200.

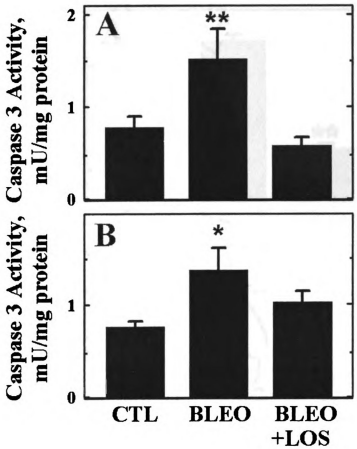

Figure 5.3. The AT1-selective receptor antagonist LOS inhibits BLEO-induced activation of caspase 3. A: BLEO was instilled intratracheally into normal mice with and without LOS in the intratracheal instillate (see Materials and Methods). Six hours later, the lungs were perfused to remove blood, excised, and the enzymatic activity of caspase 3 was measured in lung homogenates. B: Lung explants were prepared from normal mouse lung tissue perfused before excision (see Materials and Methods). BLEO (25 mU/ml) was applied in serum-free culture medium for 24 hours in the presence or absence of LOS (10^{-6}mol/L). Bars are the means ± SEM of $n = 6$; *, $P < 0.05$ *versus* control (CTL); ** , $P < 0.05$ *versus* BLEO±LOS by analysis of variance and Student-Newman-Keul's test.

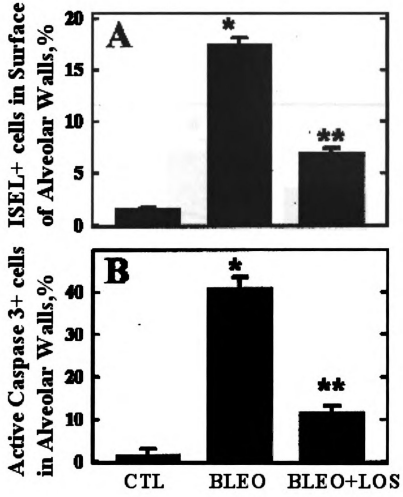

Figure 5.4. AT1 receptor blockade inhibits DNA fragmentation and caspase-3 activation in lung epithelial cells 14 days after BLEO instillation. Normal mice were given a single intratracheal instillation of BLEO in the presence or absence of LOS in the instillate. LOS also was administered thereafter daily intraperitoneally. Fourteen days later, lung sections were prepared and labeled by ISEL (A) or by IHC for the active form of caspase 3 (B). Labeling was quantitated in cells within the alveolar surfaces (see Figure 1C). Bars are the means ± SEM of $n = 6$; *, $P < 0.01$ *versus* control (CTL); **, $P < 0.05$ *versus* BLEO by analysis of variance and Student-Newman-Keul's test.

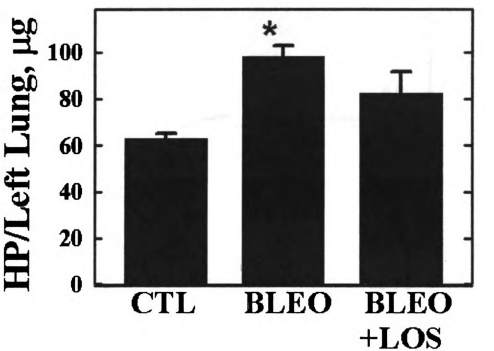

Figure 5.5. AT1 receptor blockade inhibits lung collagen accumulation at 14 days after BLEO instillation. Normal mice were administered BLEO in the presence or absence of LOS as described in Figure 5.3. Fourteen days later, total lung collagen was determined by assay of total hydroxyproline (HP) in hydrolyzed lung tissue (see Materials and Methods). Bars are the means ± SEM of $n = 6$; *, $P < 0.05$ *versus* control (CTL) by analysis of variance and Student-Newman-Keul's test.

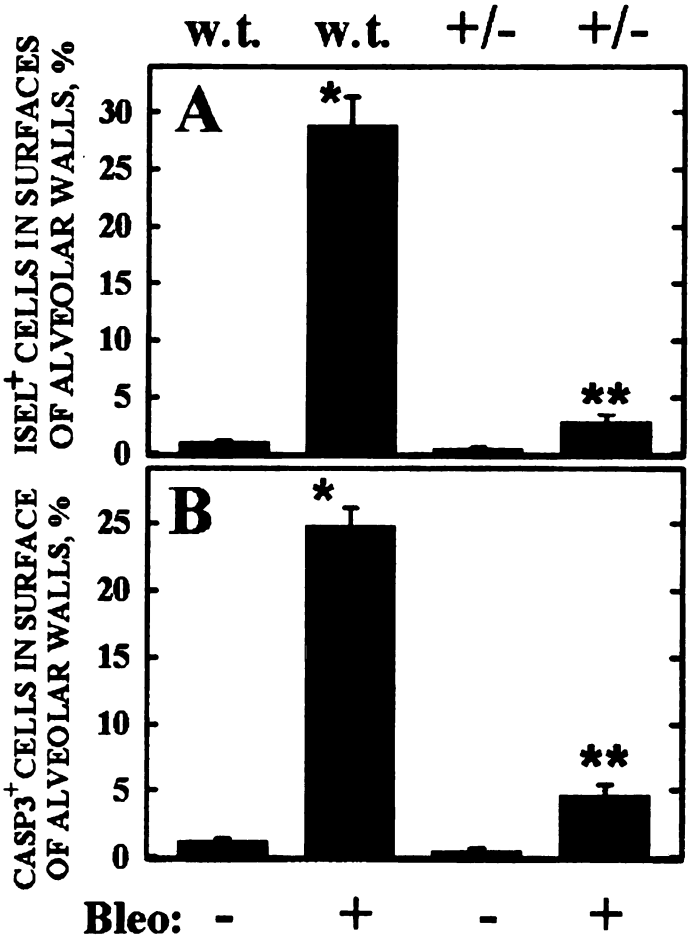

Figure 5.6. Mice deficient in angiotensin receptor AT1a exhibit reduced DNA fragmentation and caspase-3 activation in lung epithelial cells 14 days after BLEO instillation. Normal [wild type (w.t.)] or heterozygous AT1a knockout mice (+/-) were administered BLEO intratracheally as in Figure 5.3. Fourteen days later, lung sections were prepared and labeled by ISEL (A) or by IHC for the active form of caspase 3 (B), which were quantitated as described in Figure 5.4 and Materials and Methods. Bars are the means ± SEM of $n = 5$; *, $P < 0.001$ *versus* wild-type unchallenged (w.t. - BLEO); **, $P < 0.01$ *versus* wild-type challenged (w.t. + BLEO) by analysis of variance and Student-Newman-Keul's test.

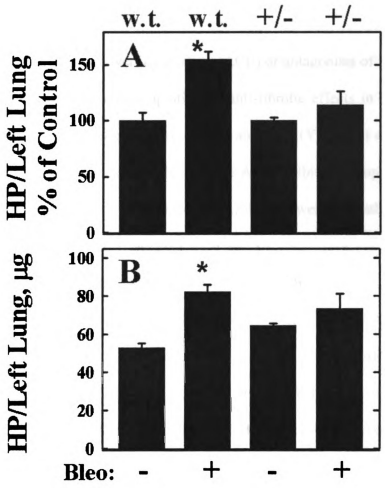

Figure 5.7. Mice deficient in angiotensin receptor AT1a exhibit reduced lung collagen accumulation in response to BLEO instillation. Normal [wild type (w.t.)] or heterozygous AT1a knockout mice (+/-) were administered BLEO intratracheally as in Figure 5.3. Fourteen days later lung tissue was fast-frozen, hydrolyzed, and total collagen was measured by hydroxyproline assay (HP) as described in Materials and Methods. A: HP data are expressed as a percentage of the corresponding control (- BLEO). B: Data are expressed as the absolute amount of HP per left lung. Bars are the means ± SEM of $n = 5$; *, $P < 0.01$ *versus* untreated (- BLEO) by analysis of variance and Student-Newman-Keul's test.

DISCUSSION

Inhibitors of angiotensin-converting enzyme (ACE) or antagonists of ANG receptor AT1 have been shown to have anti-apoptotic and anti-fibrotic effects in the heart (Sun and Weber, 1998), kidney (Mezzano et al., 2001), and liver (Yoshiji et al., 2001). The first reports of anti-fibrotic actions in the lung by the ACE inhibitor captopril, published many years ago (Molteni et al., 1985; Ward et al., 1990), were recently extended by the demonstration that the AT1 antagonists LOS and L158809 have even more potent anti-fibrotic potential in the lungs than do ACE inhibitors (Molteni et al., 2000). The present work extends those observations by showing that at least one of the mechanisms by which AT1 antagonists act is through the inhibition of apoptosis in AECs. Other potential mechanisms by which AT1 antagonists might act on the lung *in vivo*, a topic recently reviewed by Marshall (Marshall, 2003), include decreased vascular tone, decreased vascular permeability, and altered fibroblast activity. At least some of these actions could be envisioned to be related to the ability of ACE inhibitors or AT1 antagonists to reduce blood pressure. Indeed, the AT1-null mice used here were shown earlier to exhibit reductions in systemic blood pressure of about 12 mm Hg for heterozygous animals at baseline. Thus, it is possible that some of the anti-fibrotic actions of LOS or AT1a deletion might be related to lowered systemic or pulmonary hydrostatic pressures, and the data herein do not strictly exclude that possibility. On the other hand, the ability of LOS to prevent caspase-3 activation by BLEO in cultured lung explants (Figure 5.3B) argues against the involvement of decreased blood pressure in the inhibition of apoptosis because no hydrostatic pressure changes occur in explants manipulated *ex vivo*.

Moreover, the induction of LOS-inhibitable caspase 3 activities in lung explants, depleted of blood by previous PBS perfusion, and argues against a primary role for blood-derived cells in the initiation of the apoptosis. This experiment also supports the theory that the inhibitory effect of LOS on apoptosis was not mediated by an indirect action on infiltrating inflammatory cells. The initial protocol for the reported *in vivo* studies was designed to determine whether blockade or deletion of the AT1 receptor, throughout the time course of the 14- day BLEO model, was capable of inhibiting or blocking the apoptotic and fibrotic responses. The success of this strategy, particularly in light of the many known functions of angiotensin discussed in preceding paragraphs, raises the interesting question of whether the blockage of fibrogenesis was due to the acute or delayed consequences of AT1 blockade. Although a time course study of various LOS administration protocols was not performed, quantitation of the number of erythrocytes reaching the alveolar airspaces by 6 hours after BLEO (a crude index of lung barrier collapse) suggested that LOS did not prevent acute, transient barrier collapse (data not shown) despite its ability to reduce caspase 3 activation at the same sampling time (Figure 5.3). This observation, although very preliminary, is consistent with the theory that the blockage of apoptosis in AECs is a key to the subsequent blockade of collagen deposition. In contrast, blockage of receptor AT1 may also inhibit mitosis of lung fibroblasts (Marshall et al., 2000) and reduce collagen synthesis by the same cells (Marshall et al., 2004) relatively delayed effects that might be independent of AEC apoptosis at early time points. Moreover, endothelial cells also express receptor AT1 and undergo apoptosis in response to angiotensin, albeit at relatively high concentrations (Dimmeler et al., 1997; Li et al., 1999) and other cell types resident in the lung are known

to respond to angiotensin in ways currently under intense study (Harrison et al., 2003). Thus, it is possible that the acute early effects of AT1 blockade on AEC apoptosis are not necessary for inhibition of collagen deposition at later time points, and this study does not exclude that possibility. On the other hand, earlier work has shown that AECs undergoing apoptosis in response to Fas ligand, tumor necrosis factor-α or BLEO begin secreting angiotensin into the extracellular space within hours of exposure (Wang et al., 1999; Wang et al., 2000; Li et al., 2003), at least *in vitro*. Those studies also showed that the autocrine production of angiotensin and its binding to receptor AT1 on AECs were required for apoptosis in response to these agents; this mechanism can explain the ability of LOS to block AEC apoptosis *in vivo* in the present study. Moreover, previous studies with apoptosis inhibitors support the contention that the acute apoptotic response is a pivotal event in the BLEO model. Wang and colleagues (Wang et al., 2000) showed that the ACE inhibitor captopril or the caspase inhibitor ZVAD-fmk had essentially equal ability to block the appearance of apoptotic epithelial cells in rats exposed to intratracheal BLEO and to prevent subsequent collagen deposition (Wang et al., 2000). That report, which was confirmed by Kuwano and colleagues (Kuwano et al., 2001) in studies of mice exposed to BLEO and/or ZVADfmk, suggested that the blockade of fibrogenesis by captopril was indeed related to inhibition of apoptosis, rather than the many other effects of ACE inhibition *in vivo* (Marshall, 2003). Later work confirmed that the ZVAD compound had no inhibitory effect on angiotensin converting enzyme itself (Filippatos and Uhal, 2003). Thus, the present data are consistent with the ability of ACE inhibition by captopril to block both AEC apoptosis and collagen deposition in rats (Wang et al., 2000), and extend this concept to angiotensin receptor blockade in mice. The data herein

also are in agreement with recent reports that LOS inhibits BLEO-induced collagen deposition in rat lung (Fang et al., 2002) and that AT1a-null mice show reduced liver fibrosis in response to carbon tetrachloride (Kanno et al., 2003). The AT1 receptor is expressed as two isoforms, AT1a and AT1b, for which no selective antagonists have yet been developed (Filippatos et al., 2001). Subtype AT1a is known to be expressed in the lungs of mice, but AT1b was not detected in mouse lung by reverse transcriptase-polymerase chain reaction (Burson et al., 1994). Although it is possible that cells of minor abundance in the lung, such as type II cells, might express AT1b in quantities not detected in earlier studies, the primary isolates of type II pneumocytes from Wistar rats did not reveal AT1b expression by reverse transcriptase- polymerase chain reaction despite the use of two different primer sets and high-amplification cycle numbers (data not shown). In any case, the finding that deletion of only one allele of the AT1a gene significantly supports the notion that AT1a is the only active AT1 receptor subtype on the alveolar epithelium of mice.

