# REGULATION OF ALVEOLAR EPITHELIAL CELL SURVIVAL BY ANGIOTENSIN1-7 AND *MAS*

**By**

**Xu Gao**

**A THESIS**

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Physiology

2011

ABSTRACT

REGULATION OF ALVEOLAR EPITHELIAL CELL SURVIVAL BY ANGIOTENSIN II AND *MAS*

By

Xu Gao

Apoptosis of alveolar epithelial cells (AECs) is believed to be a critical event in the pathogenesis of lung fibrosis. It was previously shown that apoptosis of AECs requires autocrine generation of angiotensin (ANG) II. ANG II can also be degraded to ANG1-7 by angiotensin converting enzyme-2 (ACE-2). ACE2 was recently found to be protective but significantly downregulated in experimental lung fibrosis and patients with IPF. In other organ systems, ANG1-7 has been found to inhibit the actions of ANGII through the ANG1-7 receptor *mas*. Therefore, it was hypothesized that ANG1-7 in the lungs might antagonize the actions of ANG II in the regulation of AEC apoptosis. To test this theory, the AEC cell line MLE-12 and primary cultures of human AECs were stimulated by the profibrotic apoptosis inducers ANG II or bleomycin (BLEO). Caspase-3 activation and nuclear fragmentation were used as markers of apoptosis and were measured along with JNK phosphorylation. Exposure to ANG II or BLEO induced caspase-3 activation, nuclear fragmentation, and JNK phosphorylation in cultured AECs. Pretreatment with ANG1-7, at a concentration of 0.1μM, prevented JNK phosphorylation and apoptosis. In addition, pretreatment with A779, a specific blocker of the ANG 1-7 receptor *mas*, successfully prevented ANG1-7 induced blockade of JNK phosphorylation and nuclear fragmentation. These data indicate that ANG1-7 prevents AEC apoptosis, and its actions are mediated through the ANG1-7 *mas* receptor.

To Mom and Dad for their unconditional care, love, and support.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Bruce D. Uhal, who was a source of guidance and support through my undergraduate education. I would not have been able to do this without his encouragement and effort. I also wish to thank and acknowledge the individuals involved in my Thesis Guidance Committee: Dr. Gloria I. Perez and Dr. Arthur J. Weber, for contributing their expertise and ideas. I would also like to thank Amal Abdul-Hafez, Trang Dang, and Chris Plasecki for their help and friendship in the laboratory. Finally, I would like to thank my family and friends for their motivational support through it all.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin-Converting Enzyme |
| ACE-2 | Angiotensin Converting Enzyme-2 |
| AEC | alveolar epithelial cell |
| AGT | Angiotensinogen |
| Ang 1-7 | Angiotensin 1-7 |
| Ang II | Angiotensin II |
| Ang III | Angiotensin III |
| Ang IV | Angiotensin IV |
| $AT_1$ | Type 1 Angiotensin receptor |
| $AT_2$ | Type 2 Angiotensin receptor |
| ATI | Type I alveolar epithelial cell |
| ATII | Type II alveolar epithelial cell |
| ECM | extracellular matrix |
| EMT | epithelial-mesenchymal transition |
| HIF-1 | Hypoxia Inducible Factor -1 |
| IL | Interleukin |
| ILD | Interstitial Lung Disease |
| IPF | Idiopathic Pulmonary Fibrosis |
| JNK | c-Jun N-terminal kinase |
| MAPK | mitogen activated protein kinase |
| NFkB | nuclear factor-kappa B |

| | |
|---|---|
| PI3K | Phosphoinositide 3-kinase |
| RAS | Renin Angiotensin System |
| ROS | Reactive Oxygen Species |
| RT-PCR | Reverse Transcriptase-Polymerase Chain Reaction |
| siRNA | Small interfering RNA |
| SNP | Single Nucleotide Polymorphism |
| SP-C | surfactant protein C |
| TGF-b1 | Transforming Growth Factor-beta 1 |
| TNF-a | tumor necrosis factor-alpha |

# Chapter 1

# GENERAL INTRODUCTON

## IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrotic lung disease resulting from injury to the lung and an ensuing fibrotic response that leads to the thickening of the alveolar walls and the obliteration of the alveolar space (Fonseca et al., 2000). The American thoracic Society and the European Respiratory Society recently issued a consensus statement that defined IPF as a distinctive type of chronic fibrosing interstitial pneumonia of unknown etiology limited to the lung and associated with the histology of unusual interstitial pneumonia (American Thoracic Society, European Respiratory Society, 2002). IPF is classified as a specific presentation of idiopathic interstitial pneumonia, which is in turn a type of interstitial lung disease (American Thoracic Society, European Respiratory Society, 2002).

### Epidemiology

IPF is the most common of the idiopathic interstitial pneumonias and is associated with the worst prognosis (American Thoracic Society, 2002). Deaths from IPF are estimated to be nearly 14 times greater than those from asbestos (Wells and Mannino, 1996*)*. Differences in disease definition, classifications, diagnostic approach, and epidemiological design of the studies greatly influence the findings. In the Unite States, a recent study estimated prevalence to range from 14.0 to 42.7 cases per 100,000 population, depending on the criteria used for diagnosis (Raghu et al., 2006). This is higher than the previously reported prevalence of 3 to 6 per 100,000 persons (Cherniack et al., 1991). The median survival rate of IPF is estimated to be from 3 to 5 years (Selman et al., 2001).

Respiratory failure is the most frequent cause of death, and has been reported to account for over 80% of all fatalities (Martinet et al., 2005).

Two thirds of IPF patients are older than 60 years of age with a mean age of 66 at diagnosis (Johnston et al., 1997). The incidence, prevalence, and death rate of IPF increase with age (Coultas et al., 1994). IPF is reported to be more common in males than in females (Hubbard 1996). Some studies also suggest that the risk of developing IPF increases with environmental or occupational exposure to dusts, organic solvents, or urban pollution (Iwai, 1994). Other risk factors associated with pulmonary fibrosis include smoking, gastro-esophageal reflux disease, commonly prescribed drugs, diabetes mellitus, infectious agents, and genetic factors (Zisman et al., 2005).

**Clinical Features**

The clinical features for IPF are not specific and can also occur in various other pulmonary disorders. The symptoms usually vary with the extent of lung damage, the rate of disease progression, and the development of complications, such as lung infections or cor pulmonale. IPF usually presents with inspiratory crackles, dyspnoea on exertion, and dry, non-productive cough. Common symptoms also include weight loss and fatigue. In more than 80% of patients, bi-basilar, end-expiratory rales are found upon physical examination (American Thoracic Society, 2000). In addition, up to half of all patients develop digital clubbing where ends of the fingers become thick or club-shaped (Johnston et al., 1997). At late stages of the disease, patients also experience shortness of breath at rest as well as cyanosis of the lips and fingers. Pulmonary hypertension may also present late in the course (Panos et al., 1990).

There are currently no laboratory tests specific for the diagnosis of IPF. Laboratory evaluations are done to rule out alternative causes of interstitial lung disease such as sarcoidosis or connective tissue disease-pulmonary fibrosis. Pulmonary function tests show restrictive impairment, reduced diffusing capacity for carbon monoxide, and arterial hypoxemia exaggerated or elicited by exercise (Selman et al., 2001). Greater than 90% of patients have abnormal chest radiographs that show diffuse bilateral interstitial or reticulonodular infiltrates in the basilar and subpleural regions of the lung (Kazerooni et al., 1997; Wells et al., 1993). High-resolution computerized tomography scanning shows variable but limited ground-glass opacity subpleural honeycombing. Extensive honeycombing, septal thickening, and a lack of ground glass opacities are usually indications of a poor prognosis.

The diagnosis of idiopathic pulmonary fibrosis must include: 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 (American Thoracic Society, 2000).

**Histological Features**

The histological features of IPF are described as usually interstitial pneumonia (UIP), which can be seen in other diseases as well. The histological hallmark of usual interstitial pneumonia is heterogeneous appearance of normal-appearing lung alternating with areas of peripheral fibrosis, interstitial inflammation, and honeycomb changes, on low magnification (American Thoracic Society, 2000). The inflammatory component is

typically light with mostly lymphocytes and plasma cells. At higher-power magnification, the border between the fibrotic and normal lung, called the fibroblastic foci, can be seen with accumulations of dense, relatively acellular, collagen bundles. Fibroblastic foci are the regions of fibroblastic proliferation and represent the primary site of ongoing injury. Fibroblastic foci are rich in extracellular matrix and mesenchymal cells with cell phenotypes ranging from proliferating fibroblasts to fully differentiated smooth muscle cells. The most abundant cell type is the myofibroblast, which contribute to active contraction, distorted architecture and excess collagen deposition (Kuhn & McDonald 1991). Although not pathognomonic, the number of fibroblastic foci may be an important prognostic factor and correlate with a worse prognosis (Nicholson et al., 2002, King et al., 2001).

**Genetic factors**

Evidence suggests that genetic factors may play an important role in the pathogenesis of IPF. It has been estimated that up to 4% of IPF is familial and appears to be inherited as an autosomal dominant trait (Lawson & Lloyd, 2006). The familial form of IPF has been reported in monozygotic twins raised in different environments, in genetically related members of several families, in consecutive generations in the same families, and in family members separated at an early age (Steele et al., 2005). Genetic studies found two mutations of the SP-C gene to be associated with familial lung fibrosis (Thomas et al., 2002). These mutations lead to misfolding of the protein resulting in abnormal surfactant production and injury in type II alveolar epithelial cells (Thomas et al., 2002). Angiotensin II is a growth factor that plays a key role in the physiopathology of IPF. A nucleotide

substitution of an adenine instead of a guanine (G-6A) in the proximal promoter region of the angiotensin II precursor, angiotensinogen, has been associated with an increased gene transcription rate. A recent case-control study found that the distribution of G-6A genotypes and alleles did not significantly differ between cases and controls (Molina et al., 2008). The G-6A polymorphism of the angiotensinogen gene was found to be associated with idiopathic pulmonary fibrosis progression but not with disease predisposition (Molina et al., 2008). The G-6A polymorphism of the angiotensinogen gene could have a predictive significance in idiopathic pulmonary fibrosis patients.

**Therapeutic options**

The two main types of therapeutic agents used to treat IPF are the anti-inflammatory medications and the anti-fibrotic agents. Most therapies are, however, largely ineffective with limited evidence to suggest that any one treatment improves survival or disease progression. The conventional management of IPF with anti-inflammatory medications is based on the concept that pulmonary fibrosis is an inflammatory disorder and the suppression of inflammation prevents progression to fibrosis. These therapies continue to be used despite the lack of evidence of inflammation in the pathogenesis of IPF (Raghu et al., 2004). Clinical studies indicate that response to steroids is poor in IPF and the use of other immunosuppressive or aggressive cytotoxic agents only offer a marginal benefit at best (King et al., 2001). Recently, more anti-fibrotic agents are being used in the treatment of IPF based on the concept that the disease is a fibrotic condition with a lack of significant inflammatory component (Selman et al., 2004). A meta-analysis of the combined results of multiple studies showed that interferon-γ1b (IFN-γ1b) therapy may be efficacious in

improving survival and is associated with reduced mortality in patients with IPF (Bajwa et al., 2005). However, this benefit of IFN-γ1b therapy was not seen in patients with more severe disease (Martinez et al., 2005).

Currently, the only option for patients with end stage pulmonary fibrosis and those who fail to respond to medical treatment is lung transplantation. Patients are referred for transplantation when progressive symptoms have a vital capacity less than 60-70% and a corrected diffusion capacity below 50-60% of predicted (The American Society for transplant physicians, 1998). The five-year survival rate for patients following transplant is estimate to be 50-60% (American Thoracic Society, 2000). A recent study estimated that lung transplantation reduces the risk of death by 75% (Thabut et al., 2003).