In an earlier report describing the mechanisms by which angiotensin induces apoptosis in primary cultures of AECs, Papp and colleagues (Papp et al., 2002) showed that blockage of AT1 signaling through protein kinase C (PKC) with the specific PKC inhibitor chelerythrin could attenuate the apoptotic response to angiotensin. This finding is consistent with the known role of PKC in AT1 signaling in a variety of cell types (Li et al., 1999), but the pathways from PKC to the effector caspase 3, which is also required for this response (Papp et al., 2002), are currently unknown. Given that AEC apoptosis in response to Fas ligand, tumor necrosis factor- α, or BLEO all require the autocrine production and binding of angiotensin to AT1 (Wang et al., 1999; Wang et al., 2000; Li

et al., 2003), the report of Papp and colleagues (Papp et al., 2002) suggests that PKC inhibitors would also block AEC apoptosis in response to these agents *in vivo* as well. This prediction was not tested in the present study, but will be an interesting topic for future inquiry.

In summary, BLEO-induced apoptosis of lung epithelial cells in mice was significantly inhibited by the AT1- selective angiotensin receptor antagonist LOS or by targeted deletion of the gene for angiotensin receptor subtype AT1a. Both methods of reducing AT1 action also reduced or abrogated lung collagen accumulation in response to BLEO challenge. These data agree with earlier demonstrations of the anti-fibrotic action of ACE inhibitors and AT1-selective antagonists in rat models of lung fibrosis, and with *in vitro* studies showing a role for receptor AT1 in mediating apoptosis of AECs. They also suggest the possibility that AT1 antagonists may hold potential for the treatment of lung fibrosis in humans; this possibility is supported by the recent finding that patients with pulmonary fibrosis have a higher frequency of the D allele of angiotensin-converting enzyme (Morrison et al., 2001), a deletion polymorphism that confers higher levels of ACE.

Chapter 6

ATTENUATION OF BLEOMYCIN-INDUCED PULMONARY FIBROSIS BY INTRATRACHEAL ADMINISTRATION OF ANTISENSE OLIGO-NUCLEOTIDES AGAINST ANGIOTENSINOGEN mRNA

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ABSTRACT:

Apoptosis of alveolar epithelial cells (AECs) is believed to be critical for the development of bleomycin-induced pulmonary fibrosis. Angiotensin II is generated by AECs undergoing apoptosis and by human lung myofibroblasts isolated from IPF patient biopsies (Am J Physiol. 277:L1245-L1250, 1999; Am J Physiol. 277:L1158-L1164, 1999). Previous studies showed that apoptosis of alveolar epithelial cells in response to bleomycin could be abrogated by antisense oligonucleotides against angiotensinogen (ANGEN) mRNA and requires angiotensin II synthesis de novo (Am J Physiol Lung Cell Mol Physiol 284: L501-L507, 2003). Here we hypothesized that blockade of local pulmonary ANG II synthesis by intratracheal administration of antisense oligonucleotides against ANGEN mRNA might attenuate bleomycin-induced apoptosis of AECs and pulmonary fibrosis. Male Wistar rats received 8 U/kg of bleomycin sulfate or vehicle intratracheally. Endogenous lung ANGEN was upregulated in vivo as early as 3 hours after bleomycin instillation in rat lungs by RT-PCR, in situ hybridization and immunohistochemistry staining methods. ANGEN mRNA and angiotensin peptides were localized in alveolar wall cells in the alveolar corners, tentatively identified as type II cells, and also colocalized with alpha-Smooth Muscle Actin (α-SMA). Labeled antisense administered by intratracheal instillation was specifically accumulated in the lung compared to liver and kidney, and localized primarily in the epithelium of airways and cells within alveolar walls. Intratracheal instillation of 75ug antisense reduced bleomycin–induced pulmonary fibrosis detected by hydroxyproline assay; decreased ANGEN and active caspase-3 protein detected by western blot and reduced the ISEL positive cells, but had no effect on the serum ANG II level. These data are consistent with

the hypothesis that lung-derived ANGEN is involved in bleomycin-induced pulmonary fibrosis.

INTRODUCTION:

Idiopathic Pulmonary fibrosis (IPF) is a pathological condition resulting from injury to the lung and an ensuing fibrotic response leading to thickening of the alveolar walls and the obliteration of the alveolar space without known etiology (Fonseca C et al., 2000). In IPF the normal architecture and functional integrity of the lung are destroyed. The main histological features of the fibrotic lung are persistent and unrepaired epithelial damage, proliferation and accumulation of fibroblast/ myofibroblast cells, and increased collagen deposition (Selman et al., 2001). Unfortunately, despite years of research on its pathogenesis, the treatment of IPF has not been successful. The traditional therapeutic approaches using potent anti-inflammatory agents supplemented with an immunosuppressive agent could only offer a marginal benefit at best (King et al., 2000). Recent NHLBI Workshops have concluded that our current understanding of the pathogenesis of the IPF is incomplete (Mason et al., 1999; Crystal et al., 2002). Current evolving hypothesis about pathogenesis is that IPF results from the epithelial microfoci injury and a failure of reepithelization (Selman et al., 2001, Gauldie et al., 2002). Haschek and Witschi first proposed this hypothesis more than 20 years ago, who believe that epithelial damage drives fibrogenesis and efficient epithelialization would prevent fibrogenesis (Haschek et al., 1979; Witschi H., 1990).

Recent studies in our lab and others using bleomycin-treated rat and mouse models strongly suggested a role of epithelial apoptosis as the profibrotic event in fibrogenesis. The evidences include: First, apoptosis of AECs was found in both patients with IPF (Uhal, et al., 1998) and animal models (Hagimoto, et al., 1997). Second, induction of

apoptosis in the epithelium is sufficient to initiate a fibrotic response (Hagimoto N. et al., 1997). Third, several labs showed that blockade of the apoptosis could prevent fibrotic response (Wang, et al., 2000; Kuwano et al., 2001). These studies support the "Witschi Hypothesis" regarding the pathogenesis of pulmonary fibrosis that is PF results from the epithelial injury and failure to reepithelization. Results obtained from studies on human lungs are consistent with those on animal models. Fragmented DNA, the hallmark of apoptosis, in bronchiolar and AECs were found in lung biopsies from patients with IPF (Kuwano et al, 1996). Our lab confirmed this result by showing the simultaneous double labeling of fragmented DNA and alpha-smooth muscle actin (α-SMA), the marker for myofibroblast, in biopsies from patients with IPF (Uhal et al., 1998). Fragmented DNA in the alveolar epithelium was found frequently and immediately adjacent to a-SMA-positive interstitial cells. Thus, epithelial apoptosis colocalizes with myofibroblast where collagen deposition is severe, at least in patients with IPF.

Recent work from this laboratory has shown that exposure of cultured AECs to Fas ligand (Wang et al., 1999), tumor necrosis factor-α (Wang et al., 2000), or BLEO (Li et al., 2003) all induce expression of angiotensinogen mRNA and protein, and its cleavage to the peptide angiotensin II (ANGII). Moreover, apoptosis of cultured AECs in response to these apoptosis inducers was abrogated by antisense oligonucleotides against ANGEN mRNA (Li et al., 2003; Uhal et al., 2003; Filippatos and Uhal, 2003). For all these reasons, it was hypothesized that blockade of local pulmonary ANG II synthesis by administration of antisense oligonucleotides against ANGEN mRNA might attenuate bleomycin-induced apoptosis of AECs and pulmonary fibrosis. To test this theory, normal rats and explants were subjected to intratracheal BLEO administration and

quantitation of apoptosis and lung collagens. We report here the prevention of both BLEO-induced AEC apoptosis and lung collagen accumulation in whole animals and explants by administration of the antisense oligonucleotides against ANGEN mRNA.

MATERIALS AND METHODS

Reagents and Materials

Phosphorothioated control and antisense oligonucleotides against angiotensinogen (18-mers) were synthesized and purchased from Genemed Synthesis (San Francisco, CA). Alkaline phosphataseconjugated streptavidin, digoxigenin-labeled deoxyuridine trisphosphate (dig-dUTP), and biotinylated deoxyuridine trisphosphate (bio-dUTP) were obtained from Boehringer Mannheim, Indianapolis, IN. BLEO was obtained from Sigma Chemical Co., Saint Louis, MO. Reagents for detection of alkaline phosphatase and other secondary reagents for *in situ* end labeling (ISEL) of DNA or Western blotting were from sources described earlier (Wang et al., 2000). All other materials were of reagent grade and were obtained from Sigma Chemical Co.

Animals, Induction of Pulmonary Fibrosis, and Surgical Procedures:

Induction of Lung Injury and Fibrosis

Adult male Wistar rats, 150-200 g, were housed in a satellite facility of University Laboratory Animal Resources, Michigan State University. Animals under pentobarbital anesthesia received a single intratracheal instillation of bleomycin sulfate (BLEO) at 8 U/ kg body weight, in 400 µl of sterile saline. The 400-µl dose was instilled at end-expiration, and the liquid was followed immediately by 2ml of air to ensure delivery to the distal airways. Control animals were instilled with an equal volume of sterile saline by using previously published protocols (Wang et al., 2000). And lung tissue was perfused with PBS and harvested at 3 hours, 6 hours, 24 hours, or 2,7,14 days after instillation of bleomycin. ANGEN mRNA was detected by semiquantitative RTPCR and

in situ hybridization. Double immunolabeling was performed by using Anti-ANG I, lectin or Anti-alpha smooth muscle actin (α-SMA) antibody to identify the cellular source of ANGEN/ANG I protein. ANG II in perfused lung tissue was measured by ELISA kit specific for ANG II.

Intratracheal delivery of antisense in vivo

Fluorescent (BODIPY)-labeled 18-mer antisense phosphorothioated oligonucleotides against ANGEN were synthesized and used in a series of instillations of bare oligonucleotide at 4 different doses: 150, 75, 25 and 10ug in 400ul PBS, each of which were instilled one time I.T. into Wistar rats. 2 hours later, the lungs, livers and kidneys were excised. One half tissue was immediately frozen in liquid nitrogen and the other half was processed by a standard protocol for the preparation of frozen sections. Frozen tissues in dry ice were sent to the histology lab at Michigan State University for frozen cryostat sectioning. In order to demonstrate individual cells, all sections were incubated with PI to stain the cell nuclei. Sections then were examined under fluorescence microscope.

In some studies antisense oligonucleotides against ANGEN mRNA and scramble oligonucleotides was added to the intratracheal instillate at the dose of 75ug/rat. 14 days after instillation of BLEO with or without oligonucleotides, all animals were sacrificed for histology, detection of collagen or DNA fragmentation, and caspase 3 activation in epithelial cells.

Surgical Procedures

Immediately before sacrifice, animals were given intraperitoneal injections of sodium pentobarbital and the trachea was cannulated. After perfusion by PBS, the left lung was

ligated at the hilus, excised distal to the ligation, and immediately frozen in liquid N2 for hydroxyproline assay of total collagen (see below). The remaining lung tissues were carefully removed and were instilled with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 20 cm of H2O constant pressure, then immersed in the same fixative for 30 minutes followed by storage in 70% ethanol. The fixed tissues were washed with PBS three times for 15 minutes and were then embedded in paraffin. Five μm sections of lung were deparaffinized by passing through xylene, xylene: alcohol 1:1, 100% alcohol, and 70% alcohol for 10 minutes each. Ethanol was removing by rinsing with distilled water.

Lung Explant Culture

Normal rat lungs were removed and perfused with PBS to flush the blood out. Tracheas were cannulated and DMEM contains bleomycin (25mU/ml) alone or with oligonuclitides (40nm) were instilled. Medium filled lungs were then cut into 1 mm square pieces. As suggested in other lung tissue culture study (Taylor et al., 2000), the minced lung chunks were plated into transwell polycarbonate inserts (0.4 um pore) and covered by a thin layer (1 mm) of culture medium with 10% fetal bovine serum (FBS) or 1% insulin-transferrin-selenium (ITS) to facilitate gas exchange. We used two different culture medium to test whether culture medium with ITS has the same effect of that with FBS which could contain angiotensin system components like ANGEN.

The explants were exposed to bleomycin (25mU/ml), BLEO plus saralasin (SAR: 50ug/ml) or BLEO plus ANGEN antisense oligonucleotides (AS: 40nM) and Vit C (0.1mM). The culture medium was changed every other day and at the same time added fresh reagents. The explants were cultured in a 5% CO_2 incubator at 37 °C for 24 hours

or 14 days, and then harvested by transfer into liquid N2 and storage at -80°C until for hydroxyproline assay to quantitate collagen amount (Woessner, 1961) and caspase-3 enzyme assay.

Identification and Quantitation of Apoptotic Cells and Total Lung Caspase 3 Activity

Localization of DNA Fragmentation

ISEL of fragmented DNA was conducted by a modification of the method of Mundle and colleagues (Mundle et al., 1994). Briefly, ethanol was removed from deparaffinized lung sections by rinsing in distilled water for at least 10 minutes. The slides were then placed in 3% hydrogen peroxide (Sigma Chemical Co.) for 30 minutes at 20°C, rinsed with PBS, and incubated with Proteinase K (Sigma) in standard saline citrate for 15 minutes at 37°C. Samples were rinsed once in water, three times in 0.15mol/L PBS for 4 minutes each, and were then incubated in standard saline citrate (0.3 mol/L NaCl and 30 mmol/L sodium citrate in water, pH 7.0) at 80°C for 20 minutes. After four rinses in PBS and four rinses in buffer A (50 mmol/L Tris/HCl, 5 mmol/L MgCl, 10 mmol/L B-mercaptoethanol, and 0.005% bovine serum albumin in water, pH 7.5), the sections were incubated at 18°C for 2 hours with ISEL solution (0.001mmol/L digoxigenin-dUTP; 20 U/ml DNA Polymerase I; and 0.01 mmol/L each of dATP, dCTP, and dGTP in buffer A). Afterward the sections were rinsed thoroughly five times with buffer A and three additional times in PBS. Detection of incorporated dUTP was achieved with by incubation for 2 hours at 37°C with AP-conjugated antidigoxigenin (Boehringer Mannheim) at 1/400 dilution. Bound AP-antibody was then detected with the Fast Blue

chromogen system and the sections were mounted with Fluoromount solution (Southern Biotechnology, Birmingham, AL).

For quantitation of ISEL- positive epithelial cells, the number of positive cells within the surfaces of the alveolar walls was counted in a minimum of six randomly selected x 400 microscopic fields per lung section. Positive cells within the alveolar airspaces, or otherwise clearly not within the surface of the alveolar wall, were not scored. The counts of positive nuclei per field were expressed as a percentage of the total number of nuclei in the same microscopic field. Sections from each of at least five rats per treatment group were analyzed by an investigator blinded to sample identity.

Caspase 3 Enzyme Activity

Assay of total lung caspase-3 enzyme activity was conducted with a commercially available kit (Molecular Probes, Eugene, OR). Fast-frozen lung was homogenized in assay kit buffer and was analyzed according to the manufacturer's instructions on a Biotek FL600 fluorescence plate reader. In all samples, specificity of the reaction for caspase 3 was verified by abrogation of the signal with a caspase-3 selective irreversible inhibitor (data not shown).

Quantitation of Lung Collagen

For quantitation of total lung collagen, tissues frozen in liquid N2 were dried to constant weight in preweighed tubes at 80°C. The weighed dry tissue was hydrolyzed in 6 N HCl and was subjected to determination of hydroxyproline as described earlier by Woessner

(Woessner, 1961) The efficiency of the hydrolysis was verified with rat-tail collagen by comparison to standard hydroxyproline (Sigma Chemical Co.).

Detection of ANGEN mRNA in lung section by In Situ Hybridization (ISH)

Based on published methods (Panoskaltisis-mortari et al., 1995), modification was done so that digoxigenin-labled DNA probes will be substituted for riboprobes. For this reason, denaturation, hybridization and wash conditions was modified to favor RNA/DNA hybridization rather than RNA/RNA. Then lung sections were incubated with biotin-conjugated anti-digoxigenin antibodies, followed by using the streptavidin- AP and NBT/BCIP detection system to amplify the chromogen signal.

Immunohistochemistry (IHC) for Activated Caspase 3, ANGEN/ANGI, lectin, alpha- smooth muscle actin (αSMA).

IHC was performed with an antibody that recognizes only the active form of caspase 3 (BioVision, MountainView, CA) and anti- ANGEN/ANGI antibody (Santa Cruz, CA). Deparaffinized lung sections were blocked with a solution of 3% bovine serum albumin in PBS for 1 hour; the primary antibody was then applied overnight at 4°C in 3% bovine serum albumin/PBS. After washing in PBS, the antibody was detected with a biotin-conjugated secondary antibody and avidin-linked chromogen system. For double labeling, lectin (Vector Laboratories, CA) and alpha- smooth muscle actin (Sigma, Missouri) antibody directly conjugated with FITC were applied on some sections.

Western blot analysis to detect and quantitate pulmonary angiotensinogen and active caspase 3

The lungs were perfused and snap-frozen in liquid nitrogen and stored at -80°C until protein extraction. Lung protein extraction was performed as following. Briefly, the frozen lungs were pulverized in a chilled mortar and placed in NP40 tissue lysis buffer contain protease inhibitors cocktail. The samples were further centrifuged at 15,800 *g* for 10 min at 4°C. The supernatants were collected, and protein levels were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The protein samples (60 μg) were electrophoresed on 12% SDS-PAGE in a Mini-Protean II Electrophoresis Cell (Bio-Rad). Protein molecular weight markers (Invitrogen) were run parallel to each blot as an indicator of the molecular weight. The separated proteins were transferred at 150 V for 1.5 hours onto PVDF membrane (Bio-Rad) in a Mini Trans-Blot chamber with transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). The PVDF membrane was blocked for 1 h using 5% no fat dry milk in Tris-buffered saline (TBS). For detection of angiotensinogen, a 1:100 dilution of ANG I/II antibody (Santa cruz, CA) as used and for detection of active caspase-3, 1 ug/ml antibody (Bio Vision, CA) as used. After being washed, the membrane was incubated with horseradish peroxidase linked with the secondary antibody (anti-goat immunoglobulin G for ANGEN; anti-rabbit immunoglobulin G for active caspase-3), as recommended by the manufacturer. Finally, the washed blots were exposed to an enhanced chemiluminescence (ECL) detection system (Amersham) and recorded on an autoradiograph (Amersham film).

Attention: Images in this dissertation are presented in color.

RESULTS:

Local pulmonary angiotensin synthesis was upregulated in vivo after bleomycin instillation.

Semiquantitative RTPCR (Fig. 6.1) showed upregulation of ANGEN mRNA in the lungs as early as 3h after i.t. instillation of bleomycin. In situ hybridization of ANGEN mRNA (Fig. 6.2) demonstrated that positive labeling (dark purple, shown by arrowhead) increased 6h (Fig. 6.2: Bx200 magnification, Cx400 magnification) and 14 days (Fig.6.2: E, Fx200 magnification) after BLEO instillation compared to the corresponding controls (Fig. 6.2: A, Dx200 magnification). 6h after BLEO instillation, the positive labeling is mainly localized at the corners of the alveolar, which typically are the positions for type II alveolar cells (Fig. 6.2BC). 14 days after BLEO instillation, the positive labeling is mainly localized at fibrotic foci (Fig. 6.2E) where myofibroblast / fibroblast accumulate and the corners of the alveolar (Fig. 6.2F) near the fibrotic region.

Immunohistochemistry (IHC) staining (Fig. 6.3) by using anti-ANG I antibody, which cross-reacted with ANGEN [see Fig. 6.10 western blot: rat liver ANGEN (first lane) was recognized by anti-ANG I Ab], showed that at 24h after i.t. BLEO ANGEN /ANG I (purple) was found in alveolar walls cell at the corners that did not label with lectin (compare Fig 6.3 A &B x400, same filed with matched arrowhead), consistent with the identity of those alveolar wall cells as type II cells because lectin labels bronchial epithelium and type I alveolar epithelial cells but not type II cells (Fehrenbach et al., 2000). In severely affected areas of the parenchyma of bleomycin- induced fibrotic rat lungs, regions of ANGEN / ANG I labeling (Fig. 6.3 Cx200, black box) were observed to coincide with a loss of lectin labeling (Fig. 6.3 Dx200, white box: double label of same

section as C), consistent with our theory that alveolar epithelium dies in regions rich in ANG peptides. Enlargement of the white boxed region (Fig. 6.3 Ex400) reveals a-SMA immunoreactivity in the middle of the box on the adjacent serial section, suggestive of myofibroblasts.

Western blot for ANGEN showed that ANGEN protein in the lungs which were perfused by PBS, 14 days after bleomycin instillation were significantly higher than that in control lung instilled with saline (Fig. 6.10). (Bars are the means± SEM of n=5, $p<0.05$ vs. CTL by t test).

A specific ELISA for ANG II detected that the pulmonary concentrations of ANGII in the lungs which were perfused by PBS, 6h and 14 days after bleomycin instillation were significantly higher than that in control lung instilled with saline (Fig. 6.4). (Bars are the means± SEM of n=5, $p<0.05$ vs. CTL by t test).

Antisense oligonucleotides against ANGEN mRNA blocked bleomycin induced apoptosis and collagen accumulation in lung explants (ex vivo).

BLEO increased caspase 3 activity ($P < 0.05$), when applied in vitro at 25 mU/ml for 24 hours to rat lung explants that were depleted of blood before explant culture. Fig 6.5 showed that application of BLEO in vitro increased caspase-3 activity in the explants in 24 hours but Antisense oligonucleotides against ANGEN mRNA (40nM) inhibited the increase. Fig. 6.6A showed that there was more collagen accumulation in explants cultured in 10% FBS, treated with bleomycin compared with control group treated without bleomycin [Results were shown as μg hydroxyproline (HP) per mg of dry lung tissue. Bars are the mean ± SEM of at least 4 separated samples; * = $p<0.05$ versus

control]. Moreover, Fig 6.6B showed that ANGEN antisense oligonucleotides reduced BLEO-induced collagen accumulation in explants cultured in 1% ITS (Bars are the mean $\pm$ SEM; * = $p<0.01$ versus BLEO+AS).

Blockade of lung-derived ANGEN suppressed apoptosis of AECs and pulmonary fibrosis in vivo.

The feasibility of intratracheal delivery of antisense oligos specifically to the lung:

Figure 6.7 shows the distribution of BODIPY fluorescence in homogenates of the frozen lung, liver and kidneys after normalization to total tissue protein. At the dose of 150ug, lung tissue retained 30-fold more fluorescence, per unit protein, than liver and 37-fold more than kidney. At the dose of 75ug, the lungs retained 6-fold more than liver and 13-fold more than kidney.

Sections from the rat treated with 75ug of oligonucleotide revealed that BODIPY fluorescence (green) was localized primarily to the epithelium of airways (Fig. 6.8B, arrow) and in isolated cells of the alveolar walls (Fig. 6.8B, arrowhead). At higher magnification (Fig. 6.8C, D), some alveolar wall cells were found to concentrate the BODIPY-oligonucleotide (arrows) while others were stained with very little of the BODIPY-oligonucleotide (arrowheads).

Inhibition of Apoptosis and Collagen Deposition by Antisense in vivo:

The ability of Antisense to inhibit lung epithelial apoptosis was also observed *in vivo* at 14 days after BLEO administration. In Figure 6.12, intratracheal BLEO increased the abundance of ISEL-positive cells, but AS inhibited the increase. Measurement of lung collagen accumulation in the same animals by hydroxyproline assay (Figure 6.9B)

revealed an increase in total lung collagen by 14 days after intratracheal BLEO, but AS reduced the increase to a value not significantly different from the control (CTL). Fig. 6.9 A showed macroscopic photographs of the lungs 14 days after different treatment. Note the almost normal appearance of the lung in the Bleo +AS treated rat compared with the Bleo- and bleo+SCR treated rat lungs, which are smaller with many patchy bleeding spots. Histology of the whole lung sections obtained 14 days after intra tracheal bleomycin administration revealed typical changes of PF in bleomycin treated rats without antisense administration. The normal alveolar architecture appeared to be distorted with collapsed alveolar spaces and thickening of nearby alveolar septa. In antisense treated rats challenged with bleomycin, fibrosis-like changes were less extensively present. In rats treated with sterile saline, the histology was normal.

Active caspase-3 was detected by immunohistochemistry (IHC) and western blots (WB) 14 days after bleomycin instillation. Active caspase-3 shown by WB by using anti-active caspsase-3 (p17 fragment) antibody was induced by bleomycin and suppressed by antisense treatment (Figure 6.11, upper panel). Active caspase–3 was localized in alveolar epithelial cells and alveolar macrophages (Figure 6.11, lower panel).

The Antisense oligonucleotides against ANGEN mRNA inhibited DNA fragmentation detected by in situ end labeling (ISEL) of fragmented DNA in lung epithelial cells 14 days after BLEO instillation (Figure 6.12, Figure 6.13). ISEL positive cells are blue. In CTL group, ISEL-positive nuclei were not observed (Figure6.12A). In bleomycin alone treated group, ISEL-positive nuclei were observed in cells within the alveolar walls, many ISEL-positive nuclei were observed in septal wall cells at the alveolar corners

(Figure6.12B). Administration of antisense decreased ISEL positive cells in response to bleomycin, but scramble did not (Figure6.12 C, D).

Effect of antisense treatment on angiotensin peptides:

Intratracheal instillation of 75ug antisense decreased pulmonary ANGEN expression (fig 6.10) but had no effect on the serum ANG II level. The plasma ANGEN protein did not decreased in response to intratracheal instillation of antisense.

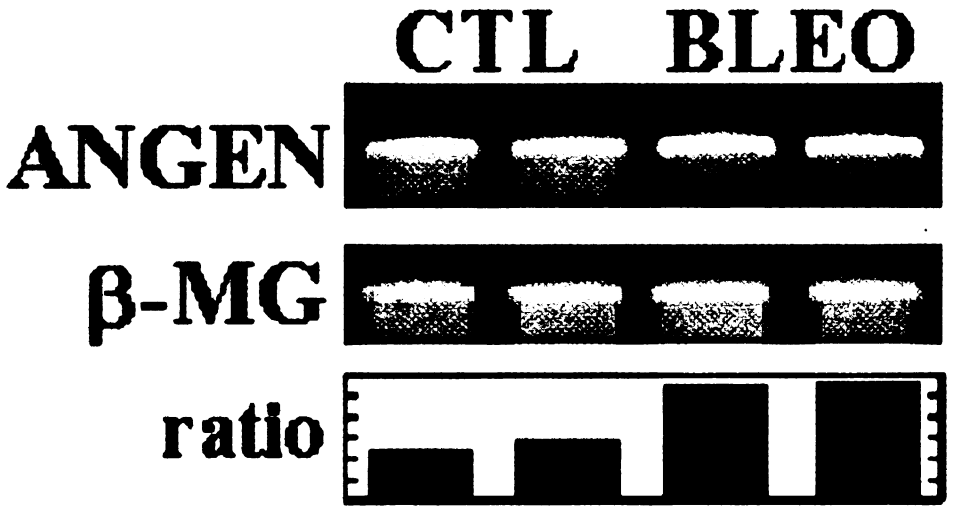


Fig. 6.1: Semiquantitative RT-PCR of angiotensinogen (ANGEN) mRNA in lungs 3hours after Bleo exposure. Normal rats were intraltracheeally instilled with Bleo (8U/Kg) or saline (CTL) for 3hours and total RNA was isolated. RT-PCR was performed as described before (see Material and Methods) with primers specific for rat ANGEN, β -Microglobulin (β -MG) as control mRNAs.

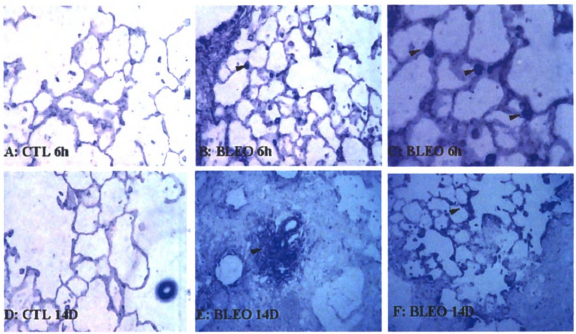


Fig. 6.2: In situ hybridization of ANGEN mRNA demonstrated that positive labeling (dark purple, shown by arrowhead) increased 6h (B x 200 magnification, C x 400 magnification) and 14 days (E, Fx200 magnification) after BLEO instillation compared to the corresponding controls (A, D x 200 magnification). 6h after BLEO instillation, the positive labeling is mainly localized at the corners of the alveoli, which typically are the positions for type II alveolar cells (BC). 14 days after BLEO instillation, the positive labeling is mainly localized at fibrotic foci (E) where myofibroblast / fibroblast accumulate and the corners of the alveolar (F) near the fibrotic region.