To develop new therapies, recent studies have focused on targeting angiotensin II (ANG II) and its role in the pathogenesis of pulmonary fibrosis. These studies showed that inhibition of the RAS using angiotensin converting enzyme inhibitors (ACEi's), angiotensin receptor blockers or receptor deletion was able to prevent experimental lung fibrosis (Wang et al., 2000b, Marshall et al., 2004, Uhal et al., 2007a, Molteni et al., 2000, Molina-Molina et al., 2006) and improve lung function in IPF patients (Woo et al., 2003).

## PATHOGENESIS OF FIBROGENESIS

Until recently, pulmonary fibrosis has been considered to be an inflammatory disorder where lower respiratory tract inflammation leads to derangements of the alveoli resulting in scaring of the lung parenchyma (Gallin et al., 1992). Inflammation results in the loss of functional alveolar-capillary unit, the accumulation of collagen, and the formation of the honeycomb lung. Recent studies, along with the limited success of anti-inflammatory treatments, suggest the existence of alternative hypothesis regarding the pathogenesis of IPF. The current hypothesis is that IPF results from alveolar epithelial injury and abnormal wound repair (Selman et al., 2001). This theory is supported by evidence from human lung biopsies where nascent fibrotic foci were colocalized with unrepaired or abnormal epithelia (Uhal et al., 1998).

### Alveolar Epithelial Cells

The alveolus is the basic functional unit of the lung where gas exchange occurs between the air and the capillaries. The site of gas exchange is composed of the capillary endothelium and alveolar epithelium separated by a thin interstitium composed of connective tissue, ECM and a few fibroblasts (Fonseca et al., 2000). The alveolar epithelium is composed of two types of alveolar epithelial cells (AECs) that are morphologically and functionally distinct. The first is the thin and elongated type I (AT I) cells that are terminally differentiated and cover 95% of the alveolar surface to facilitate gas exchange. AT I cells are metabolically active and express cell surface receptors for a variety of substances, including extracellular matrix (ECM) proteins, growth factors, and cytokines. The second are type II (AT II) alveolar epithelial that reside in alveolar corners

and are cuboidal in shape with rounded nuclei. AT II cells secrete surfactant, facilitate trans-epithelial movement of water, function as antigen presenting cells and serve as the progenitor of both types of epithelial cells (Uhal, 1997). AT II cells are important in the maintenance and repair of the alveolar epithelium by replacing lost cells and restoring normal tissue architecture and lung function. The regeneration of a continuous epithelium is vital in maintaining barrier function and in limiting airway hyper-activity. This process is most critical in response to lung injury and other insults. Epithelial injury and blunted epithelial repair are sufficient to promote pulmonary fibrotic processes (Adamson et al., 1988).

One important step in the healing process is the rapid re-epithelialization of the denuded area through epithelial cell migration, proliferation and differentiation. The inhibition of alveolar epithelial repair in animal models increases the severity of subsequent fibrosis (Haschek and Witschi, 1979), suggesting that the efficient repopulation of the denuded alveolar basement membrane acts to suppress subsequent fibroblast proliferation and extracellular matrix accumulation. IPF alveolar epithelium show significant loss of AT I cells, hyperplasia of AT II cells and altered expression of adhesion molecules and MHC antigens (Kasper, 1996). AT II cells in IPF have decreased capacity to restore damaged AT I cells, resulting in epithelial cuboidalization and the presence of transitional reactive phenotypes (Kasper, 1996), abnormalities in pulmonary surfactant (McCormack et al., 1991), and alveolar collapse **(**Burkhardt, 1989).

The epithelial cells overlying fibroblastic foci are found to be hyper- and dysplastic, with abnormal morphology and gene expression patterns (Kasper et al., 1996). These cells secrete a variety of profibrotic cytokines and participate in a bidirectional communication

network with neighboring fibroblasts where each cell type influences the proliferation/survival of the other. AEC apoptosis was also detected adjacent to myofibroblasts-containing fibroblastic foci, the presumed primary sites of epithelial injury in IPF. Ongoing apoptosis is believed to be a key component in the progression of IPF and appears to be essential for the development of transforming growth factor-beta (TGF-β) induced lung fibrosis (Lee et al., 2004; Uhal et al., 1998).

**Coagulation and fibrinolysis**

The disruption of blood vessels and extravasation of blood into the wound after tissue injury reestablishes hemostasis while also providing a provisional extracellular matrix in which the repair process can begin. AT II cells and macrophages convert the leaked in fibrinogen into fibrin, which then becomes scaffold for fibroblast migration (Gross et al., 1991). The efficient and orderly resolution of the fibrin matrix will reconstitute a normal alveolar space. Continued deposition of fibroblasts and dysregulated coagulation, however, will lead to destruction of the alveolar space and contribute to pulmonary fibrosis. Whether fibrin is deposited or resorbed in the alveolar space is critical in the development of fibrosis and is determined by the balance of procoagulatory, fibrinolytic, and antifibrinolytic activities in the lungs. Fibrin removal is slowed in IPF lungs as evidenced by increased procoagulant and anti-fibrinolytic activities found in bronchoalveolar lavage fluid from IPF patients (Gunter et al., 2000).

During wound repair, the fibrinolytic process is required to cleave a path for epithelial cell migration. The plasminogen activation system mediates this process and is crucial in tissue remodeling. Plasminogen, when activated to plasmin by tissue-type

plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), is the primary fibrinolytic enzyme responsible for degrading fibrin clots and promoting wound reepithelialization. The overexpression of uPA has been shown to decrease fibrosis in a murine model, indicating a protective role of uPA in the development of pulmonary fibrosis (Sisson et al., 2002). Plasminogen activator inhibitors (PAIs) negatively regulate plasminogen. Animal models have shown that the overexpression of PAIs inhibits plasmin activity and promotes the formation of fibrosis while the lack of PAIs increase plasmin activity and prevents fibrosis (Eitzman et al., 1996). Alveolar epithelial cells synthesize uPA and its receptor uPAR as well as PAI-1 (Hasegawa et al., 1997; Simon et al., 1992). PAI-1 is strongly induced by TGF-β and may play a role in TGF-β induced fibrosis.

In normal repair, epithelial cells are able to dissolve the fibrin barrier and migrate throughout the denuded wound surface. The proximity of uPA and its receptor greatly increases the rate of plasminogen activation on the alveolar epithelium and the clearance of fibrin from the alveolar spaces (Hattori et al., 1989). Alveolar epithelial cells in the IPF lung, however, seem to play a role in increasing procoagulant and antifibrinolytic activities. BAL fluid from IPF patients was found to contain significantly more PAIs but the same amount of uPA as compared to normal individuals (Kotani et al., 1995). This suggests that the IPF alveolar microenvironment prefers a pro-coagulant and anti-fibrinolytic state that promotes ECM accumulation and inhibits alveolar reepithelialization.

**Profibrotic Cytokines**

Alveolar epithelial cells in IPF also express several cytokines and growth factors that promote fibroblast migration and proliferation as well as extracellular matrix

accumulation. Damaged AECs produce platelet-derived growth factor (PDGF) (Antoniades et al., 1990), tumor necrosis factor alpha (TNF-α) (Kapanci et al., 1995), endothelin-1 (Giaid et al., 1993), and are the primary producers of TGF-β (Khalil et al., 1996). TGF-β has profound effects on epithelial cells and fibroblasts and is a central regulator of pulmonary fibrosis. TGF-β promotes epithelial cell apoptosis (Hagimoto et al., 2002), epithelial-to-mesenchymal transition (EMT) (Kim et al., 2006), epithelial cell migration (Yu et al., 2008), collagen synthesis, fibroblast proliferation and transformation into myofibroblasts (Scotton and Chambers, 2007). Studies with animal models found that overexpression of TGF-β leads to extensive and progressive fibrosis with limited inflammation (Sime et al., 1997). PDGF released by epithelial cells is a potent mitogen and chemoattractant for fibroblasts. PDGF mRNA and protein were found to be upregulated in the epithelial cells of IPF patients (Antoniades et al., 1990). Hyperplastic AT II cells in pulmonary fibrosis synthesize TNF-α, which promotes DNA synthesis and proliferation of fibroblasts (Battegay et al., 1995). Endothelin-1 also plays a role in stimulating fibroblast proliferation and transdifferentiation into myofibroblasts (Shahar et al., 1999). Together, these cytokines released by AECs after injury promote proliferation, migration, and matrix deposition by fibroblasts.

**Myofibroblasts**

Fibroblasts are highly versatile and are able to interconvert between several distinct but related cell types. This phenotypic plasticity of fibroblasts is crucial to tissue repair after injury. After lung injury, fibroblasts are responsible for secreting ECM proteins to provide a tissue scaffold for epithelial cell migration during repair. Additionally, the dissolution of this scaffold along with apoptosis of fibroblasts and myofibroblasts are

critical for restoration of normal tissue structure (Desmouliere et al., 1995; Lorena et al, 2002).

In IPF, AECs lose their ability to provide normal and homeostatic fibroblast-suppressive functions and instead initiate the pathologic process by producing most of the factors that induce the fibroblast phenotypic changes. In the fibroblastic foci of the injured lung, fibroblasts continually modify their interactions with the microenvironment. They first become migratory, then proliferative, and finally become profibrotic and secret abundant ECM (Kuhn et al., 1989). Activation of fibroblasts by the secreted factors produce myofibroblasts that express contractile proteins and have ultrastructural features that are intermediate between fibroblasts and smooth muscle cells (Gabbiani et al., 1971). The contractile function of myofibroblasts is important in the re-epithelialization process by bringing wound margins closer together. Myofibroblasts also have high synthetic capacity for ECM proteins (Ignotz and Massague, 1986), growth factors/cytokine (Finlay et al., 2000), growth factor receptors (Thannickal et al., 1998), integrins (Heino et a. 1989), and oxidants (Thannickal et al 1989).

In addition to sending activation signals to mesenchymal cells, AECs themselves can also undergo phenotypic transition to fully differentiated mesenchymal cells such as fibroblasts and myofibroblasts (Zavadil et al., 2005). This process is called epithelial to mesenchymal transition (EMT). Epithelial cells undergoing EMT are able to lose polarity, disassemble cell adhesion systems, produce cell-motility machinery, and move from one location to another (Zavadil et al., 2005). EMT plays a pivotal role in cellular transdifferentiation during embryonic morphogenesis and tumor progression. In the adult, injury to epithelial cells induces EMT and contributes to fibrosis in a number of organs

(Iwano et al., 2002; Saika et al., 2004). EMT has been investigated as a mechanism underlying fibrosis in renal and lens epithelium. Injured renal tubular epithelial cells during kidney fibrosis migrate through damaged basement membranes into the interstitium and fully transdifferentiate into fibroblasts and myofibroblasts (Fan et al., 1999; Kalluri et al., 2003). About 36% of new fibroblasts come from EMT of local epithelium in kidney fibrosis (Iwano et al., 2002).

The stimulatory input of epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factors (FGF), and especially TGF-β factors stimulate epithelial cells to lose polarity, express basement membrane-degrading matrix metalloproteinases (MMPs), undergo cytoskeletal rearrangements, and express machinery necessary for motility, leading to migration and complete transition to a mesenchymal phenotype (Grunert et al., 2003; Kalluri et al., 2003; Savagner et al., 2001). It was recently demonstrated *in vitro* that TGF-β1 induces EMT in AECs (Willis et al., 2005).

In addition to the highly synthetic phenotype of fibroblasts in IPF, there is also an imbalance in the production of MMPs and tissue inhibitors of metalloproteinases (TIMPs) that control MMP activity (Ramos et al., 2001; Selman et al., 2000). MMPs are a family of highly regulated zinc-dependent peptidases and have been implicated in the remodeling of ECM and cell migration (Nagase and Woessner, 1999). TIMP-2 is expressed by myofibroblasts within the fibrotic foci and may induce mesenchymal cell proliferation. This in part explains the survival of mesenchymal cells in the fibroblast foci in IPF, in contrast to the expected cell death as observed in normal wound healing (Desmouliere et al., 1995). In addition, TIMP-2 has been found to contribute to the irreversible structural remodeling in IPF (Fukuda et al., 1998).