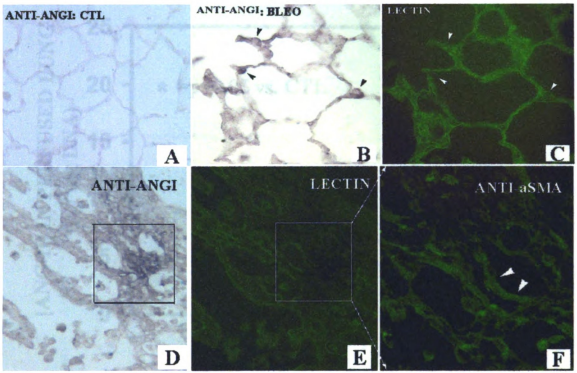
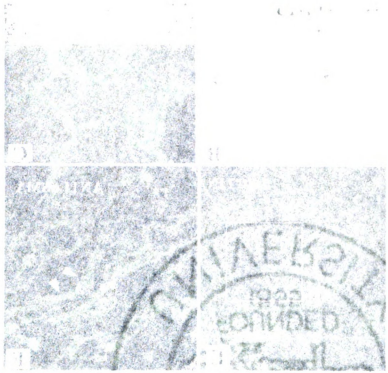


Fig. 6.3: The sections were subjected to IHC with antibodies against the ANGI, lectin and a-SMA 24 hours (B, C) and 7 days (D, E, F) after intratracheal instillation of bleomycin. 24h after BLEO instillation ANGEN /ANG I (purple) was found in alveolar walls cell at the corners that did not label with lectin (compare B&C x400, same field with matched arrowhead). In severely affected areas of the parenchyma of bleomycin- induced fibrotic rat lungs, regions of ANGEN / ANG I labeling (Dx200, black box) were observed to coincide with a loss of lectin labeling (Ex200, white box: double label of same section as D). Enlargement of the white-boxed region (F x400) revealed a-SMA immunoreactivity in the middle of the box on the adjacent serial section, suggestive of myofibroblasts.



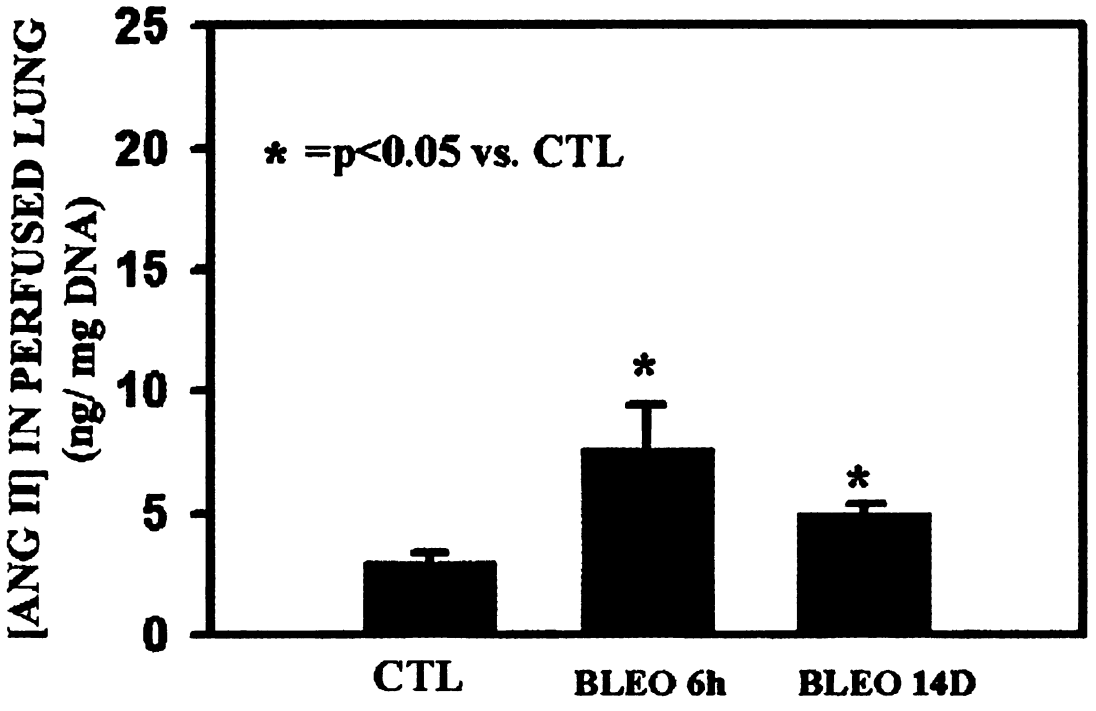


Fig. 6.4 Pulmonary angiotensin II (ANG II) increased in bleo-induced lung injury. 6 hours and 14 days after bleo instillation, lungs were perfused, homogenized, and analyzed by ELISA specific for ANG II. Values are means of at least 5 separate determinations.

* Significantly different from CTL, $P < 0.05$ (by Student's *t*-test).

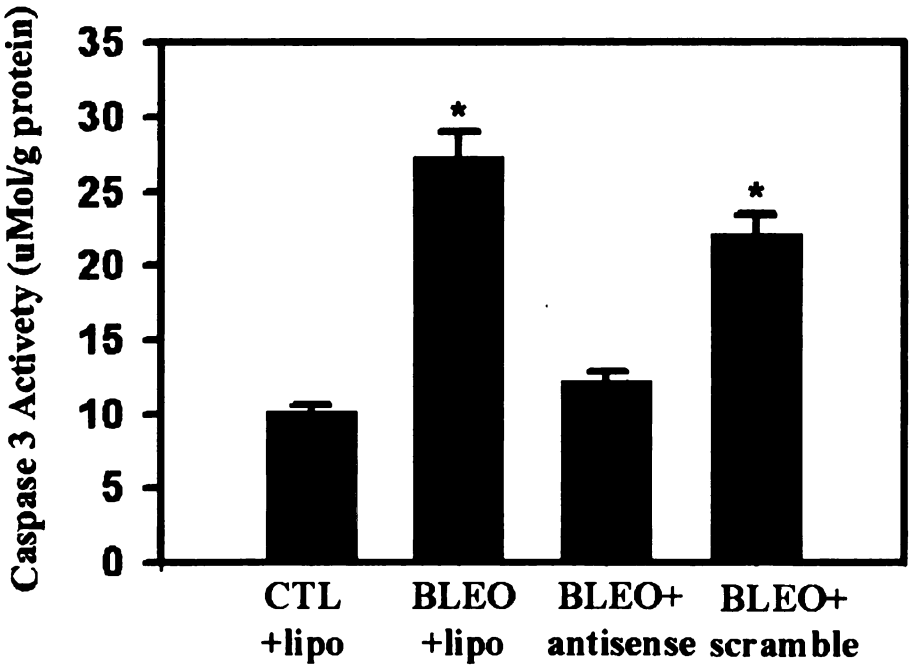


Fig. 6.5 The Antisense oligonucleotides against ANGEN mRNA inhibit BLEO-induced activation of caspase 3. Lung explants were prepared from normal rat lung tissue perfused before excision (see Materials and Methods). BLEO (25 mU/ml) was applied in serum-free culture medium for 24 hours in the presence or absence of antisense (40nM). Bars are the means ± SEM of $n = 3$; *, $P < 0.05$ *versus* control (CTL+lipo) by analysis of variance and Student-Newman-Keul's test.

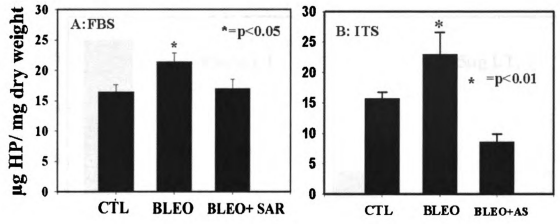


Fig. 6.6 The Antisense oligonucleotides against ANGEN mRNA and non-selective AT receptor antagonist saralasin inhibit BLEO-induced collagen accumulation in lung explants. Lung explants were prepared from normal mouse lung tissue perfused before excision (see Materials and Methods). BLEO (25 mU/ml) was applied in 10% FBS (A) or 1% ITS (B) for 14 days in the presence or absence of SAR (50ug/ml) or antisense (40nM). Bars are the means ± SEM of $n = 3$; *, $P < 0.05$ *versus* control (CTL) in A; *, $P < 0.01$ *versus* CTL in B by analysis of variance and Student-Newman-Keul's test.

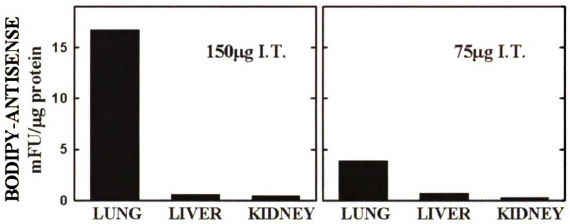


Fig. 6.7 Distribution of fluorescence in lung, liver and kidney 2 hous after instillation of BODIPY labeled oligonucleotides. Normal rats were instilled with different dose of BODIPY labeled oligonucleotides. Fluorescence intensity was measured in the homogenates of the frozen lung, liver and kidneys after normalization to total tissue protein.

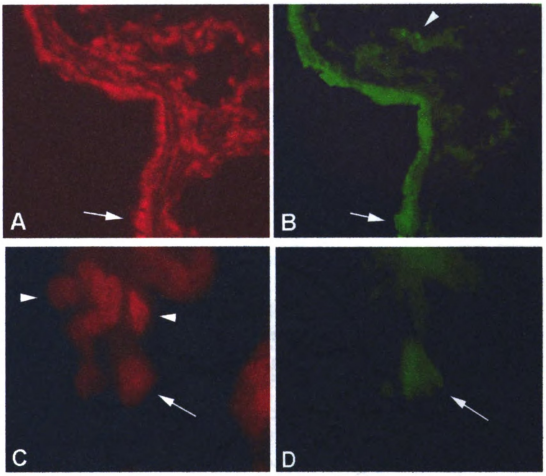
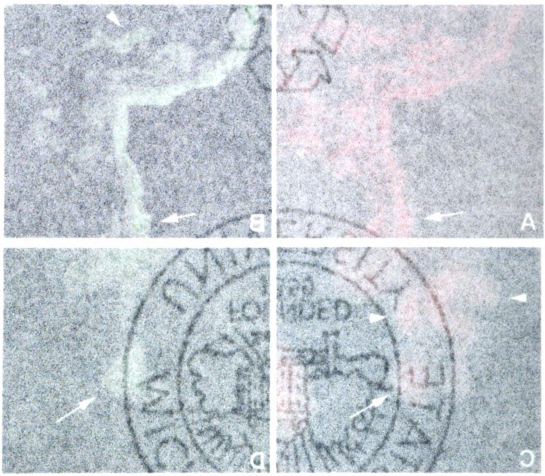


Fig. 6.8 Localization of the intratracheal instilled fluoscence-labelled antisense against ANGEN mRNA on the lung sections from rats treated with 75ug of oligonucleotides. Panels with red fluorescence show PI staining, and panels with green fluorescence show BODIPY staining. Panel A, B (10 x10) show the same field from the same section. And so do panel C, D (40 x 10). Panel A, B revealed that BODIPY fluorescence was localized primarily to the epithelium of airways (arrow) and in isolated cells of the alveolar walls (arrowhead). Panel C, D revealed that some alveolar wall cells were stained with high intensity of BODIPY-oligonucleotides (arrows) while others were stained with very little of the oligonucleotides (arrowheads).


B
A
D
C

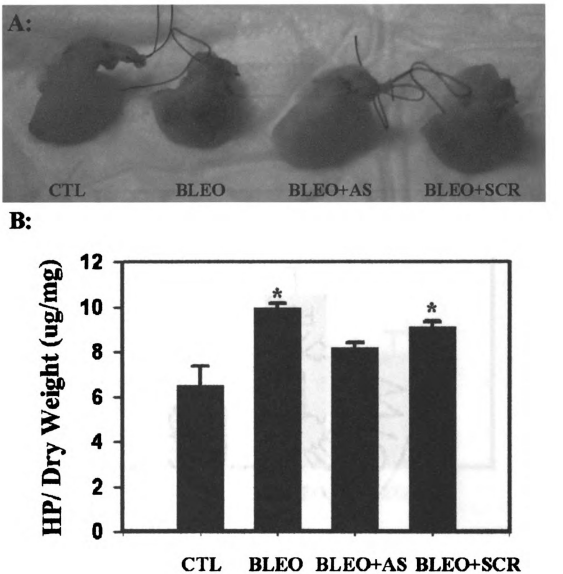


Fig. 6.9 A: Macroscopic photographs of the lungs 14 days after different treatment. Note the almost normal appearance of the lung in the Bleo +AS treated rat compared with the Bleo- and bleo+SCR treated rat lungs, which are smaller with many patchy bleeding spots. B: Quantitation of total lung collagen by hydroxyproline (HP) assay. At 14 days post-Bleo, collagen was quantitated by HP assay applied to hydrolyzed lung tissue. See METHODS for details. * $P < 0.05$ vs. CTL by ANOVA.