Myofibroblasts have also been shown to secrete angiotensin peptides that may induce apoptosis of adjacent alveolar epithelial cells (Uhal et al., 1998). The signaling crosstalk between AECs and fibroblasts is critical in determining whether fibrosis occurs after lung injury. The various roles and phenotypes of fibroblasts and myofibroblasts make them key effector cells in the pathogenesis of fibrosis.

**Basement Membrane Disruption and ECM Remodeling**

The basement membrane plays an important role in maintaining the integrity and differentiation of the alveolar epithelium. The basement membrane is a complex structure composed of type IV collagen, laminin, entactin, fibronectin, and heparin sulfate-chondroitin proteoglycans (Yurchenco and Schittny, 1990). The disruption of the basement membrane contributes to the pathogenesis of fibrosis. MMP proteins produced by the myofibroblasts in IPF have been shown to degrade different components of the basement membrane, type IV collagen in particular (Segura et al., 2000). In addition, MMP proteins in IPF have been found to coincide in areas with denuded alveolar basement membrane (Hayashi et al., 1996; Selman et al 2000). These findings suggest that myofibroblasts play a role in the degradation of the basement membrane to facilitate their migration into the alveolar spaces. This disrupted basement membrane may also contribute to the failure of an orderly repair of damaged AT I cells.

Fibroblasts and myofibroblasts also play a central role in the synthesis, deposition, and remodeling of the ECM. Increase in the deposition of ECM, including fibrillar collagens, fibronectin, elastic fibers, and proteoglycans, results in the aberrant tissue remodeling observed in IPF (Selman et al., 1996). This remodeling eventually leads to

extensive structural disorganization in the lung microenvironments and progressive loss of the alveolar-capillary units.

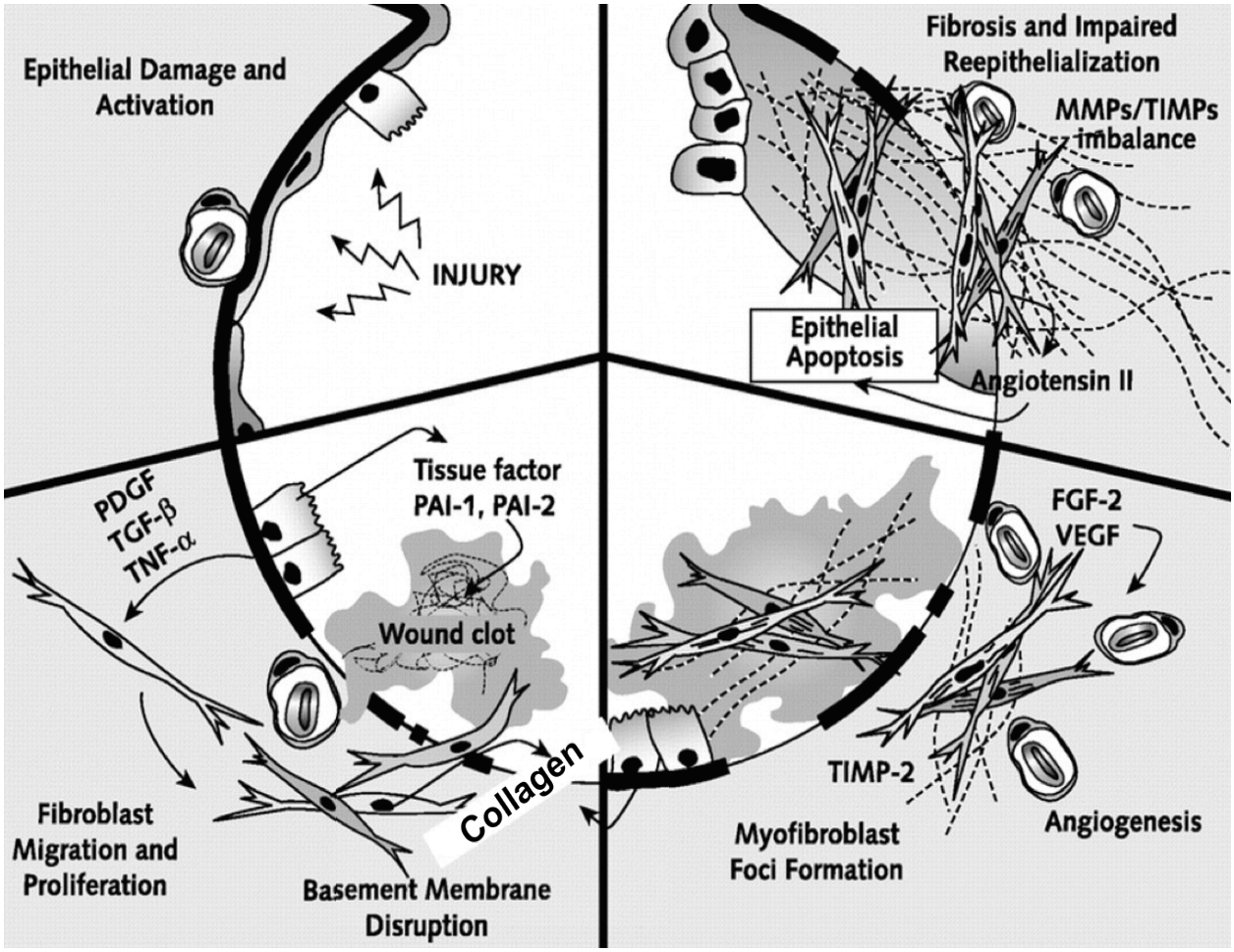


**Figure 1.1.**

**Pathogenesis of idiopathic pulmonary fibrosis [Figure 7.1 modified from (Selman et al., 2001)].** Alveolar epithelial cell damage (top left) induces an antifibrinolytic environment in the alveolar spaces, which then enhances wound clot formation. Alveolar epithelial cells also secret growth factors that induce migration and proliferation of fibroblasts and differentiation into myofibroblasts (bottom left). Myofibroblasts secret extracellular matrix proteins, mainly collagens. Neovascularization is induced by angiogenic factors TIMP-2, FGF-2, and VEGF (bottom right). An imbalance between

interstitial collagenases and tissue inhibitors of matrix metalloproteinases (MMPs) provokes the progressive deposit of extracellular matrix (top right). Myofibroblasts also produce angiotensin II, which provokes alveolar epithelial cell death, further impairing re-epithelialisation. PDGF: platelet-derived growth factor; TGF: transforming growth factor; TNF: tumour necrosis factor; PAI: plasminogen activator inhibitor; TIMP: tissue inhibitors of metalloproteinases; VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor.

## APOPTOSIS IN IDIOPATHIC PULMONARY FIBROSIS

Apoptosis is programmed cell death that is a critical physiological process in the development and maintenance of tissue homeostasis. Apoptosis is important in almost all cell types to provide a balance between cellular proliferation and turnover. In IPF, apoptosis affects the development of fibrosis through both increased apoptosis of epithelial cells resulting in inefficient reepithelialization and resistance to apoptosis of fibroblasts resulting in increased fibrosis (Antoniou et al., 2007).

### Apoptosis in General

#### *Morphological and Biochemical Changes*

The cell undergoes a highly regulated series of characteristic morphological and biochemical changes during apoptosis that distinguish it from normal and necrotic cells. The first morphological changes during apoptosis are cytoplasmic shrinking, loss of cell-cell contacts and active membrane blebbing (Allen et al., 1997). In addition, the cytoplasm and nuclear chromatins condense and fragment into membrane-bound vesicles. Phosphatidylserine (PS) externalizes at the intact cell surface and promotes signal recognition by phagocytic cells. Endogenous DNase degrades chromosomal DNA and cleaves the internucleosomal regions into double stranded DNA fragments. Another marker of apoptosis is the activation of cytosolic caspases. Caspases are cysteine proteases that cleave and degrade a specific subset of cellular proteins in cells undergoing apoptosis. The entire process of apoptosis occurs in a predictable sequence that concludes with the engulfment of apoptotic bodies by other cells.

***Caspases***

Caspases are a family of aspartate-specific cysteine proteases that are present in all animal cells as inactive zymogens. In apoptosis, caspases function as both effectors in cell disassembly and as initiators in initiating this disassembly in response to proapoptotic signals. Initiator caspases are the first to be activated in a death pathway. They then activate effector caspases through a caspase cascade. Proteolytic processing at conserved aspartic acid (Asp) residues triggers caspases to assume active states. During activation, the zymogen pro-proteins are cleaved at a site known as the linker region to generate the large and small subunits of the active enzymes. One small and one large subunit make up one catalytic unit of the active caspase enzyme. Each catalytic unit contains one active site and a dimer of identical catalytic units make up one active caspase enzyme. The N-terminal prodomain regulates activation of the proenzyme and is highly variable in sequence and length. Caspases are unusually highly specific and recognize the Asp residues they cleave within tetrapeptide motifs (Nicholson and Thornberry, 1997). This strict specificity ensures that caspases are signaling proteases intended for specific protein cleavage and not for protein degradation.

Caspase proteins collaborate in proteolytic cascades where caspases activate themselves and each other. Caspase enzymes cleave their substrates at Asp residues, and are also activated by proteolytic processing at Asp residues. Activated upstream initiator caspases then activate downstream effector caspases. The N-terminal domains of proform initiator caspases are large and function as protein interaction modules that allow them to interact with various proteins that trigger caspase activation. The proforms of downstream

effector caspases contain only short N-terminal prodomains that appear to serve no function. The effector caspases depend mostly on initiator capases for their proteolytic processing and activation. Consequently, the sequence of the cleavage sites of zymogen effector caspases generally matches the preferred tetrapeptide specificities of the initiator caspases. Additionally, cleavage sites of proteins that were identified as caspase substrates that undergo processing during apoptosis also match with the preferred specificities of the effector caspases (Thornberry et al., 1997). Most caspases in mammals and higher eukaryotes are directly involved in cell death except for a few, such as caspase-1, 4, and 5 that are involved in processing of pro-inflammatory cytokines in humans. The tetrapeptide specificities of these cytokine-processing proteases do not match the cleavage sites of most of the proteins known to undergo cleavage during apoptosis, but instead they match with the sequences of the cleavage sites within pro-cytokines (Salvesen and Dixit, 1997).

Initiator capases can by activated by a variety of mechanisms to initiate the apoptotic cascade. The underlying biochemical mechanisms of initiator caspase activation are all remarkably similar and can be explain by the induced proximity model (Salvesen and Dixit, 1999). The zymogen forms of unprocessed caspases are not completely inactive but rather possess weak protease activities that, in some cases are at approximately 1% of the level of the fully active enzymes. Protein interactions can bring the zymogens close together and allow them to trans-process each other to produce fully active proteases.

### *Mechanisms of Apoptosis*

Apoptosis can be induced by various physiological stimuli such as deformation of the cell membrane, growth factor withdrawal, inflammatory cytokines, osmotic shock or

UV irradiation, as well as a number of molecules and agents. When stimulated, the cells undergo the activation of pro-apoptotic signaling pathways that transduce a signal to the interior of the cell and activate the apoptotic machinery.

The mechanism of apoptosis begins with the initiation phase, during which various apoptotic stimuli lead to activation of caspases, followed by the execution phase where the activated caspases induce cell death. The caspase cascade can be activated by many pathways, two of which have been studied in detail. Both of these pathways lead to the activation of the signaling cascade that results in the cleavage of inactive pro-caspase molecules into active caspase proteins.

*The Extrinsic Pathway/Receptor-mediated Pathway*

The first is the extrinsic or death receptor pathway that involves the activation of death receptors present in the cell membrane, such as Fas and tumor necrosis factor (TNF) receptors I and II (Figure 1.2). Death receptors are protein interaction modules that consist of a compact bundle of six α-helices (Huang et al., 1996). Death receptors bind to each other with specificity directed by differences in their surface residues. Fas receptor can be activated by either the Fas ligand (FasL) on the surface of cytotoxic lymphocytes, or by a soluble form of FasL (sFasL) that can be cleaved from cell membranes by MMP-7 and MMP-3 (Matsuno et al., 2001; Powell et. al 1999). Upon ligation to FasL, the Fas receptor forms microaggregates at the cell surface. This allows the adaptor molecule, Fas-associated protein with death domain (FADD), to be recruited to the cytosolic tail of the Fas receptor. FADD contains a death effector domain (DED) at its amino-terminus and a Death Domain (DD) at its carboxy-terminus. Caspase-8 contains two DEDs at its amino-terminus. FADD

binds Fas receptor and at least three molecules of procaspase-8 through homotypic interactions. Within this death inducing signaling complex (DISC), procaspase-8 is able to undergo activation by autocatalytic cleavage via the induced proximity mechanism. During activation, the N-terminal prodomain of procaspase-8 is cleaved off and the active protease is released into the cytosol. The active caspase-8 then cleaves and activates downstream caspases-3, 6, and 7 as well as the pro-death protein Bid. The Bid cleavage fragment then targets the mitochondria and leads to a mitochondrial amplification of the caspase pathway.

*The Intrinsic Pathway/Mitochondrial Pathway*

The intrinsic pathway responds to ionizing radiations, chemotherapeutic drugs, mitochondrial damage, and environmental cues. Death triggers increase mitochondrial permeability, leading to the release of cytochrome *c* and the subsequent recruitment and activation of caspase-9 (Zou et al., 1999). The release of cytochrome *c* triggers the formation of the apoptosome complex. The apoptotic protease-activating factor 1 (Apaf1) is the main component of the apoptosome. Apf-1 is a multidomain protein that consists of three functional regions: an N-terminal caspase-recruitment domain (CARD); a nucleotide-binding and oligomerization domain (NOD); and a string of WD40 repeats in the C-terminal of the protein. In the absence of an apoptotic signal, Apaf-1 exists in a monomeric form. Cytochrome *c* binding allows the compact Apaf-1 molecule to stretch out and polymerize upon biding ATP (Acehan et al., 2002). Studies of the apoptosome through electron cryomicroscopy reveal it as a seven-spoked wheel with the caspase-9 recruitment domain, CARD of Apaf-1, in the central hub (Renatus et al., 2001). Caspase-9 is activated through dimerization of monomers within the apoptosome. In contrast to the activation of

caspase-8, the N-terminal prodomains of caspase-9 is not cleaved and the caspase-9 enzyme must remain bound to Apaf-1 to be fully active (Rodriguez et al., 1999). Activated caspase-9 is then released from the complex to cleave and activate downstream caspases-3, 6, and 7 (Budihardjo et al., 1999). Members of the B-cell lymphoma (Bcl)-2 family proteins, including the anti-apoptotic Bcl-2/Bcl-xL and the pro-apoptotic Bax/Bid, regulate the release of cytochrome c from the mitochondria and act to suppress or promote apoptosis. Activated Bax/Bid migrate from the cytosol to the mitochondria to initiate release of cytochrome *c* from the mitochondria and promote apoptosis. Bcl-2/Bcl-xL are primarily localized in the outer member of the mitochondrial and are able to inhibit the release of cytochrome *c* to suppress apoptosis. The balance of anti-apoptotic proteins and pro-apoptotic proteins determines the release of cytochrome *c* and activation of downstream caspases and therefore the cell's susceptibility to apoptotic stimuli. In addition, both Bcl-2/Bcl-xL and Bax/Bid are transcriptional targets of the tumor suppressor protein p53, which induces cell cycle arrest and apoptosis in response to DNA damage (Earnshaw et al., 1999)

*Caspase Substrates*

Activation of caspases leads to downstream cleavage of key structural components of the cytoskeleton and nucleus as well as proteins involved in the apoptosis signaling pathway. Pro-and anti-apoptotic regulatory proteins are among the first to be cleaved by activated caspases. Caspase-3 cleavage of Bcl-2/Bcl-xL inactivates the anti-apoptotic properties of these proteins by producing a pro-apoptotic product that increases mitochondrial permeability and release of cytochrome *c* (Li et al., 1998). Caspase-8 can

also induce cytochrome *c* release by cleaving pro-apoptotic Bid (Lou et.al, 1994). These cleavage events allow the extrinsic pathway to communicate with and utilize the mitochondrial pathway to amplify apoptotic signals.

*Other Pathways*

The MAPK pathway allows signals to be transmitted from the cell membrane to the nucleus in response to various stimuli. The MAPK pathway also induces the phosphorylation of intracellular substrates such as protein kinases and transcription factors to regulate cellular functions such as cell growth, differentiation, and apoptosis (Kyriakis and Avruch, 1996). The MAPK family of kinases include: the extracellular signal-regulated kinases (ERKs), ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK); stress-activated protein kinases (SAPKs), also referred to as c-Jun, $NH_2$-terminal kinases (JNKs), which include p54 SAPK (SAPKα/,β, JNK2) and p45 SAPK (SAPKγ, JNK1); and the p38 kinases (α, β, γ, and δ) (Widmann et al., 1999). Activation of the SAPK/JNKs and p38 kinases play a role in apoptosis and activation of the ERK1/ERK2 pathways are involved in cell survival (Earnshaw et al., 1999).

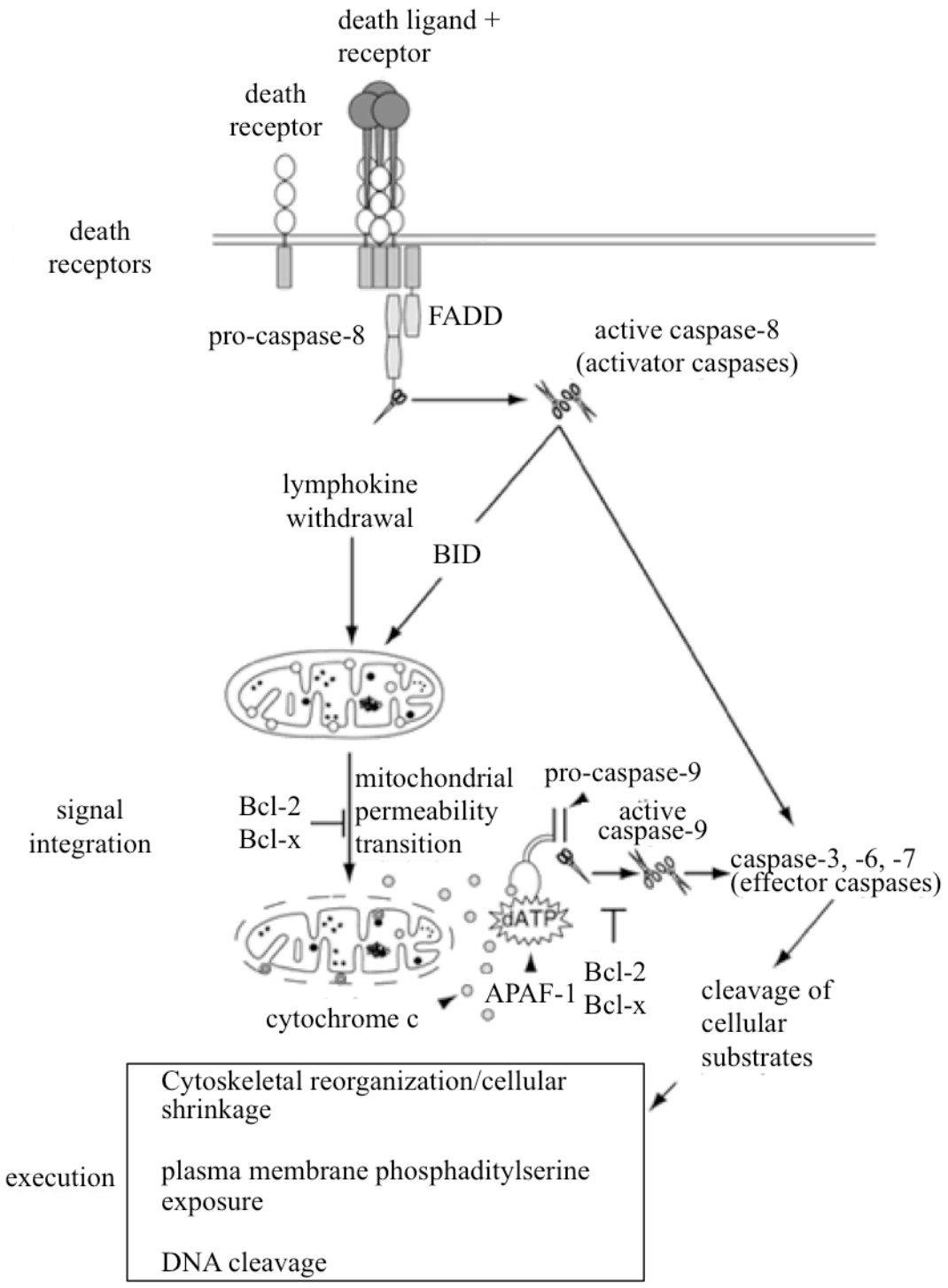


**Figure 1.2.**

**Induction of apoptosis [Figure 11.9C.1 modified from (Siegel and Lenardo, 2002)]**

## Alveolar Epithelial Cell Apoptosis in IPF

Alveolar epithelial cell injury and death have consistently been found in human IPF. The survival and recovery of epithelial cells is key to normal repair of damaged epithelia. Fibrosis occurs through the pathological repair process of tissue remodeling. Often, the degree of remodeling is closely associated with the patient's prognosis. Hence, a greater understanding of epithelial cell apoptosis may lead to the development of effective strategies for treatment.

### *Evidence of Apoptosis*

Alveolar epithelial cell injury is a characteristic feature of IPF. In the normal lung, ATI cells makeup 40% of the AEC population and cover 95% of the alveolar surface (Mason and Shannon, 1997). The number of AT1 cells are markedly decreased in the IPF lung after injury and cell death. Recently, increased apoptosis of ATII cells has been found in areas of IPF that do not have established apoptosis (Barbas-Filho et al., 2001), and in epithelial cells that overlay myofibroblasts (Uhal et al., 1998). Additionally, increased expression of proapoptotic proteins and decreased expression of anti-apoptotic proteins has also been found in the IPF lung (Plataki et al., 2005). Experiments using caspase inhibitors also demonstrate the involvement of apoptosis in fibrosis. The tripeptide broad-spectrum caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk), inhibits the intracellular activation of caspase-like proteases in vivo , and attenuates bleomycin-induced pulmonary fibrosis in mice (Kuwano et al., 2001; Wang et al., 2000). Thus, there is growing evidence that subpopulations of AECs in IPF are undergoing

apoptosis in association with the dysfunctional repair of the damaged alveolar epithelium.

### *Induction of Pulmonary Fibrosis*

Studies of animal models have provided further understanding of whether excessive AEC apoptosis is sufficient to lead to fibrogenesis. The induction of AEC apoptosis using aerosolized anti-Fas antibody was found to associate with the development of pulmonary fibrosis in mice (Hagimoto et al., 1997). Targeted transgenic overexpression of bioactive TGF-β1 to the murine lung produces a transient wave of epithelial apoptosis that is followed by mononuclear-rich inflammation, tissue fibrosis, myofibroblast hyperplasia, and honeycombing (Lee et al., 2004). Furthermore, a null mutation of early growth response gene (EGF)-1 or caspase inhibition can rescue the fibrotic phenotype (Lee et al., 2004).