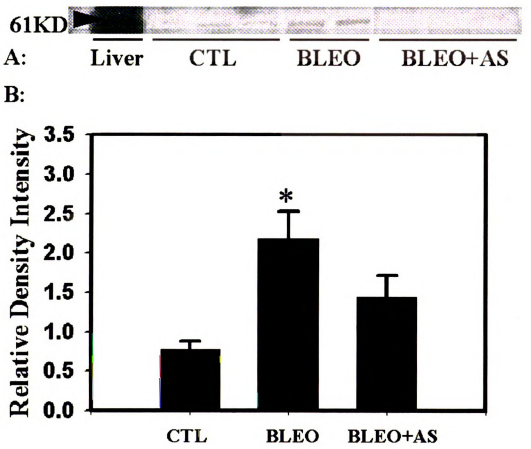


Fig. 6.10 Bleomycin increased angiotensinogen protein expression, which was suppressed by the treatment of antisense in perfused lung tissues. 14 days after instillation of bleomycin or vechicle, lungs were perfused and homogenized for the western blot analysis. (A) Representative immunoblot is shown and (B) densitometric evaluation of blot data. Data are presented as mean ±SE (n=5). *: $P<0.05$ versus CTL

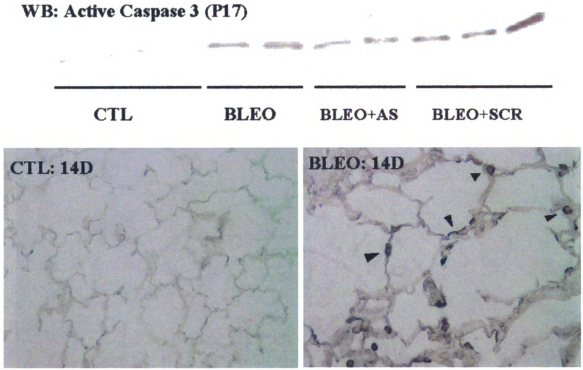


Fig 6.11. Detection of active caspase-3 by immunohistochemistry (IHC) and western blots (WB) 14 days after described treatment. Male Wistar rats received indicated treatment intratracheally. Lung tissues were harvested 14 days after treatment and subjected to IHC and WB by using anti-active caspsase-3 (p17 fragment) antibody. *Upper penal (WB)*: Active caspase-3 was induced by bleomycin and suppressed by antisense treatment. *Lower penal (IHC):* active caspase–3 was localized in alveolar epithelial cells and alveolar macrophages.

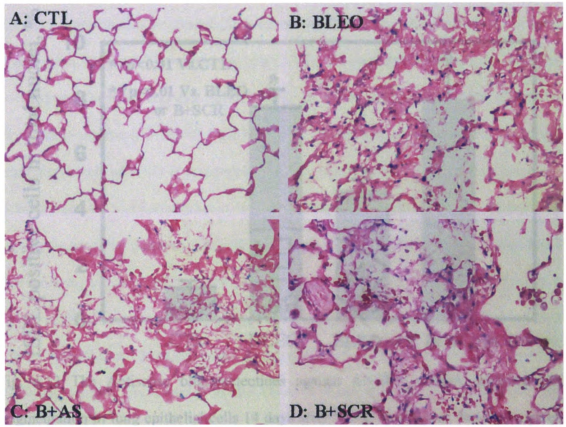


Fig 6.12. Detection of apoptotic cells by in situ end labeling (ISEL) of fragmented DNA 14 days after described treatment. Male Wistar rats received indicated treatment intratracheally; lung tissues were harvested 14 days after treatment and subjected to ISEL coupled to a fast blue detection system (see METHODS). Positive reaction is blue. *A*: in CTL group, ISEL-positive nuclei were not observed. B: in bleo group, ISEL-positive nuclei were observed in cells within the alveolar walls, many ISEL-positive nuclei were observed in septal wall cells at the alveolar corners. C: administration of antisense decreased ISEL positive cells in response to Bleo. D: administration of scramble nucleotides did not decreased ISEL positive cells in response to Bleo.

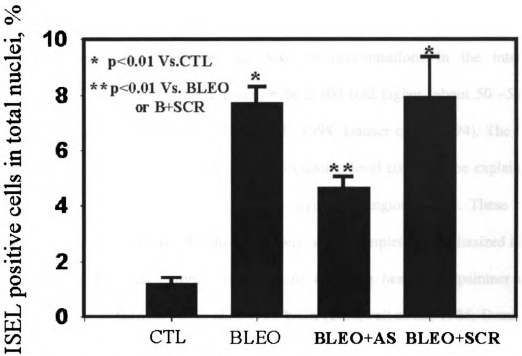


Fig. 6.13 The Antisense oligonucleotides against ANGEN mRNA inhibited DNA fragmentation in lung epithelial cells 14 days after BLEO instillation. Rats were given a single intratracheal instillation of BLEO in the presence antisense or scramble ONT in the instillate. Fourteen days later, lung sections were prepared and labeled by ISEL. Labeling was quantitated in cells within the alveolar surfaces. Bars are the means ± SEM of $n = 5$; *, $P < 0.01$ *versus* control (CTL); **, $P < 0.01$ *versus* BLEO or BLEO +SCR by analysis of variance and Student-Newman-Keul's test.

DISCUSSION:

Recently a few studies suggested the existence of "local" angiotensin systems in various organs and tissues. For example, the ANG II concentrations in the interstitial compartment of heart and eye were found to be 5-100 fold higher (about 50 –500pM) than that in plasma (~5-10pM) (van Kats et al., 1998; Danser et al., 1994). The higher interstitial levels of ANG II compared to the circulating level could not be explained by diffusion and/or receptor-mediated uptake of circulating angiotensin II. These results thereby suggest that tissue angiotensin II is largely, if not completely, synthesized locally. Furthermore, cultured cells from various organs including heart (Lindpaintner et al., 1988), vascular endothelium (Li et al., 1999), brain (Campbell et al., 1986; Dzau et al., 1982; Ohkubo et al., 1986) and lung (Filippatos et al., 2001) were shown to express the RAS components. In contrast to the classical endocrine system of RAS in which angiotensin II is delivered to tissues via circulating blood, local angiotensin systems can be from either "intrinsic "(independent of the endocrine RAS) or "extrinsic" (relying on the endocrine RAS as its components sources) sources.

The evidence that local angiotensin system is involved in pulmonary fibrosis is as follows: 1) Enzymes needed for the production of local angiotensin peptide were upregulated in pulmonary fibrosis. Polymorphisms that confer higher levels of ACE predispose patients to lung fibrosis (Morrison et al., 2001). Cathepsin D was upregulated in both animal and human fibrotic lung (Kasper et al., 1996; Koslowski et al., 2003). ACE is upregulated in both human and animal fibrotic lungs (Venkatesan et al., 1997; Specks et al., 1990). 2) Angiotensin system antagonists block experimental lung fibrosis (Ward et al., 1990; Uhal

et al., 2000 and 2002; Molteni et al., 1985 and 2000; Fang et al., 2002; Li et al., 2003; Marshall et al., 2004; Otsuka et al., 2004).

For the pulmonary angiotensin system, where the ANGEN, the only precursor of angiotensin II, comes from is unknown. Two cells types in the lung under certain conditions, so far, have been found to be able to produce ANGEN in vitro. In vitro primary AECs could synthesize and secrete ANGEN that is converted to ANG II when undergoing apoptosis induced by Fas ligand (Wang et al., 1999), TNF-alpha (Wang et al., 2000), amiodarone (Bargout et al., 2000) and bleomycin (Li et al., 2003). Studies done in our lab showed that primary myofibroblasts isolated from fibrotic human lungs (IPF biopsies) expressed the ANG II precursor ANGEN mRNA and protein (Wang et al., 1999), suggesting that human lung myofibroblast can synthesize ANGEN in culture. Furthermore, the concentration of ANG II detected by Enzyme-Linked Immunosorbent Assay (ELISA) was found to be significantly higher in culture medium of myofibroblast isolated from fibrotic human lungs than that from normal lungs. Preincubation of the medium with purified renin and ACE increased the ELISA-detectable ANG II concentration roughly eight fold, indicating that there are abundant ANGEN synthesized constitutively which waits to be converted to ANG II. However, no in vivo studies have been published to directly prove the existence of a local angiotensin system produced in the lung independently of the endocrine angiotensin system during the development of pulmonary fibrosis. Therefore, the present study was performed to answer a part of unknown question. We showed that endogenous lung ANGEN was upregulated in vivo as early as 3 hours after bleomycin instillation in rat lungs by RT-PCR, in situ hybridization and immunohistochemistry staining methods. ANGEN mRNA and

angiotensin peptides were localized in alveolar wall cells in the alveolar corners, tentatively identified as type II cells, and also colocalized with alpha-Smooth Muscle Actin (αSMA)(Fig 6.3). Those data suggested that bleomycin activated the production of the local angiotensin systems in vivo. Moreover, those data are consistent with our earlier finding of epithelial cell death in the vicinity of alpha- SMA-positive myofibroblast in fibrotic human lung and support the theory that alveolar epithelial death is caused by the ANG peptide.

We hypothesized that lung derived angiotensinogen is required for development of PF. Using whole animal models of bleomycin- induced PF, we cannot discriminate the lung-derived angiotensin system from the endocrine angiotensin system. However, using explants as the ex vivo model we can rule out the contributions of endocrine angiotensin system and hemodynamics to the development of bleomycin-induced PF as blood circulation is removed in explants. Furthermore, as lung explants provide an in vitro condition at the organ level, it will provide a better mimic of the in vivo conditions compared to cell cultures. This study showed that bleomycin could induce apoptosis and fibrosis in lung explants, which could be blocked by antisense oligonucleotides against ANGEN mRNA (Fig. 6.6), suggesting lung-derived ANGEN is required for bleomycin-induced pulmonary fibrosis in serum free condition.

The previous studies in our lab demonstrate that apoptosis of AECs in response to BLEO can be abrogated by antisense oligonucleotides against angiotensinogen (ANGEN) mRNA. We use the same oligonucleotide sequences, delivered by intratracheal (I.T.) instillation, for in vivo experiments to attempt blockade of BLEO-induced apoptosis and lung fibrosis in the intact animal. The success of such an experiment will depend on

specific delivery to the lung, so we sought to determine the distribution of the oligonucleotides in the lungs and other tissues after I.T. instillation. Our data showed that a single I.T. dose of 150ug antisense oligonucleotide can provide delivery primarily to lung with relatively little accumulation in liver or kidney. Moreover, the intratracheal instillation of antisense at dose of 75ug provided delivery of oligonucleotides primarily to lining cells of the airways and alveolar walls, suggesting that this is a feasible approach for in vivo studies. The initial concern was that a single dose of the antisense oligonucleotides against ANGEN mRNA is not sufficient to exert its effects on pulmonary fibrosis considering the decay of its efficacy over time in vivo although a single dose of the same antisense oligonucleotides against angiotensinogen mRNA reduces hypertension for a long time in various hypertension animal model (Gyurko et al., 1993; Wielbo et al., 1995; Wielbo et al., 1996; Peng et al., 1998; Kagiyama et al., 2001; Phillips, 2001). The half-life of ANGEN antisense oligonuclotide is not known although there are a few reports regarding the half-lives of similar oligonucleotides available. Some studies showed that the half-life of a 15-mer phosphorothioate oligonucleotide was 9 hours in human serum, 4 days in tissue culture media (Li et al., 1996) and 19 hours in cerebrospinal fluid (Campbell et al., 1990). Other studies reported that the half-life of phosphorothioate oligonucleotide following inhalation delivery to lung was more than 20 hours in mice (Templin et al., 2000) and 30 hours in rabbit (Ali et al., 2001). Regardless of this concern, intratracheal instillation of 75ug antisense once reduced bleomycin–induced pulmonary fibrosis detected by hydroxyproline assay, decreased pulmonary ANGEN and active caspase-3 level detected by western blot and ISEL positive cells, but had no effect on the serum ANG II level. These data are

consistent with the hypothesis that lung-derived ANGEN is involved in bleomycin-induced pulmonary fibrosis.

Summary and Conclusion

The present study demonstrated that the upregulation of de novo ANGEN synthesis found in vitro occurs in vivo. Administration of antisense oligos against ANGEN mRNA, presumably by blockade the synthesis of lung-derived ANGEN, blocked BLEO-induced apoptosis and lung fibrosis. Those data also suggest that lung-derived ANGEN is the additional therapeutic target within the pulmonary angiotensisn system and antisense oligonucleotides against ANGEN mRNA may hold potential for the treatment of lung fibrosis in humans.

Chapter 7

GENERAL DISSCUSSION

1. Angiotensin Receptor AT1 Mediates Bleomycin-induced Apoptosis and Lung Fibrosis

Our study demonstrated at various levels including cellular, organ and whole animal level that AT1 receptor mediates bleomycin-induced apoptosis and lung fibrosis.

1.1 An Essential Role by Angiotensin Receptor AT1 in Bleomycin-Induced Apoptosis in Alveolar Epithelial Cells (AECs):

We demonstrated that BLEO induced apoptosis of primary AECs in a dose dependent manner in vitro. This result is consistent with the in vivo studies from other labs showing that AECs apoptosis is found in bleomycin-induced pulmonary fibrosis model (Hagimoto et al., 1997). To confirm the nature of the cell death induced by BLEO was apoptosis, our study also showed that fragmented DNA induced by bleomycin was blocked by the broad-spectrum caspase inhibitor ZVAD-fmk, the caspase 3-selective inhibitor DEVD-fmk and endonuclease inhibitor ATA. Furthermore, we found that the ANG receptor subtype AT1-selective blocker losartan blocked nuclear fragmentation induced by bleomycin, suggesting that angiotensin receptor subtype AT1 mediates BLEO-induced apoptosis as it does in ANGII-induced apoptosis (Papp et al., 2002). Similarly, our study using the human AECs showed that BLEO-induced apoptosis of A549 cells was inhibited by the AT1-selective blocker L158809 but not by the AT2-selective antagonist PD123319 (Li et al., 2003). Moreover, BLEO-induced apoptosis was

also prevented by a neutralizing ANGII antibody (anti-ANGII). Furthermore, the total enzymatic activity of caspase 3 was elevated by exposure of A549 cells to BLEO and the increase was inhibited by non-selective AT receptor antagonist saralasin (Li et al., 2003). The findings that bleomycin-induced apoptosis of AECs could be blocked by the AT1-selective antagonists but not by the AT2-selective antagonist support the contention that there is an essential role by AT1 receptor in Bleomycin-induced apoptosis in AECs.