### *Mechanism of Alveolar Epithelial Apoptosis*

*p53 and p21 upregulation*

Lung tissues from patients with IPF reveal upregulation of p53 and p21 in lung epithelial cells (Kuwano et al., 1996). In normal cells, p53 responds to DNA damage and mediates cell cycle arrest, DNA repair, and apoptosis. p53 expression is upregulated in response to a variety of stresses. ATII cell apoptosis is associated with upregulation of p53 and p21 expression in diffuse alveolar damage (Guinee et al., 1996). ATII cells of patients with IPF also express high levels of p53 in association with DNA strand breaks (Kuwano et al., 1996; Plataki el al., 2005;). p53 and p21 are also overexpressed in AECs treated with bleomycin (Mishra et al., 2000; Kuwano et al., 2000). p21 is a critical downstream effecter in the p53 pathway of growth control in mammalian cells and is induced after exposure to

DNA-damaging agents (el-Deiry et al., 1993). p21 enhances cell survival by promoting DNA repair or by modifying cell death caused by exposure to hyperoxia (O'Reilly et al., 2001). Transfer of the p21 gene through adenovirus to epithelial cells attenuates bleomycin-induced pulmonary fibrosis in mice (Inoshima et al., 2004). p21 also regulates activation of caspase-3. The formation of a procaspase-3-p21 complex is essential for cell survival (Suzuki et al., 1998; Suzuki et al., 1999), suggesting that p21 may act as a key regulator of DNA replication and repair after lung damage.

*Fas-FasL Pathway Activation*

Fas is expressed on the luminal surface of a subset of AT II cells (Fine et al., 1997). In patients with acute lung injury, alveolar epithelial damage is in part associated with the local upregulation of the Fas-FasL pathway and subsequent activation of the apoptotic cascade in epithelial cells (Albertine et al., 2002). In lung tissues of IPF patients, FasL mRNA and protein expression are upregulated in infiltrating inflammatory cells and Fas protein is overexpressed in the epithelial cells (Kuwano et al., 1999). BAL fluid obtained from patients with IPF or ARDS could induce apoptosis on small airway epithelial cells through the Fas-FasL pathway (Matute-Bello et al.,1999; Hagimoto et al., 2002).

Bleomycin is used to induce pulmonary fibrosis in animal models of lung injury and fibrosis. FasL mRNA in this model is upregulated in infiltrating lymphocytes. In addition, excessive apoptosis is detected in the bronchiolar and alveolar epithelial cells where Fas protein expression is upregulated (Hagimoto et al., 1997) Neutralization of FasL prevents the development of fibrosis in this model (Kuwano et al., 1999). These findings indicate that the Fas-mediated apoptotic pathway is essential in the bleomycin-induced pulmonary

fibrosis model. Furthermore, the inhibition of caspases may provide a new approach to treating pulmonary fibrosis.

*TGF-β Induction of Apoptosis*

TGF-β is a strong chemotactic attractant for monocytes and macrophages as well as a potent promoter of ECM production. There is a consistent increase in TGF-β production in epithelial cells and macrophages in lung tissues of IPF patients (Khalil et al., 1991) and in bleomycin-induced pulmonary fibrosis in rodents (Raghow et al., 1989). Gene transfer of a TGF antagonist reduces the fibrotic response to bleomycin (Kolb et al., 2001). TGF-β1 directly induces epithelial cell apoptosis via Fas and caspase-3 activation, and by enhancer the FasL-Fas interaction (Hagimoto et al., 2002). The Bax-mediated, Bid-activated pathway is involved in the pathogenesis of pulmonary fibrosis. TGF-β1 significantly stimulates Bax and Bid expression and causes the release of MMP-12 and tissue inhibitor of matalloproease-1 in mice (Kang et al., 2007). Bid-deficient mice are protected from developing fibrosis after TGF-β1 activation, indicating that Bid is required for AEC apoptosis and bleomycin-induced fibrosis in mice (Budinger, 2006). In an experiment using mice, TGF-β1 overexpression in lung epithelial cells induced apoptosis and fibrosis. This induction was attenuated when a caspase inhibitor was administered from day 0 but not from day 5 after TGF-β1 overexpression (Lee et al., 2004), indicating that TGF-β1-induced epithelial cell apoptosis is a critical early event in pulmonary fibrosis.

*Oxidative Stress*

Oxidative stress has been proposed to significantly contribute to epithelial cell damage in IPF (Kuwano et al., 2003). A number of studies demonstrate the increase of oxidative stress in IPF. Patients with IPF exhibit an increase in the spontaneous production of oxidants in the alveolar epithelial lining fluid (Saleh et al., 1997) as well as a significant reduction in antioxidant capacity in the plasma and BAL fluid (Rahman et al., 1999). Apoptosis plays a key role in hyperoxic lung injury (Albertine et al., 2002). Hyperoxia can affect both ATI and endothelial cells as well as induce DNA damage in ATII cells (Roper et al., 2004). Hyperoxia amplifies ventilator-induced cytokine production, neutrophil influx, and apoptosis through activation of the JNK and ERK pathway (Ward et al., 1983). It was traditionally thought that alveolar inflammatory cells produce the reactive oxygen species (ROS) that induce AEC injury/apoptosis in IPF (Cantin et al., 1987; Stausz et al., 1990). However, recent studies suggest that structural cells of the lung, particularly activated myofibroblasts, produce sufficient amounts of extracellular ROS to induce injury/apoptosis of adjacent epithelial cells. Bleomycin induces AEC apoptosis and fibrosis through an increase of ROS (Wallach-Dayan et al., 2006*)*.

*Alveolar Epithelium-Fibroblast Crosstalk*

Severe injury to the lung epithelial cells followed by insufficient repair disturbs normal epithelial-fibroblast interactions. The lack of reepithelialization after damage allows fibroblasts to proliferate and eventually resulting in pulmonary fibrosis. Epithelial cells control fibroblasts through the release of cytokines. At the same time, fibroblasts can also affect alveolar epithelial cells by releasing factors that disrupt their normal repair.

Abnormal fibroblasts isolated from fibrotic human lungs produce factors that can induce apoptosis and necrosis of AECs *in vitro* (Uhal et al., 1995).

## THE RENIN ANGIOTENSIN SYSTEM

### General RAS

The traditional view of the renin angiotensin system (RAS) characterizes it as an endocrine system with important roles in the regulation of fluid homeostasis, electrolyte metabolism and blood pressure. Angiotensinogen (AGT), an α-glycoprotein, is released from the liver and cleaved by renin in the circulation to form the decapeptide, Angiotensinogen I (ANG I). Renin is an aspartyl protease secreted by the granular cells in the juxtaglomerular apparatus of the kidney in response to decreased renal perfusion and plasma sodium concentration. ANG I is activated by angiotensin converting enzyme (ACE) to form the octapeptide angiotensin II (ANG II). ACE, a membrane-bound metalloproteinase, is primarily expressed in high concentrations on the surface of endothelial cells in the pulmonary capillaries. Alternative pathways involving enzymes other than renin and ACE have also been shown to generate ANG I and ANG II. For example, ANG II may be generated by chymase released from mast cells in the vascular tissue under certain pathological conditions. ANG II is considered the main effector peptide of the RAS and exerts its actions through specific cell surface angiotensin receptors.

The main receptors of ANG II are $AT_1$ and $AT_2$, both belong to a superfamily of seven transmembran G-protein couple receptors. The $AT_1$ receptor mediates most of the well-known actions of ANG II, such as vasoconstriction, sodium retention, aldosterone release, and cell growth and proliferation, etc. Actions mediated by the $AT_2$ receptor are less well characterized but appear to counteract $AT_1$ effects by promoting vasodilation, cell differentiation, and inhibiton of cell growth and apoptosis (de Gasparo et al., 2000).

In additional to ANG I and ANG II, several functional peptides can be generated through alternative cleavage pathways. These include angiotensin III (ANG III), angiotensin IV (ANG IV), and angiotensin 1-7 (ANG 1-7). ANG III is generated from ANG II by aminopeptidase A and plays a similar role as ANG II. Cleavage of ANG III by aminopeptidase M generates ANG IV, which is involved in the vascular inflammatory response (Ruiz-Ortega, 2007). ANG 1-7 heptapeptide is generated through cleavage of ANG II by angiotensin-converting enzyme 2 (ACE2). ANG 1-7 was found to have actions opposing those of ANG II and could act as a part of a counterbalancing mechanism in controlling the actions of ANG II in specific tissues (Ferrario et al., 2006).

**Local RAS**

Recent evidence of RAS components expression in most organs and tissues suggests the existence of a "local" RAS system that responds to physiological or pathophysiological stimuli in an autocrine, panracrine and/or intracrine manner. This local system contains all the components necessary to produce ANG II and other angiotensin peptides along with their respective receptors, as well as renin/prorenin receptors. Most, if not all of the renin found in local RAS is derived from renal renin. Local RAS systems may operate independently of or interact with "circulating RAS" to exert novel and tissue specific actions. These include cellular actions such as regulation of cell growth, differentiation, proliferation and apoptosis, reactive oxygen species (ROS) generation, hormonal secretion, tissue inflammation, and fibrosis (Leung et al., 2007; Montani & Van Vliet 2004).

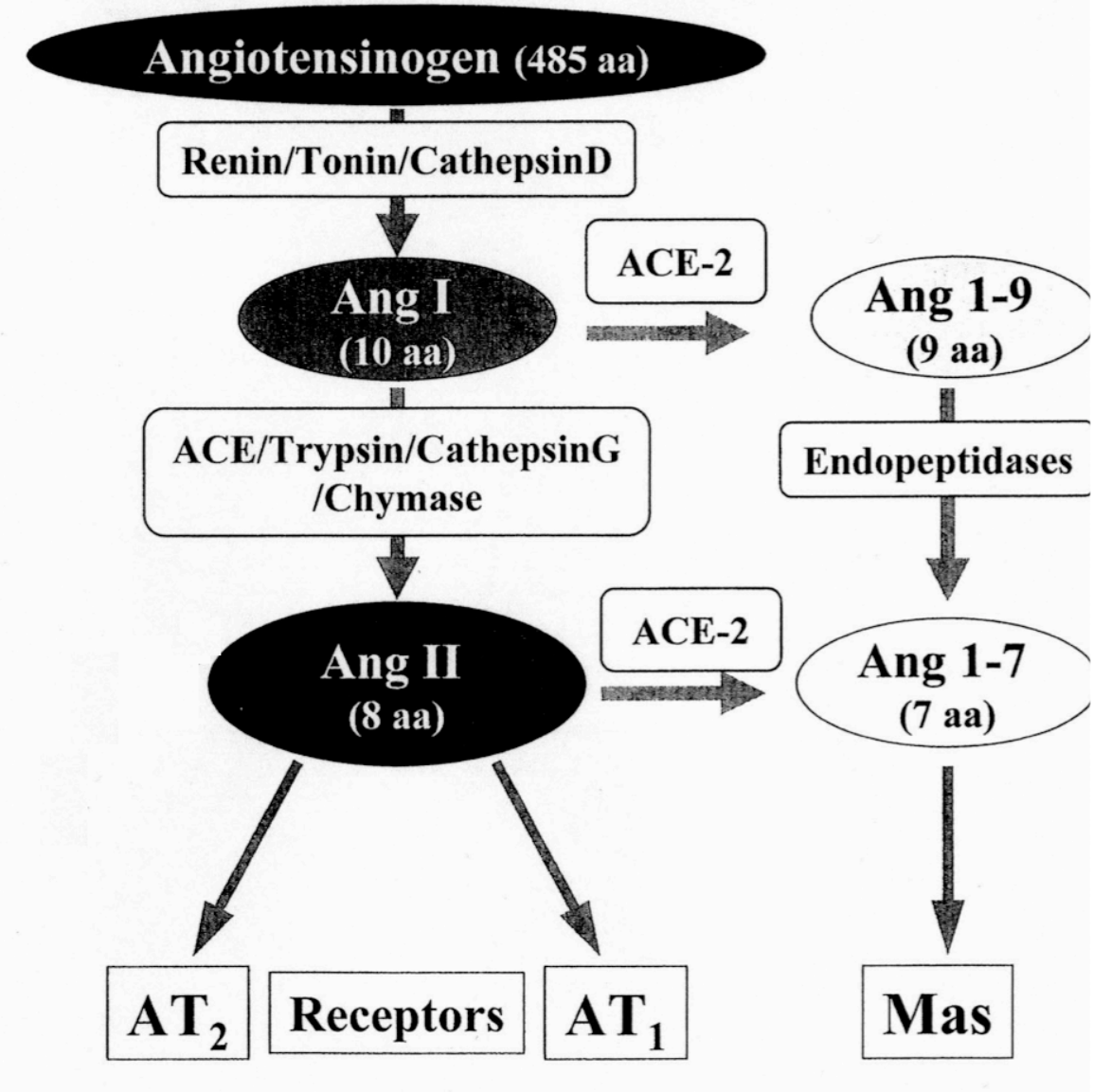


**Figure 1.3.**

**The Renin Angiotensin System (RAS).** The diagram illustrates the enzymatic cleavage of angiotensinogen protein into angiotensin I (ANG I), angiotensin II (ANG II), angiotensin 1-9 (ANG 1-9), angiotensin 1-7 (ANG 1-7). Also shown are the known receptors for angiotensin peptides: $AT_1$ (angiotensin receptor 1), $AT_2$ (angiotensin receptor 2), Mas. ACE: angiotensin converting enzyme; ACE-2: angiotensin converting enzyme-2; aa: amino acid.

## BLEOMYCIN-INDUCED EXPERIMENTAL PULMONARY FIBROSIS MODEL

The bleomycin-induced experimental pulmonary fibrosis model is one of many animal models of pulmonary fibrosis that have been developed to address the pathogenesis of pulmonary fibrosis. Bleomycin is an antineoplastic antibiotic that was isolated from a strain of *Strptomyces verticillus* (Umezawa et al., 1966). Bleomycin is often used to treat squamous cell carcinomas and various lymphomas (Ichikawa et al., 1967, 1969; Yogoda et al., 1972; Blum et al., 1973). Instead of being just a single peptide, bleomycin consists of a family of complex glycopeptides with different amine groups (Umezawa et al., 1966, 1967; Umezawa 1973, 1974). The mechanisms of the antineoplastic actions of bleomycin are cell cycle-dependent and complex. Bleomycin can intercalate between DNA base pairs, leading to DNA unwinding and impaired protein synthesis. Bleomycin also reduces molecular oxygen to superoxide and hydroxyl radicals, producing radical oxygen species that then attack DNA and cause strand cleavage (Sausville et al., 1978).

Treatment with bleomycin has very little side effects on bone marrow (Kimura et al., 1972; Boggs et al., 1974) and immunocompetence (Dlugi et al., 1974; Lehane et al., 1975). Despite these advantages, bleomycin has severe toxic side effects on the lung and skin organ systems (Thrall and Scalise, 1995). After parenteral administration, bleomycin concentrations are highest in the lungs and skin, likely due to the lack of a hydrolase, which inactivates bleomycin (Umezawa et al., 1972).

The bleomycin-induced pulmonary fibrosis animal model is currently the most commonly used model in studying the pathogenesis of fibrotic lung disease. Pulmonary fibrosis induced by bleomycin is dependent on dosage and administration route, most common of which are parenteral and intratracheal. Various animals, such as mice and rats,

have been used in models of bleomycin-induced pulmonary fibrosis (Thrall and Scalise). The development of lung injury in these models is similar regardless of species and route of administration. The lung injury has three stages (Thrall and Scalise, 1995): (1) the acute inflammatory stage where the inflammatory mediator systems are activated and pulmonary edema appears; (2) The subacute stage where collagen is synthesized and the level of net lung collagen elevates; (3) the chronic stage that is dominated by the metabolism of connective tissue towards reepithelialization. The bleomycin animal models are valuable tools in studying the pathogenesis of fibrotic lung disease.

## PULMONARY ANGIOTENSIN SYSTEM IN IDIOPATHIC PULMONARY FIBROSIS

Recent evidence suggests the existence of a local intrinsic RAS system in the distal lung parenchymal that plays a central role in the signaling of apoptosis in alveolar epithelial cells (Wang et al., 1999). ANG II in the lung, derived from its precursor AGT, drives fibrosis through upregulation of collagen gene expression in lung fibroblasts (Marshall et al., 2004; Marshall et al., 2000), induction of apoptosis in alveolar epithelial cells (Li et al., 2003), and other profibrotic actions (Marshal, 2003).

AGT in the local system is synthesized within the lung tissue itself by alveolar epithelial cells and myofibroblasts. Primary cultures of human or rat AECs can synthesize and secrete AGT, which is converted to ANG II when undergoing apoptosis induced by Fas ligand (Wang et al., 1999), TNF-α (Wang et al., 2000), amiodarone (Bargout et al., 2000) and bleomycin (Li et al., 2003). Myofibroblasts isolated from lungs of IPF patients were shown to synthesize AGT mRNA and protein constitutively (Wang et al., 1999). Intratracheal administration of antisense oligonucleotides against AGT mRNA prevented bleomycin-induced lung fibrosis in rats without affecting circulating levels of ANGEL protein, suggesting that local pulmonary synthesis of ANG II from the precursor AGT within the lung is required for lung fibrogenesis (Li et al., 2007).

The local angiotensin systems often utilize components of endocrine RAS system in its pathways. For example, synthesis of local ANG II in the heart and vessel walls reply on the uptake of circulating renin through either diffusion into the interstitial space or through binding to prorenin receptors (Re, 2004). Renin mRNA has not been detected in the lungs. Other proteases in the lung, such as cathepsin D (Cat D), are capable of cleaving AGT to

ANG I. Cat D expression is upregulated in the fibrotic lungs of both rat and human. Cat D is also overexpressed in the lungs of patients with pulmonary fibrosis (Kasper et al., 1996), and is induced in lung cells during apoptosis (Kasper et al., 1999). It is possible that production of ANG I from AGT in the lungs is mediated by Cat D.

The decapeptide ANG I is inactive until it is cleaved into ANG II by the dipeptidyl carboxypeptidase ACE. ANG II is thought to be responsible for most of the physiological and pathophysiological effects of the RAS. ACE in the pulmonary vascular endothelium is primarily responsible for the conversion of ANG I to ANG II in the circulation (Oparil et al., 1971). ACE2, the first known homologue of ACE, was recently discovered to be another regulator of the RAS system (Donoghue et al., 2000). ACE2 was first described for its ability to cleave ANG I into ANG 1-9, a peptide with no known function (Donoghue et al., 2000). It was later found to also degrade ANG II to the biologically active peptide, ANG 1-7 (Vickers et al., 2002). *In vitro* studies demonstrate that the catalytic efficiency of ACE2 for ANG II is 400 times greater than for ANG I (Vickers et al., 2002), suggesting that ACE2 is primarily involved in the conversion of ANG II to ANG 1-7. ACE2 expression has been identified in the heart, kidney, testis, gastrointestinal tract, brain, and lung (Hammer et al., 2002). Despite its similarities to ACE, the *in vitro* enzymatic activities of ACE2 is unaffected by ACE inhibitors (Donoghue et al., 2000; Tipnis et al., 2000).

Both receptors of ANG II, AT1 and AT2, are found to be expressed in the lung. In the human lung, AT1 receptor mRNA and proteins were found on vascular smooth muscle cells, macrophages and in the stroma underlying the airway epithelium (Bullock et al., 2001). AT2 receptor RNA and protein were highly expressed on the bronchial epithelial

cell brush border, endothelial cells, and underlying mucous glands of the epithelium (Bullock et al., 2001). A study of the rat lung also found AT1 to be expressed on alveolar macrophages, alveolar type II cells, vascular smooth muscle cells, endothelial cells and fibroblasts of the rat lung (Otsuka et al., 2004). The AT1 expression in the lung was significantly upregulated after induction of fibrosis (Otsuka et al., 2004), suggesting that ANG II promotes lung fibrosis via the AT1 receptor.

Although AGT in the lung can be synthesized by myofibroblasts, they have a limited ability to convert the AGT to ANG II (Wang et al., 1999). Alveolar epithelial cells, on the other hand, are capable of proteolytically processing AGT to ANG II as well as undergoing apoptosis in response to the ANG II produced (Wang et al., 1999). Thus, it is theorized that the death of AECs adjacent to underlying myofibroblasts in the fibrotic lung is due to production of AGT by the myofibroblasts and its subsequent conversion to ANG II by the epithelial cells.

The local RAS system can also induce apoptosis of AECs without myofibroblasts. Purified ANG II was shown to induce apoptosis of AECs in primary culture through the AT1 receptor (Papp et al., 2002). The induction of AEC apoptosis by Fas ligand (Wang et al., 1999), TNF-α (Wang et al., 2000), amiodarone (Bargout et al., 2000), and bleomycin (Li et al., 2003) were blocked by AGT antisense mRNA oligonucleotides, ACE inhibitors, and ANG II receptor antagonists, indicating that AEC apoptosis as induced by these agents require angiotensin synthesis de novo. The inhibition of bleomycin-induced AEC apoptosis with either an ACE inhibitor or ANG receptor antagonist was observed both *in vitro* and *in vivo*, indicating that ANG II may be required for apoptosis in both cultured cells and the intact lung.

Angiotensin system antagonists have been shown to attenuate lung fibrosis in experimental models. Applications of ACE inhibitors, which presumably prevent ANG II production, have been shown to block pulmonary fibrosis in animal models induced by various agents. Treatment with the ACE inhibitor captopril, for example, effectively inhibited monocrotaline (Molteni et al., 1985) as well as γ irradiation-induced lung fibrosis in rats (Ward et al., 1990). Captopril was also shown to inhibit the proliferation of human lung fibroblasts *in vitro* (Nguyen et al., 1994). The specific AT1 receptor antagonist L158809 and the nonthiol ACE inhibitor enalapril were also shown to have similar effects on radiation-induced pulmonary fibrosis in rats (Molteni et al., 2000). Captopril was also found to prevent collagen deposition on bleomycin-treated rats (Wang et al., 2000). Recently, it was shown that captopril and AT1 selective antagonist losartan block the amiodarone induced pulmonary fibrosis in rats (Uhal et al., 2002). Together, these results suggest that ANG II plays an important role in lung fibrosis through the AT1-receptor.

The ACE inhibitor captopril as well as caspase inhibitors have been found to block pulmonary fibrosis through the inhibition of apoptosis. Caspases play key roles in apoptosis signaling. Application of the broad-spectrum caspase inhibitor ZVADfmk, or captopril both attenuated epithelial apoptosis and collagen deposition in intratracheally administered bleomycin-induced pulmonary fibrosis in rats (Wang et al., 2000). Delivery of the same caspase inhibitor through inhalation also inhibited bleomycin-induced in mice (Kuwano et al., 2001). These results indicate that both ANG II and epithelial apoptosis are required for lung fibrogenesis.

# Chapter 2

# ANGIOTENSIN 1-7 REGULATES ALVEOLAR EPITHELIAL CELL SURVIVAL THROUGH THE RECEPTOR MAS

**ABSTRACT**

Apoptosis of alveolar epithelial cells (AECs) is believed to be a critical event in the pathogenesis of lung fibrosis. It was previously shown that apoptosis of AECs requires autocrine generation of angiotensin (ANG) II. ANG II can also be degraded to ANG1-7 by angiotensin converting enzyme-2 (ACE-2). ACE2 was recently found to be protective but significantly downregulated in experimental lung fibrosis and patients with IPF. In other organ systems, ANG1-7 has been found to inhibit the actions of ANGII through the ANG1-7 receptor *mas*. Therefore, it was hypothesized that ANG1-7 in the lungs might antagonize the actions of ANG II in the regulation of AEC apoptosis. To test this theory, the AEC cell line MLE-12 and primary cultures of human AECs were stimulated by the profibrotic apoptosis inducers ANG II or bleomycin (BLEO). Caspase-3 activation and nuclear fragmentation were used as markers of apoptosis and were measured along with JNK phosphorylation. Exposure to ANG II or BLEO induced caspase-3 activation, nuclear fragmentation, and JNK phosphorylation in cultured AECs. Pretreatment with ANG1-7, at a concentration of 0.1μM, prevented JNK phosphorylation and apoptosis. In addition, pretreatment with A779, a specific blocker of the ANG 1-7 receptor *mas*, successfully prevented ANG1-7 induced blockade of JNK phosphorylation and nuclear fragmentation. These data indicate that ANG1-7 prevents AEC apoptosis, and its actions are mediated through the ANG1-7 *mas* receptor.