1.2 An Essential Role of Angiotensin Receptor AT1 in Bleomycin-induced Apoptosis and Fibrosis in Lung Explants:

We cannot discriminate the lung-derived angiotensin system from the endocrine angiotensin system using whole animal models of bleomycin- induced PF. However, using explants as the ex vivo model the contributions of endocrine angiotensin system and hemodynamics to the development of bleomycin-induced PF can be ruled out as blood circulation is removed in explants. Furthermore, as lung explants provide an in vitro condition at the organ level, it offers a better mimic of the in vivo conditions compared to cell cultures. Our studies showed that bleomycin could induce apoptosis and fibrosis in lung explants, which could be blocked by AT1 selective antagonists losartan, suggesting that AT1 receptor is required for bleomycin-induced apoptosis and pulmonary fibrosis in serum free condition.

The ability of LOS to prevent caspase-3 activation by BLEO in cultured lung explants argues against the involvement of decreased blood pressure in the inhibition of apoptosis as no hydrostatic pressure is there in explants. Moreover, the induction of LOS-inhibitable caspase 3 activities in lung explants argues against a primary role for blood-

derived cells in the initiation of the apoptosis as blood was removed from the explants. Hence, this result also suggests that the inhibitory effect of LOS on apoptosis was not mediated by an indirect action on infiltrating inflammatory cells.

1.3 In vivo Inhibition of Apoptosis and Collagen Deposition by an AT1 Antagonist and AT1 Gene Deletion:

Our study demonstrated that losartan, the AT1 receptor antagonist, inhibited lung epithelial apoptosis and fibrosis *in vivo* 14 days after BLEO administration. The specific effect of inhibition of apoptosis was confirmed by using two different assays: ISEL labeling to mark the fragmented DNA and active caspase 3 labeling to show the activation of caspase 3. Meanwhile, measurement of lung collagen accumulation in the same animals by hydroxyproline assay revealed an increase in total lung collagen after intratracheally delivery of BLEO, which was reduced by LOS to a level not significantly different from the control.

The AT1 receptor is expressed in two isoforms, AT1a and AT1b, for which no selective antagonists have yet been developed (Filippatos et al., 2001). Subtype AT1a is known to be expressed in the lungs of mice, but AT1b was not detected in mouse lung by reverse transcriptase-polymerase chain reaction (Burson et al., 1994). Although it is possible that cells of minor abundance in the lung, such as type II cells, express AT1b in quantities not detected in earlier studies (Burson et al., 1994), the primary isolates of type II pneumocytes from Wistar rats did not reveal AT1b expression by reverse transcriptase-polymerase chain reaction despite the use of two different primer sets and high-amplification cycle numbers (from unpublished observation). Based on those studies, we

used AT1a-null mice to test the hypothesis that AT1 receptor is essential for the development of pulmonary fibrosis. Our study demonstrated that deletion of the AT1 receptor, throughout the time course of the 14-day BLEO model, was capable of inhibiting or blocking apoptotic and fibrotic responses. Furthermore, the finding that deletion of only one allele of the AT1a gene significantly reduced Bleo-induced apoptosis and collagen accumulation supports the notion that AT1a is the only active AT1 receptor subtype on the alveolar epithelium of mice.

One concern was raised when we were using AT1a deletion mice. That is whether the ANG II dependent caspase-3 activation cascade is still intact in AT1a deletion mice since we detected less active caspase-3 in response to instillation of bleomycin. The chance that caspase-3 activation cascade has been compromised is small since those mice have normal phenotype except that 12-mmHg lower blood pressure is observed. If caspase-3 activation cascade were damaged in those mice, they would have a much higher chance to develop tumors, which has not been reported in those mice. In addition, in a recently published study, Ohashi et al. (2004) demonstrated that ANG II dependent caspase-3 activity is increased in AT1a deletion mice, strongly supporting that ANG II dependent caspase 3 activation cascades is intact in AT1a deletion mice.

1.4 AT1 receptor blockade block fibrosis via suppression of apoptosis:

Considering the many known functions of angiotensin II, these results above raise the question of whether the inhibition of fibrogenesis by AT1 blockade was due to its suppression of apoptosis. For example, blockade of AT1 receptor may also inhibit mitosis of lung fibroblasts (Marshall et al., 2000) and reduce collagen synthesis by lung

fibroblasts (Marshall et al., 2004). Both effects are relatively delayed consequences of AT1 blockade that might be independent of AEC apoptosis occurring at earlier time. Moreover, endothelial cells also express AT1 receptor and undergo apoptosis in response to angiotensin, although at relatively high concentrations (Dimmeler et al., 1997; Li et al., 1999). There are also other cell types residing in the lung known to respond to angiotensin by mechanisms currently under intensive study (Harrison et al., 2003). Thus, it is possible that the acute early effects of AT1 blockade on AEC apoptosis are not required for inhibition of collagen deposition later.

Quantification of erythrocytes reaching the alveolar airspaces 6 hours after BLEO administration (a crude index of lung barrier collapse) suggested that LOS did not prevent acute, transient barrier collapse despite its ability to reduce caspase-3 activation at the same time. This observation is in agreement with the theory that the blockade of apoptosis in AECs is a key to the subsequent blockade of collagen deposition. Moreover, previous studies in our lab with apoptosis inhibitors support the contention that the acute apoptotic response is a crucial event in the BLEO model of PF (Wang et al., 2000). Wang and colleagues showed that the ACE inhibitor captopril or the caspase inhibitor ZVAD-fmk had essentially equal ability to block the appearance of apoptotic epithelial cells in rats exposed to intratracheal BLEO and to prevent subsequent collagen deposition (Wang et al., 2000). That report, which was confirmed by Kuwano and colleagues (Kuwano et al., 2001) in studies of mice exposed to BLEO and/or ZVADfmk, suggested that the blockade of fibrogenesis by captopril was indeed related to inhibition of apoptosis, rather than the many other effects of ACE inhibition *in vivo* (Marshall, 2003). Later work in our

lab confirmed that the ZVAD compound had no inhibitory effect on angiotensin converting enzyme itself (Filippatos and Uhal, 2003).

On the other hand, earlier work has shown that AECs undergoing apoptosis in response to Fas ligand, tumor necrosis factor-α or BLEO begin secreting angiotensin into the extracellular space within hours of exposure (Wang et al., 1999; Wang et al., 2000; Li et al., 2003). Those studies also showed that the autocrine production of angiotensin and its binding to AT1 receptor on AECs were required for apoptosis in response to those agents. This mechanism can explain the ability of LOS to block AEC apoptosis *in vivo* in the present study. My data also are in agreement with recent reports that LOS inhibits BLEO-induced collagen deposition in rat lung (Fang et al., 2002) and that AT1a-null mice show reduced liver fibrosis in response to carbon tetrachloride (Kanno et al., 2003). Thus, the present data are consistent with the ability of ACE inhibition by captopril to block both AEC apoptosis and collagen deposition in rats (Wang et al., 2000), and extend this concept to angiotensin receptor blockade in mice.

1.5 Potential intracellular mechanisms underlying AT1 mediated apoptosis:

In an earlier report describing the mechanisms by which angiotensin induces apoptosis in primary cultures of AECs, Papp and colleagues (Papp et al., 2002) showed that blockage of AT1 signaling through protein kinase C (PKC) with the specific PKC inhibitor chelerythrin could attenuate the apoptotic response to angiotensin. This finding is consistent with the known role of PKC in AT1 signaling in a variety of cell types such as human coronary artery endothelial cells (Li et al., 1999) but the pathways from PKC to the effector caspase 3, which is also required for this response (Papp et al., 2002), are

currently unknown. Given that AEC apoptosis in response to Fas ligand, tumor necrosis factor- α, or BLEO all require the autocrine production and binding of angiotensin to AT1, (Wang et al., 1999; Wang et al., 2000; Li et al., 2003) the report of Papp and colleagues (Papp et al., 2002) suggests that PKC inhibitors would also block AEC apoptosis in response to these agents *in vivo* as well. This prediction was not tested in the present study, but will be a worthwhile topic for future inquiry.

2. Essential role for cathepsin D in bleomycin-induced apoptosis of alveolar epithelial cells

CatD is a lysosomal protease known to be ubiquitously expressed (Uchiyama et al., 2001). It has been suggested that this and other lysosomal proteases might be involved in the production of a bioactive molecule required for apoptosis of PC12 cells in response to trophic withdrawal (Isahara et al., 1999). Particularly, a role for the aspartyl protease CatD in apoptosis has been shown previously in HeLa cells exposed to interferon-γ, Fas ligand or TNF-α (Deiss et al., 1996) and in PA1 ovarian cancer cells (Wu et al., 1998). Furthermore, the activity of CatD is upregulated by the apoptosis inducer adriaourcin in PA1, ML1 leukemia cells and U1752 lung cancer cells (Wu et al., 1998). Although the aspartyl protease inhibitor pepstatin A could block apoptosis in these cell types, the exact mechanism(s) by which CatD participates in the execution of apoptosis is unclear.

Cathepsin D also is known to be one of the enzymes capable of proteolytically processing the liver-derived and serum-borne protein angiotensinogen to the peptide angiotensin I, a function normally performed in the serum by the kidney-derived enzyme renin

(Filippatos et al., 2001). Evidence from several nonpulmonary cell types has established CatD as the primary enzyme that converts angiotensinogen to ANGI within local "intrinsic" angiotensin systems, independently of renin (Filippatos et al., 2001; Weber et al., 1995).

2.1 CatD activity is upregulated in AECs apoptosis response to bleomycin and in fibrotic lungs:

It was shown that CatD activity is upregulated in AECs in fibrotic human lung (Kasper et al., 1996) and induced in the L132 lung cell line during apoptosis in vitro (Kasper et al., 1999). Consistent with those findings, in our study, western blotting of conditioned medium from rat AECs exposed to bleomycin reveals bleomycin-induced increases in several isoforms of CatD. Hence, we demonstrated that bleomycin upregulated CatD activity and release from AECs.

To investigate the mechanisms underlying the increase of CatD activity in response to bleomycin, we performed the time course study to monitor the change of the level of CatD mRNA. In other cell types such as PA1 human ovarian cancer cells, it was shown that apoptosis inducers upregulate both CatD protein and mRNA, suggesting control of activation at the level of mRNA (Wu et al., 1998). However, in our study, RT-PCR studies of AEC transcripts after bleomycin treatment failed to detect changes in CatD mRNA despite significant increases in CatD activity and immunoreactive protein by western blotting. It is likely that the relatively few sampling times chosen for realtime analyses of CatD mRNA may have missed a transient but shortlived increase in the mRNA that might be revealed by a more exhaustive time course study. On the other

hand, CatD is known to undergo activation by proteolytic mechanisms as well; in human U937 cells, CatD was shown to undergo processing from the inactive prepro-isoform (52kdal) to the active proCatD (48kdal) and an active 32kdal isoform, in response to autocatalysis of the enzyme induced by the direct binding of the apoptosis mediator ceramide (Heinrich et al., 1999). However, two of the isoforms shown to be increased in AEC media (52 and 48kdal) are larger than the primary isoform detected intracellularly in AEC (44kdal). This finding argues against proteolytic processing alone as a mechanism of CatD activation in AEC. Thus, the exact mechanism(s) by which bleomycin upregulates CatD in AEC is unknown, but will pose an interesting problem for future studies.

Pepstatin A-inhibitable CatD activity also was upregulated by amiodarone and TNF-alpha, both of which induce apoptosis in AECs (Uhal et al., 2003; Wang et al., 2000). However to determine whether the requirement for CatD is universal to all proapoptotic stimuli for AEC will require further investigation.

2.2 Bleomycin-induced apoptosis of AECs is reduced by blockade of CatD activity or synthesis:

Our data showed that bleomycin-induced nuclear fragmentation and caspase 3 activity were significantly reduced by the aspartyl protease inhibitor pepstatin A and by antisense oligonucleotides against CatD mRNA, strongly suggesting a role played by CatD in AEC apoptosis.

The other finding from our study that CatD antisense treatment did not completely block bleomycin-induced nuclear fragmentation indicates a potential role for additional

protease(s) in angiotensinogen processing and subsequent AEC apoptosis. This interpretation is reflected by the result the protease inhibitor pepstatin A, which blocks all aspartyl proteases, was also incapable of complete blockade of nuclear fragmentation despite complete inhibition of CatD enzyme activity in AEC lysates. On the other hand, the antisense treatment for CatD, which is at least theoretically specific, did not completely eliminate immunoreactive CatD detected by western blotting. Thus, it is difficult to determine whether the incomplete blockade of apoptosis is due to insufficient CatD knockdown or additional proteases activities.

2.3 CatD is involved in conversion of angiotensinogen to ANG I during bleomycin –induced AECs apoptosis.

Our previous study demonstrated that bleomycin-induced apoptosis of AECs was competely blocked by specific angiotensin receptor antagonists or ANG-neutralizing antibodies or antisense oligonucleotides against ANGEN mRNA (Li et al., 2003). Therefore, bleomycin-induced apoptosis of AECs requires the autocrine synthesis of angiotensinogen, angiotensin II and it's binding to ANG receptor AT1 (Li et al., 2003). This finding support the hypothesis that autocrine generation of ANGII is required for AEC apoptosis regardless of the initiating stimulus (Uhal, 2002). This result is also consistent with data from previous studies in our lab showing that purified ANGII itself was a potent inducer of apoptosis in AEC (Wang et al., 1999). Those data imply that AECs express enzymes capable of converting angiotensinogen to ANGII. Although Wang et al. (Wang et al., 1999) showed constitutive expression of angiotensin converting

enzyme (ACE) by AECs, the aspartyl protease required for providing the substrate for ACE (ANGI) in AEC was unknown.

As noted before, apoptosis of cultured AECs in response to bleomycin was significantly inhibited by the aspartyl protease inhibitor pepstatin A and by antisense oligonucleotides against CatD mRNA. The same inhibitors also prevented the enzymatic processing of a synthetic fragment of angiotensinogen (amino acids 1-14) containing the CatD and ACE cleavage sites, and completely blocked AEC apoptosis in response to the same peptide. For example, incubation of primary rat AECs with the fragment F1-14 alone in serum-free culture medium without CatD yielded significant production of ANGII. Our result from study using the fragment 1-14 of angiotensinogen containing the Cat D and ACE cleavage sites supports the theory that CatD is required for the conversion of angiotensinogen to ANGII by AEC. This result is also consistent with the earlier demonstration of constitutive, although low, expression of both ACE and an unidentified aspartyl protease by primary AECs (Wang et al., 1999). Moreover, the complete abrogation of AEC apoptosis in response to angiotensinogen fragment F1-14 by the nonselective and AT1-selective ANG receptor antagonists saralasin and losartan, confirmed that the induction of apoptosis was dependent on both the generation of ANGII from F1-14 and the binding of ANGII to receptor AT1. Most importantly, the findings that AEC apoptosis in response to F1-14 was essentially abrogated by either pepstatin A or by CatD antibodies, strongly suggest that the conversion of angiotensinogen to ANGI, and subsequently ANGII to induce AEC apoptosis, is dependent on CatD activity.

In summary, bleomycin upregulated CatD enzymatic activity and expression in primary cultures of rat AECs. Apoptosis of cultured AECs in response to bleomycin was significantly inhibited by the aspartyl protease inhibitor pepstatin A and by antisense oligonucleotides against CatD mRNA. The same inhibitors also prevented the enzymatic processing of a synthetic fragment of angiotensinogen (amino acids 1-14), and completely blocked AEC apoptosis in response to the same peptide. These data are consistent with earlier studies showing that apoptosis of AECs in response to bleomycin requires the autocrine synthesis and proteolytic processing of angiotensinogen to angiotensin II, and suggest that the proteolytic processing requires CatD. Therefore, our data suggest that blockade of CatD and other aspartyl proteases might provide a potential therapeutic strategy for pulmonary fibrosis by preventing AECs apoptosis and for lung injuries involving this mode of cell death.

3. Essential Roles for Angiotensinogen in Bleomycin-Induced Apoptosis and Lung Fibrosis

In our study, the same strategies were used to demonstrate the essential roles for angiotensinogen in bleomycin-induced apoptosis and lung fibrosis as for AT1 receptor. Our studies demonstrated that lung-derived angiotensinogen is involved in bleomycin-induced apoptosis and lung fibrosis at various levels including cellular, organ and whole animal level.

3.1 Essential Roles for Angiotensinogen in Bleomycin-Induced Apoptosis in Alveolar Epithelial Cells:

Our data demonstrated that BLEO induces ANGEN expression in primary AECs and A549 cells. To determine if functional ANGEN mRNA is required for the apoptotic response to BLEO, phosphorothioated antisense or scrambled-sequence control oligonucleotides against ANGEN mRNA were transfected into rat AECs and A549 cells. Our studies showed that BLEO-induced apoptosis of primary AECs and A549 cells was inhibited by the ANGEN antisense but not by the scrambled control oligonucleotides. Moreover, exposure to BLEO for 20 hours reduced the total cell number of A549 cells (attached plus detached, bottom panel), which was prevented by the ANGEN antisense. Although the mechanism by which bleomycin upregulates ANGEN mRNA was not addressed in this study, the findings that BLEO, Fas L and TNF-α all increased ANGEN mRNA (Wang et al., 1999; Wang et al., 2000) imply the involvement of a pathway common to these inducers. Regulation of ANGEN expression is mostly studied in the hepatocyte, in which the stimulatory effects of TNF-α and interleukin-6 have been shown to be mediated through the interaction of transcription factors NF-kB and STAT-3 with the Acute Phase Response Element (APR) of the ANGEN promoter (Brasier et al., 1994; Sherman and Brasier, 2001). In contrast, studies of the regulation of ANGEN expression by the cardiac ourocyte have shown that p53 is a key regulator of its expression in response to a variety of stimuli (Leri et al., 1998; Pierzchalski et al., 1997). Whether this difference reflects the distinct developmental lineages of these cell types or other factors is unknown. From this point of view, future investigations of the regulation of ANGEN expression by cells of the lung shall be quite worthwhile, particularly in light of the fact that the lung contains many different cell types that are in very close proximity but of distinct embryologic origins. As an example, the results reported here that ANGEN

expression is required for apoptosis of AECs compliment our earlier report of ANGEN expression by human lung myofibroblasts (Wang, et al., 1999), which reside immediately adjacent to apoptotic AECs in the fibrotic lung in situ.

3.2 Essential Roles for Angiotensinogen in Bleomycin-Induced Apoptosis and Lung Fibrosis in explants:

Lung-derived angiotensin system can not be discriminated from the endocrine angiotensin system if a whole animal model is used to study the role of angiotensin system in bleomycin- induced PF. However, using explants as the ex vivo model we can rule out the contributions of endocrine angiotensin system and hemodynamics to the development of bleomycin-induced PF as blood circulation is removed in explants. Furthermore, providing an in vitro condition at the organ level, lung explants will offer a better mimic of the in vivo conditions compared to cell cultures.

Our studies showed that BLEO increased caspase 3 activity when applied in vitro to rat lung explants that were depleted of blood before explant culture. Application of BLEO in vitro increased caspase-3 activity in the explants, which was inhibited by antisense oligonucleotides against ANGEN mRNA. Futhernmore, we showed that there was more collagen accumulation in explants treated with bleomycin compared with control group without bleomycin. Moreover, we showed that ANGEN antisense oligonucleotides reduced BLEO-induced collagen accumulation in explants cultured in 1% ITS. Hence, this study demonstrated that bleomycin could induce apoptosis and fibrosis in lung explants, which could be blocked by antisense oligonucleotides against

ANGEN mRNA, suggesting lung-derived ANGEN is required for bleomycin-induced pulmonary fibrosis in serum free condition.

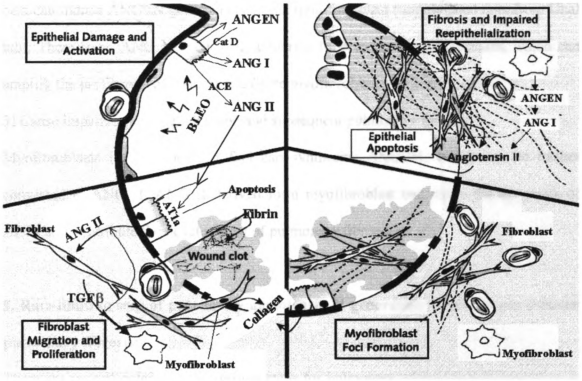
3.3 Essential Roles for Angiotensinogen in Bleomycin-Induced Apoptosis and Lung Fibrosis in vivo:

The previous studies in our lab demonstrated that apoptosis of AECs in response to BLEO was abrogated by antisense oligonucleotides against angiotensinogen (ANGEN) mRNA (Li et al., 2003). We used the same oligonucleotide sequences delivered by intratracheal (I.T.) instillation for in vivo experiments to block BLEO-induced apoptosis and lung fibrosis in the intact animal. To ensure specific delivery of the oligonucleotide to the lung, the distribution of the oligonucleotides in the lungs and other tissues after I.T. instillation was determined. Our data showed that a single I.T. dose of 150ug antisense oligonucleotide provided delivery primarily to lung with relatively little accumulation in liver or kidney. Moreover, the intratracheal instillation of antisense at dose of 75ug provided delivery of oligonucleotides primarily to lining cells of the airways and alveolar walls. Those results suggest that I.T. instillation of the oligonucleotide is a feasible approach for in vivo studies.

Intratracheal instillation of 75ug antisense once along with bleomycin reduced bleomycin–induced pulmonary fibrosis detected by hydroxyproline assay or picrosirius red staining, decreased ANGEN protein expression and active caspase-3 labeling and ISEL positive cells, but had no effect on the serum ANG II level. These data are consistent with the hypothesis that lung-derived ANGEN is involved in bleomycin-induced pulmonary fibrosis. Those data also suggest that lung-derived ANGEN is the

additional therapeutic targets within the pulmonary angiotensisn system and antisense oligonucleotides against ANGEN mRNA may hold potential for the treatment of lung fibrosis in humans.

4. Roles of Pulmonary Angiotensin System in Development of Bleomycin-induced Pulmonary Fibrosis: [Figure 7.1 modified from (Selman et al., 2001)]



Roles of pulmonary angiotensin system in development of bleomycin-induced pulmonary fibrosis can be summarized in the above figure, described as following:

1) Induce epithelial damage and activation:

Bleomycin induces apoptosis of lung AECs by upregulation of ANGEN gene expression. Synthesized ANGEN, which can be converted to ANG I by cathepsin D (Cat D). ANG I can be further converted to ANG II by ACE. ANG II can induce apoptosis of the AECs

through AT1 receptor, which will cause excessive loss of AECs and insufficient epithelial repair.

2) Cause fibroblast migration and proliferation:

ANG II synthesized by epithelial cells can cause proliferation and transdifferentiation of fibroblasts into myofibroblasts, which are the most important source of collagen in pulmonary fibrosis through upregulation of TGF-beta gene expression. In addition, TGF beta can induce ANGEN gene expression in lung fibroblast (unpublished data from Uhal lab). There is an ANG II- TGF beta antocrine loop in the lung fibroblasts, which can amplify the profibrotic of ANG II and cause myofibroblast foci formation.

3) Cause impaired reepithelialization and subsequent pulmonary fibrosis

Myofibroblasts in myofibroblast foci can synthesize ANGEN, which can be further converted to ANG II. ANG II derived form myofibroblast can cause the apoptosis of AECs and contribute the development of pulmonary fibrosis.

5. Rate-limiting step of pulmonary angiotensin II generation in bleomycin–induced pulmonary fibrosis:

There are several potential rate limiting steps for pulmonary angiotensin II generation in bleomycin induced pulmonary fibrosis including:

5.1 Renin and/or Cat D:

In other organ systems such as the cardiovascular system, renin has been shown to be the major determinant for local ANG II production, in which renin derived from kidney is taken up by the tissues via diffusion and binding to the (pro)renin receptors (Danser, 2004). However, there is no evidence suggesting that similar mechanism for the

production of the pulmonary angiotensin system exists since the local renin synthesis has never been demonstrated in the lung (Re, 2004). In our study (unpublished data), when a non-specific aspartyl protease inhibitor Pepstatin A, also an inhibitor for renin, was systemically administered, bleomycin induced pulmonary fibrosis was more severe in those animals.

Our study demonstrated that Cat D is responsible for the conversion of ANGEN to Ang I in the primary AECs. However, it is probably not the only enzyme capable of doing so as the complete inhibition of CatD by non-selective protease inhibitor pepstatin A did not completely block bleomycin-induced apoptosis in AECs. Hence, additional protease(s) may be involved in angiotensinogen processing and subsequent AEC apoptosis. Although Cat D activity and protein expression was increased in bleomycin- induced pulmonary fibrosis model in rats (Koslowski et al., 2003; Uhal et al., unpublished data), its roles in pulmonary fibrosis and pulmonary angiotensin peptides generation is still unclear in vivo. Futhermore, in bleomycin induced rat pulmonary fibrosis model, systemically administrated Pepstatin A did not protect the animals from bleomycin induced pulmonary fibrosis argues against that conversion of ANGEN to ANGI by Cat D is the rate limiting step in pulmonary angiotensin synthesis.

5.2 ACE

ACE is expressed predominantly by the pulmonary endothelial cells in a membrane-bound form and mainly responsible for generating circulating ANG II from angiotensin I. ACE inhibitors completely blocked apoptosis of AECs induced by Fas L, TNF a, bleomycin and Amiodarone, suggesting that ACE is the only enzyme responsible for converting angiotensin I to angiotensin II in vitro. The *in vivo* condition could be

different though ACE activity was elevated in bronchoalveolar lavage fluid (BALF) and/or serum in many potentially fibrotic lung diseases including idiopathic pulmonary fibrosis (Specks et al, 1990). However, the functional significance of increased ACE activity in those fluids is not clear. It is speculated that increased ACE activity indicates endothelial cell damage causing ACE to dissociate from the surface of injured endothelial cells (Marshall, 2003). There is evidence supporting that ACE is functionally relevant to pulmonary angiotensin generation although pulmonary tissue ACE activity remain unchanged in bleomycin induced pulmonary fibrosis model (Marshall et al., 2004). For example, ACE inhibitor Ramipril decreased pulmonary ANG II concentration and reduced bleomycin-induced pulmonary fibrosis in rats. This result is consistent with our previous finding that Captopril, another ACE inhibitor, attenuated bleomycin-induced pulmonary fibrosis (Wang et al., 2000). Although Ramipril completely inhibited ACE activity in the lung, pulmonary ANG II concentration only decreased by half, suggesting that ACE activity represents approximately half of the ANG II-generating capacity in the lung. Other non-ACE enzyme such as chymases could be responsible for a significant proportion of ANG II-generating activity in the lung as shown in the cardiac tissue (Urata et al., 1990). In addition, chymase was shown to be activated in the pulmonary inflammation and fibrosis induced by paraquat in hamsters (Orito et al., 2004).

Taken together, those data argue against that ACE activity underlies the rate-limiting step in ANG II generation within the lung.

5.3 ANGEN and angiotensin peptides:

ANGEN is the only known precursor for the synthesis of angiotensin peptides. Our studies demonstrated that ANGEN mRNA expression in primary AECs and A549 cells is upregulated and functional ANGEN mRNA is required for the apoptotic response to Fas L, TNF α, bleomycin and Amiodarone as apoptosis of primary AECs and A549 cells was inhibited by the ANGEN antisense but not by the scrambled control oligonucleotides (Li et al., 2003).

In lung explant where systemic angiotensin system was removed, bleomycin –induced collagen accumulation ex vivo requires the ANGEN gene expression as ANGEN antisense blocked the collagen deposition in explants. In whole animal model, circulating levels of angiotensinogen are approximately equal to the Michaelis Constant *(Km)* of renin for its substrate (about 1 uM) (Gould and Green, 1971). Therefore, the rate of angiotensin II synthesis can be regulated by changes in angiotensinogen levels. Since the normal concentration of ANGEN is near the *Km* for its reaction with renin (Gould and Green 1971), one would expect any change in ANGEN levels to be accompanied by parallel changes in the formation and actions of Ang II. For instance, transgenic mice expressing the rat angiotensinogen gene are hypertensive (Kimura et al., 1992) and mice without angiotensinogen gene expression are hypotensive (Tanimoto et al., 1994). In addition, for any given level of renin activity angiotensin II synthesis can be altered by changes in the concentration of available angiotensinogen (Poulsen and Jacobson, 1993). For example, up-regulation of ANGEN in tissue can alter tissue concentrations of angiotensin II, particularly, in tissues where are not subjected to systemic short loop or long loop feedback control (Poulsen and Jacobson, 1993). In bleomycin induced pulmonary fibrosis model, ANGEN expression is upregulated in the lung shown in

chapter 6. Elevation of local pulmonary ANGEN concentration increased pulmonary ANG II concentration. Furthermore, intratracheal delivery of antisense against ANGEN mRNA blocked the apoptosis of alveolar epithelial cells and subsequent fibrosis, which is consistent with the result that local ANG II generation is decreased.