## INTRODUCTION

Idiopathic pulmonary fibrosis is a pathological condition resulting from lung injury and an ensuing fibrotic response that leads to thickening of the alveolar walls and the obliteration of the alveolar space (Fonseca et al., 2000). The etiology of the disease is unknown. Current evolving hypothesis about pathogenesis is that IPF results from the epithelial injury and a failure of reepithelialization (Selman et al., 2001). Alveolar epithelial cell apoptosis is a critical event in the pathogenesis of pulmonary fibrosis and the regulation of AEC apoptosis is of great interest and potential clinical importance. The bleomycin animal models are valuable tools in studying the pathogenesis of fibrotic lung disease. Recent studies in our lab and other labs using the bleomycin-induced models in rats and mice suggested a role of epithelial apoptosis as the profibrotic event in fibrosis. First, apoptosis of AECs was found in both patients with IPF (Uhal, et al., 198) and animal models (Hagimoto et al., 1997). Second, induction of apoptosis in the epithelium is sufficient to produce a fibrotic response (Hagimoto et al., 1997). And lastly, several labs have shown that the blockade of apoptosis could prevent the fibrotic response (Wang et al., 2000; Kuwano et al., 2001).

Recent work from this lab demonstrated that the exposure of cultured AECs to Fas ligand (Wang et al, 1999), tumor necrosis factor-α (Wang et al., 2000) or bleomycin (Li et al., 2003) a induce expression of angiotensinogen mRNA and protein and its cleavage to the peptide Angiotensin II. They also showed that bleomycin induces fibrosis through induction of alveolar epithelial cell apoptosis (Wang et al., 2000). They found that the ACE inhibitor captopril and the broad-spectrum caspase inhibitor ZVAD-fmk were able to reduce bleomycin-induced apoptosis (Figure 2.1). Moreover, captopril and ZVAD-fmk also

prevented the bleomycin-induced accumulation of collagen (Figure 2.2). Together, these results indicate that the induction of apoptosis is required for bleomycin-induced fibrosis and that ANG II and caspases play an important role in this pathway.

In another study, our lab demonstrated that bleomycin-induced apoptosis of alveolar epithelial cells requires ANG II synthesis de novo. Li et al. showed that bleomycin-induced nuclei fragmentation was blocked by ZVAD-fmk, captopril and the nonselective ANG receptor antagonist saralasin (Figure 2.3). In addition, they also demonstrated that bleomycin-induced apoptosis of primary AECs was blocked by the caspase-3-selective blocker DEVD-fmk and the $AT_1$ receptor selective blocker losartan (Figure 2.4), suggesting that $AT_1$ receptor mediates bleomycin-induced apoptosis as it does ANGII-induced AEC apoptosis. Together, these data indicates that the production of ANGII is a necessary event in bleomycin-induced AEC apoptosis.

Bleomycin induces alveolar cell apoptosis through the JNK-dependent activation of the mitochondrial death pathway (Lee et al., 2005). Bleomycin-induced Bax activation was inhibited by the expression of a dominant negative JNK in MLE-12 cells (Lee et al., 2005). Additionally, recent evidence show that both bleomycin and ANGII induces caspase 9 activation, suggesting that ANG II, just like bleomycin, also induces apoptosis through the mitochondrial death pathway requiring the activation of phosphorylation of JNK (Figure 2.5).

Angiotensin converting enzyme 2(ACE-2) degrades ANG II into the heptapeptide ANG 1-7 (Figure 2.6). In other organ systems, ANG1-7 was found to prevent some of the actions of ANGII through the ANG1-7 receptor *mas* (Gava et al., 2009; Lara et al., 2010; Tallant et al, 2005). Precisely which lung cell types express active *mas* signaling is

currently unknown. This study reports evidence that ANG1-7 in the lung acts through the ANG1-7 receptor *mas* to inhibit ANGII signaling and AEC apoptosis.

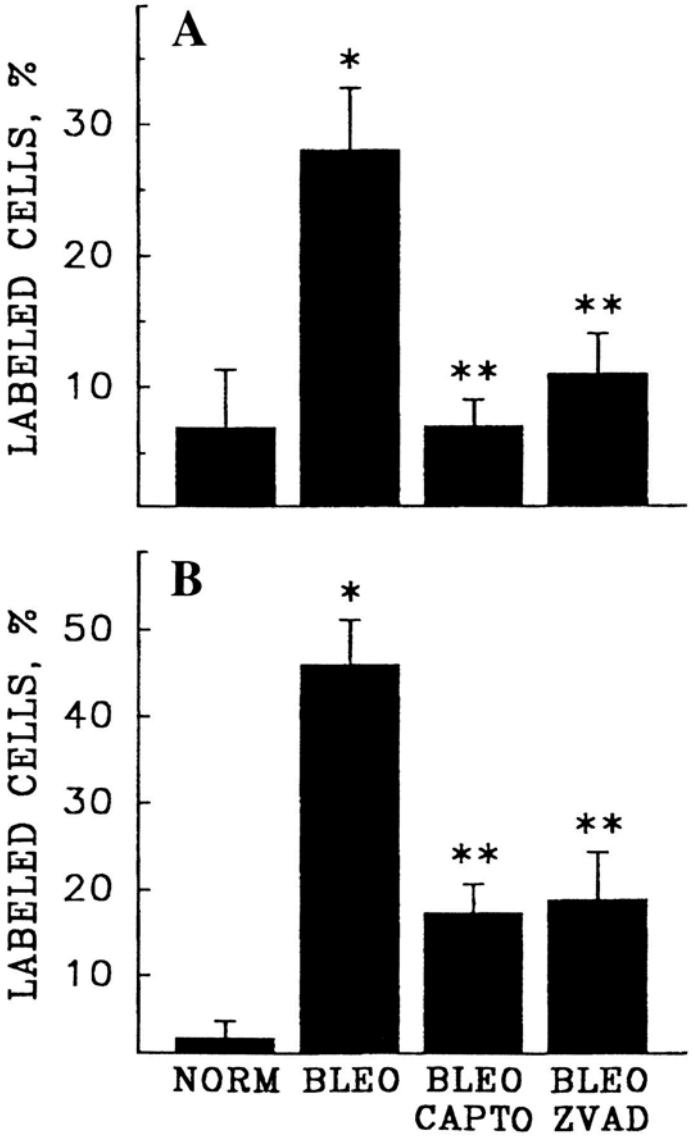


**Figure 2.1**.

**Quantitation of ISEL-positive nuclei in alveolar (A) and airway (B) epithelial cells after Bleo, CAPTO, and ZVAD-fmk administration**. NORM, animals exposed to vehicle only. ISEL-positive nuclei were scored as a percentage of the total epithelial nuclei within the alveolar and airway epithelial cell populations. See methods for details. * $P < 0.01$ vs. NORM. ** $P < 0.01$ vs. Bleo (both by ANOVA and Student-Newman-Keuls test). (Adapted from Wang et al., 2000)

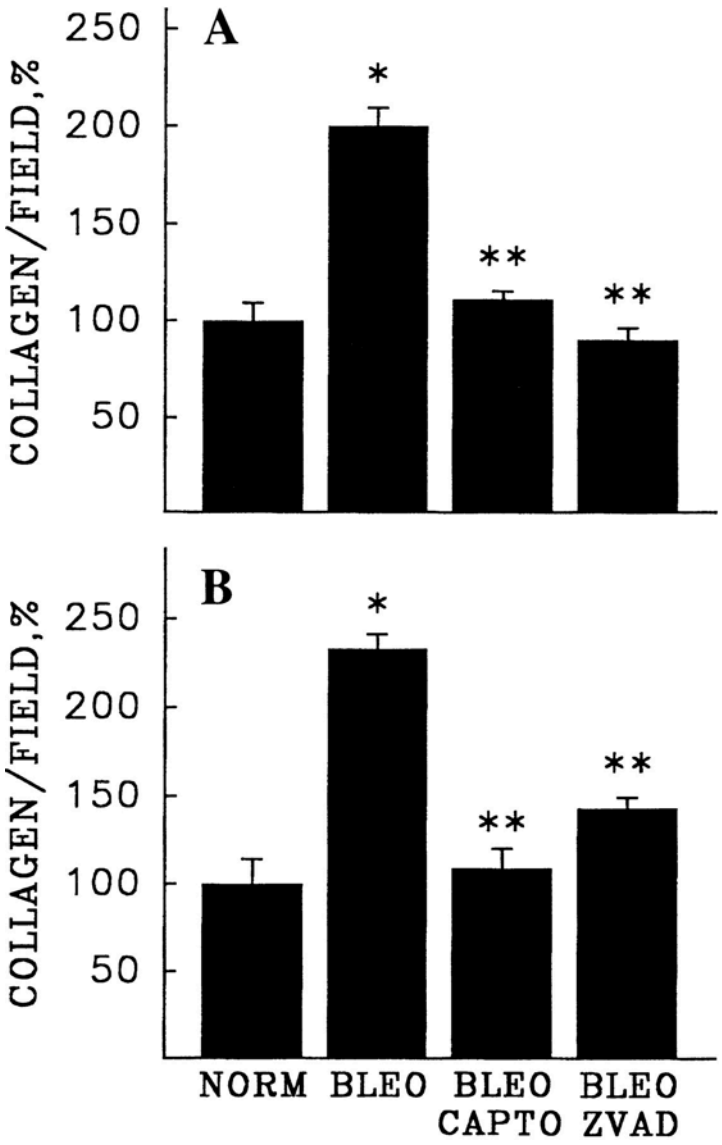


**Figure 2.2**.

**Quantitation of collagen by PR staining of alveolar (A) and peribronchial (B) lung parenchyma**. Collagen was quantitated from polarized light images as the total number of white pixels per unit area within the alveolar and peribronchial lung parenchyma. See methods for details. * $P < 0.01$ vs. NORM. ** $P < 0.01$ vs. Bleo (both by ANOVA and Student-Newman-Keuls test). (Adapted from Wang et al., 2000)