Whether the circulating ANGI and ANGII contribute to the pulmonary ANGII is unknown. There is a possibility that tissue can uptake circulating angiotensins through AT1 receptor binding and internalization (Danser, 2004). Uptake from the circulation can be quantified by measuring steady state tissue and plasma levels of 125I-Labelled Ang I and II during 125I-ANGI and II infusion. The results obtained from pigs (van Kats et al. 1997, 1998, 2001) showed that I25I-Ang II, but not 125I-Ang I, accumulated in tissues like heart, kidney and adrenal gland. The absence of significant tissue 125 I-Ang I accumulation indicated that the presence of tissue ANG I cannot be attributed to uptake from circulation. If the same thing occurs in the lung, circulating ANGI does not contribute to the local pulmonary ANGII generation.

Accumulation of 125 I-Ang II at tissue sites was largely prevented by pretreatment with an AT 1 receptor antagonist (Danser, 2004), suggesting that uptake of Ang II is mediated via AT 1 receptor-dependent endocytosis. It is unlikely that AT 2 receptors play a role in this process since AT 2 receptors do not internalize Ang II (Matsubara 1998). Similar conclusions about AT1 receptor mediates internalization of ANG II were drawn from Ang II infusion studies in rats (Zou et al. 1996a,b). However, comparison of the 125 I-labelled and endogenous angiotensin levels revealed that, despite the significant uptake of 125I-Ang II in various tissues, the majority (>90%) of tissue Ang II is not derived from circulation. Instead, it is locally synthesized from locally generated Ang I (Danser, 2004).

In the lung ANGII uptake may not exist as treatment with losartan increased rather than decreased pulmonary ANG II concentration (Marshall, 2004).

Taken together, our results together with others' indicate that elevation of ANGEN concentration in the lung is the most likely rate-limiting step for the pulmonary angiotensin II generation.

6. Therapeutic Implications:

6.1 Existing clinical trials:

There have been several clinical trials to examine the therapeutic potentials of angiotensin system antagonists, especially the ACE inhibitors, on IPF as well as other fibrotic lung diseases. The results obtained from those trials, however, are still controversial and inconclusive. For example, Kyung et al (2002) conducted a small size clinical study to investigate the clinical efficacies of angiotensin receptor antagonist on treating IPF. Fourteen patients diagnosed with IPF by open lung biopsy and by american thoracic sociatey (ATS) criteria were divided into two groups; the first group were given AT1 antagonist losartan (n=8), the second group were given placebo without losartan (n=6). Although there was no significant difference in serum angiotensin II level between the two groups, patients treated by losartan showed increased Forced Vital capacity (FVC) (by 12%). These results suggest that angiotensin II receptor antagonist may be an effective agent for treating IPF.

Uhal et al. evaluated the effects of angiotensin system antagonists on pulmonary fibrosis induced by amiodarone, an antiarrhythmic reagent in which angiotensin system is also considered to play an important role in the pathogenesis of this disease (Uhal et al.,

unpublished observation). After re-analyzing the CHF-STAT study (Survival Trial of Antiarrythmic Therapy in CHF) (Singh et al., 1997), we found that compared with those who did not receive the ACE inhibitors, there was a higher percentage without lung fibrosis (90.5% vs. 83.3%) and a lower percentage (0.98 vs. 3.3%) with severe pulmonary fibrosis of patients receiving either ACEis or ANG receptor antagonists although the difference was not statistically significant due to the low statistics power. Based on these results, we speculate that there is a "trend" for patients who did not receive ACEis drugs to develop more frequent and severe lung fibrosis. Similarly, we observed that with concurrent administration of the ACE inhibitors there was a trend toward improved survival in patients with long-term administration of amiodarone although the difference was not statistically significant due to the low statistics power ($p=0.57$). That the CHF-STAT study did not demonstrate statistical differences in pulmonary toxicity of patients receiving ACE inhibitors versus placebo (Boutitie et al., 1999) may be due to the fact that the number of patients receiving placebo was too low to reach sufficient statistical power as they consist only 9% of the total patients. Hence, a larger population of patients treated with amiodarone is needed to determine the protective effects of ACEis and angiotensin receptors blocker on amiodarone induced pulmonary toxicity (Flaherty et al., 2002).

Contradictory to those above findings, results from two other clinical trials performed by different investigators do not suggest the beneficial role of angiotension-converting enzyme inhibitors in treating IPF. The first study by Raghu et al. (Raghu et al., ATS 2004 Mtg, Orlando, FL) employed prospective, randomized, double blind trials to investigate the association of ACE inhibitors with survival and disease progression in patients with

IPF. Data were collected from 168 patients, of which 20 were on ACEis and 146 patients were not. The other recently published clinical study by Nadrous et al (Nadrous et al., 2004) retrospectively reviewed the effects of ACEis on survival of 478 patients with IPF who visited Mayo Clinic Rochester from 1994 through 1996. 57 patients (12%) received ACEI and the rest of them were not. Both studies found that the mortality rates and rates of disease progression were similar in patients with or without ACEis treatment, thus suggesting that there is no beneficial effect of ACEis on survival of patients with IPF. However, there are some pitfalls in those two studies. For example, the first study was not specifically designed for evaluating the effects of ACEis on IPF. Instead, the major purpose was to evaluate the effects of interferon gamma-1 b on treatment of IPF. As a retrospective study, the limitations in the second study include lack of blinded randomization for ACEis use, variations in the specific types and doses of ACEis prescribed, varying concurrent therapies for IPF, reliance on clinicoradiologic parameters for case definition in most patients, and incomplete information on duration of ACEis use before establishment of the diagnosis of IPF. In addition, in order to detect a 20% survival difference in a trial evaluating an agent for IPF, the minimum sample size is 700 patients (Mapel et al., 1996). In both studies, the number of patients who received ACEis was relatively small; therefore, those studies may be statistically underpowered to reliably detect a survival benefit of ACEis.

In conclusion, the results from clinical trials regarding whether the angiotensin system antagonists have therapeutic effects on IPF are still controversial. Clinical trials involving larger population with randomized and double blind designs need to be done to determine their therapeutic potentials for IPF.

6.2 AT1 antagonist may be more efficient than ACE inhibitors in term of antifibrotic effect in vivo

AT1 antagonist and ACE inhibitors both completely blocked AECs apoptosis induced by Fas L, TNF alpha and amiodarone, bleomycin in vitro. And we were not able to detect the difference between those two lines of reagents in inhibiting apoptosis. However, there are some in vivo studies suggesting that the AT1 antagonist may be a more efficient antifibrotic drug than ACE inhibitors. For example, it was shown that L-158, 809, a AT1 receptor antagonist, L-158, 809, was more effective in preventing radiation-induced pneumopathy and lung fibrosis than angiotensin-converting enzyme inhibitors captopril and enalapril (Moltine et al., 2000), the mechanism of which, however, was unknown. Theoretically speaking, AT1 receptor antagonist should be a more efficient anti-fibrotic drug than ACE inhibitors in the lung due to the following reasons: 1) AT 1 receptors mediate most of the profibrotic effects in the lung. Therefore, inhibition of AT1 receptors should exert more direct effects than ACE on AT1 receptor-mediated signal transduction. 2) AT 1 receptor antagonists increase the local concentration of ANG II (Marshall et al., 2004), which may act through AT2 receptor to exert antifibrotic function as shown in the heart (Danser, 2004). 3) ACE is not the only enzyme to convert ANG I to ANG II in the lung. Thus, ACE inhibitors might not completely block the profibrotic effect of ANG II converted by other enzymes such as chymase 4) ACE inhibitors disturb the bradykinin system, the function of which is unknown in pulmonary fibrosis. 5) Long –term administration of ACE inhibitors can induce the production of ACE, which will counteract the antifibrotic effects of ACE inhibitors (Boomsma et al., 1981).

6.3 Potential therapeutic approaches for IPF: Local administration of ANGEN antisense or AT1 antagonist for IPF:

Our studies together with others' demonstrated that activated pulmonary angiotensin system plays an essential role in the development of pulmonary fibrosis. The potential rate-limiting step in the production of the pulmonary angiotensin system is the local synthesis of angiotensinogen. The AT 1 receptor plays a major role in mediating the profibrotic effect of local ANG II in the lung. Those findings indicate that local administration of ANGEN antisense or AT1 antagonists are potential therapeutic approaches for IPF. The benefits of local administration of those regents are as follows.

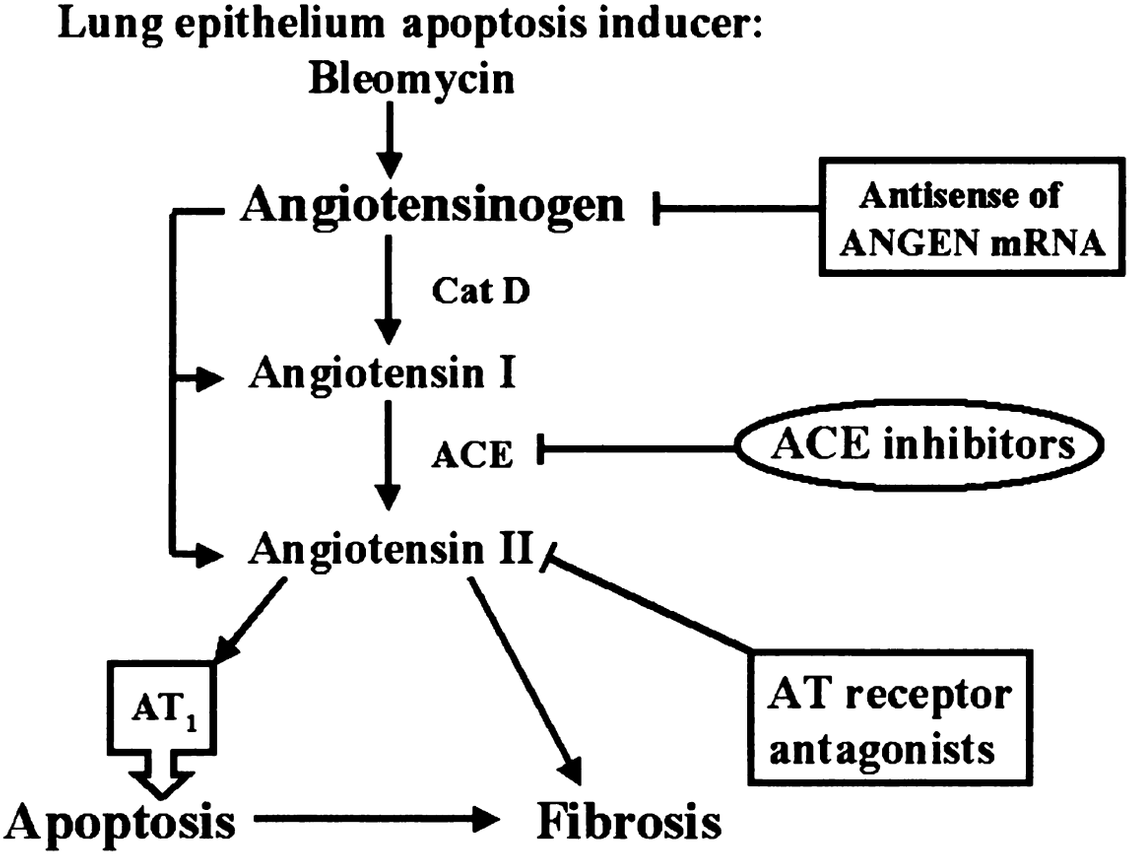
6.3.1. Local administration does not disturb the systemic angiotensin system

Lung is easier to be accessed compared to most of the other organ systems and local delivery of drugs via the airway is feasible as nebulization or aerosolation of medications via inhalation are commonly used clinically. Furthermore, in our study, intratracheal instillation of antisense oligonucleotides against ANGEN mRNA achieved lung specific delivery of the drug as the instilled antisense were contained in the lung and did not affect plasma ANGEN and ANG II level. Yet, it is notable that, there exist regional differences in alveolar ventilation resulting in the uneven delivery of the inhaled drugs. Hence, this could cause insufficient delivery to most severely fibrotic areas within the lung (Marshall et al., 2003). Direct instillation through the endo-tracheal tube or via a bronchoscope in patients with IPF could be an alternative approach to compensate for the non-selective delivery of nebulized drug in the lung.

6.3.2 Local administration is a more effective approach with fewer side effects

High tissue specificity ensures that much lower doses are needed to achieve the same effects for locally administered drugs compared to systemically administered counterparts. In addition, locally administration will cause much fewer side effects. This has been proven by the locally administered steroids for asthma treatment (Ververeli et al., 2004).

7. General Conclusions: (Fig. 7.2)



Pulmonary angiotensin system is activated in bleomycin induced pulmonary fibrosis model. During the process of bleomycin-induced AECs apoptosis, AECs synthesize ANGEN, which is converted to ANG I by cathepsin D (Cat D). ANG I is further converted to ANG II by ACE. Locally produced ANG II mediates apoptosis of the AECs

leading to excessive loss of AECs and insufficient epithelial repair. ANG II produced by apoptotic epithelial cells also directly activates the fibroblasts. Both effects lead to pulmonary fibrosis. Synthesis of ANGEN is the key upstream event in this process, which can be blocked by the antisense oligonucletides against ANGEN mRNA. Local administration of ANGEN antisense can be used, as a potential therapeutic approach for treating PF. AT1 receptor is required for bleomycin- induced apoptosis and fibrosis and thus can also be a potential therapeutic target for PF.

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