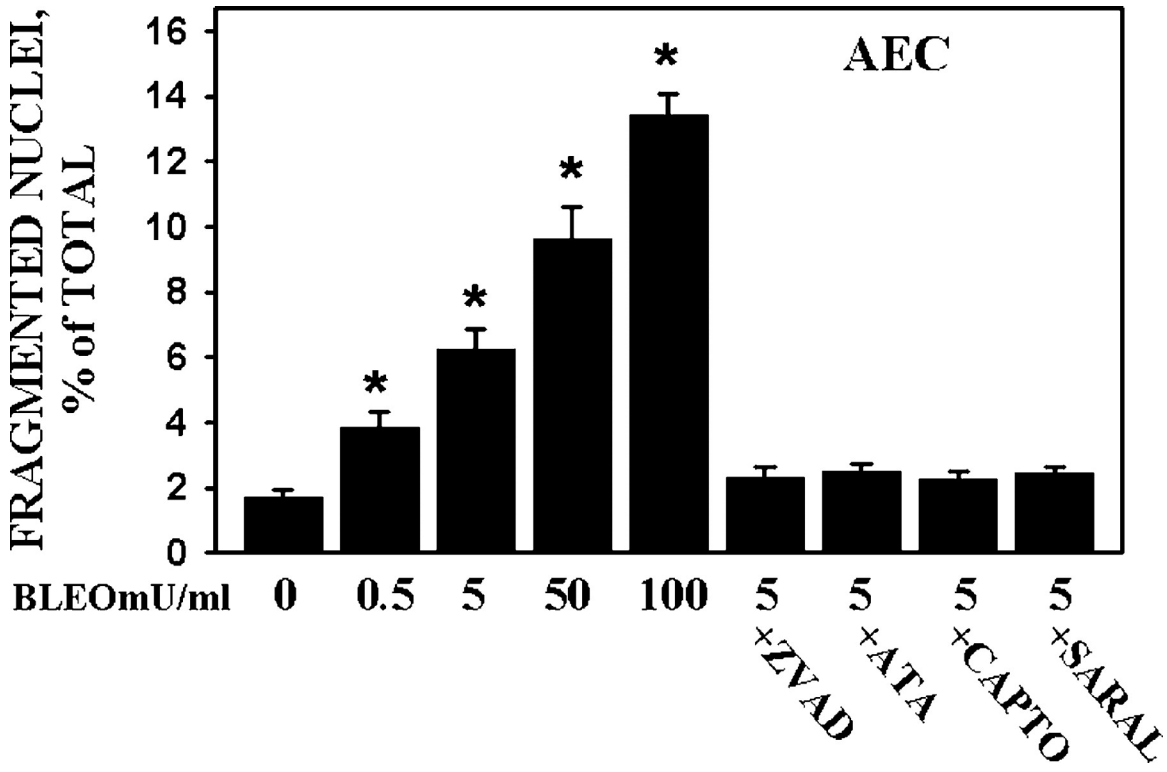


**Figure 2.3**.

**Dose-dependent induction of nuclear fragmentation by 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 (seematerials and methods). Putative inhibitors were added 30 min before addition of Bleo; nuclear fragmentation was scored as described in Fig. 1 B and materials and methods. ZVAD, N-benzylcarboxy-Val-Ala-Asp-[O-Me]-CH2F (60 μM); ATA, aurintricarboxylic acid (10 μM); Capto, captopril (500 ng/ml); Saral, saralasin (50 μg/ml). Bars are the means ± SE of at least 4 observations; *P < 0.05 vs. control (0.0 Bleo). (Adapted from Li et al., 2002)

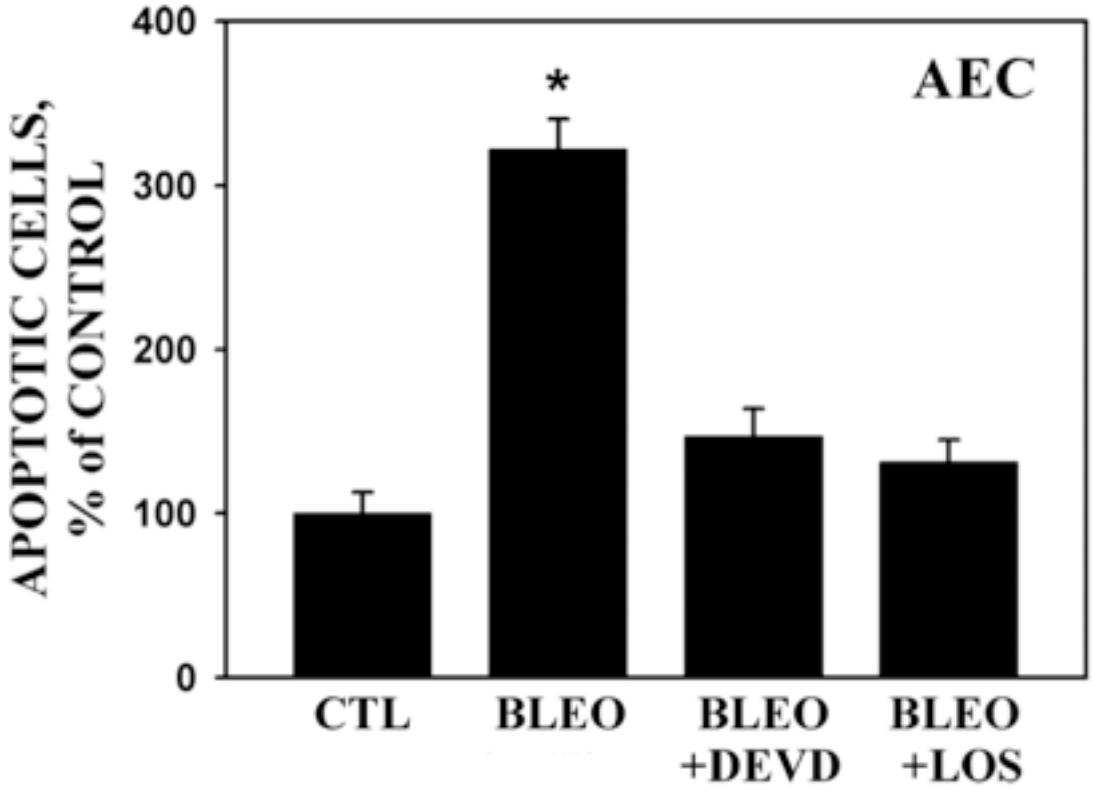


**Figure 2.4**.

**Blockade of Bleo-induced apoptosis in primary AECs by selective caspase or ANG receptor blockers**. Rat AECs were isolated and challenged with 25 mU/ml Bleo alone or in the presence of the caspase-3-selective inhibitor Asp-Glu-Val-Asp-[O-Me]-CH2F (DEVD-fmk, 60 μM) or the ANG receptor AT1-selective antagonist losartan (Los, 10−6 M). Control cultures (Ctl) received blocker vehicles only. Nuclear fragmentation was scored as described in Fig. 1 andmaterials and methods. Bars are means ± SE of at least 4 observations; *P < 0.05 vs. control. (Adapted from Li et al., 2002)

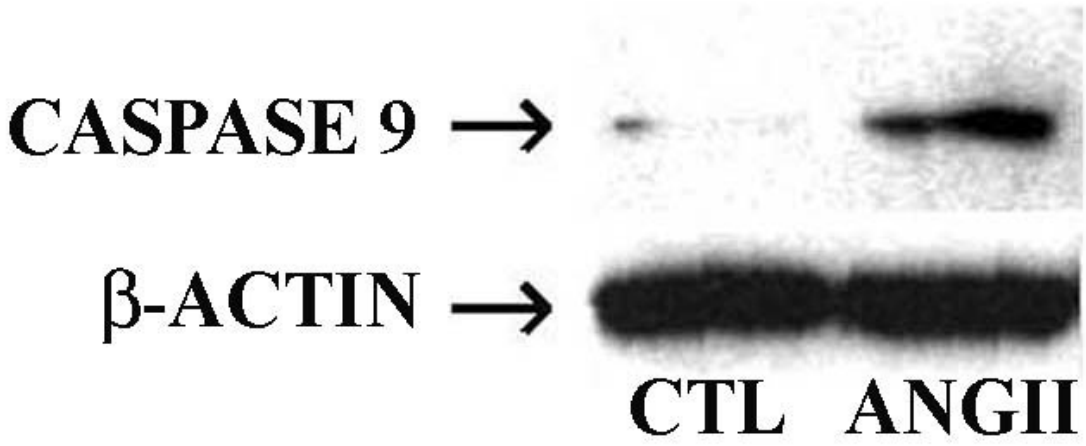


**Figure 2.5**.

**ANGII induces activation of caspase-9**. Primary cultures of rat type II alveolar epithelial cells were cultured in serum-free medium for 6 hours with or without ANGII. Cells were harvested with NP40 buffer for Western blotting. (Xiaopeng Li, 2006)

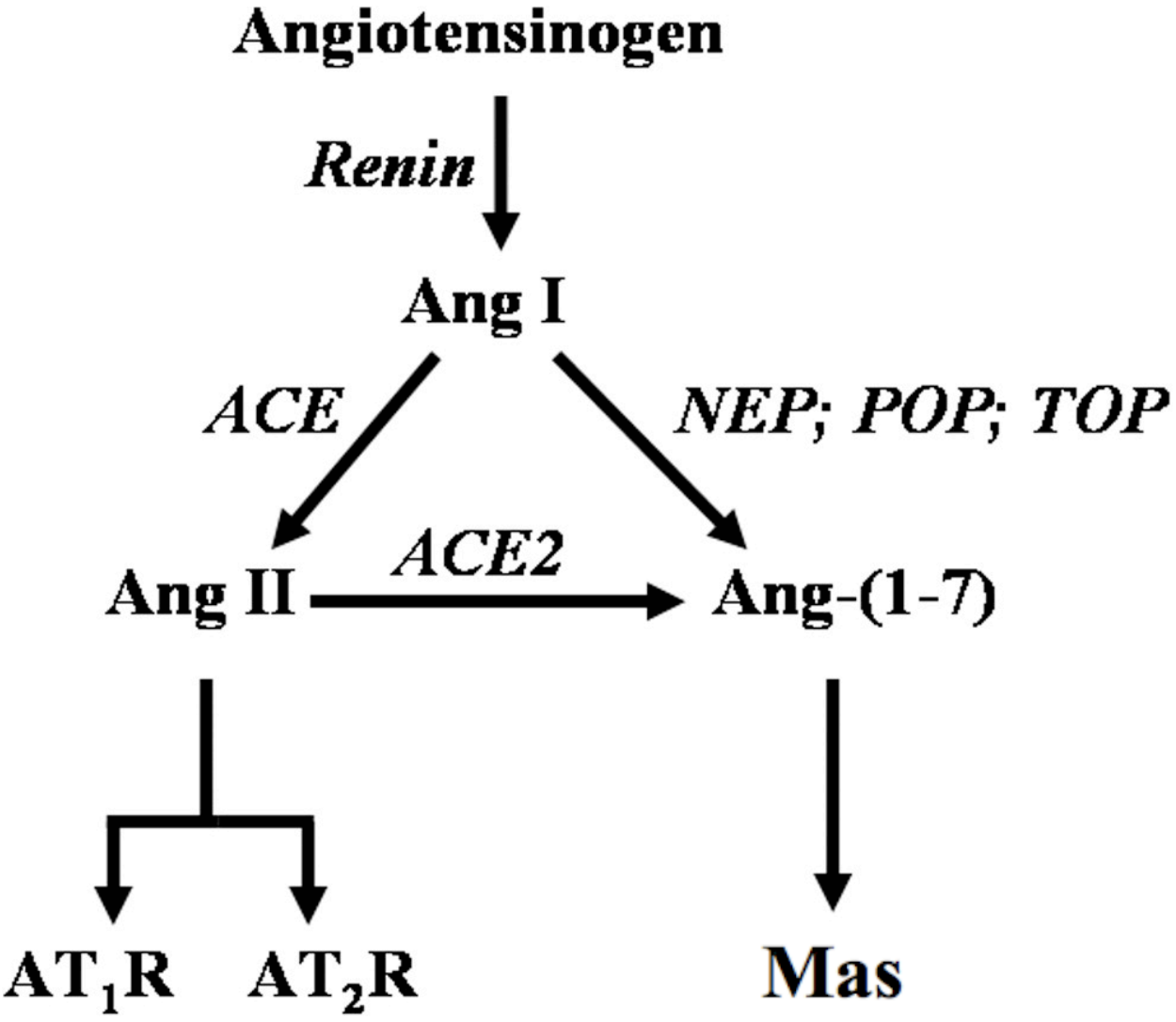


**Figure 2.6**.

**Pathways for the formation of ANG 1-7**. Renin cleaves angiotensinogen into the 10-aa peptide ANG I. ANG I is then converted to angiotensin II by ACE or to ANG 1-7 by neprilysin (NEP), prolyl-endopeptidase (POP) or thimet oliogopeptidase (TOP). ANG 1-7 can also be generated from ANG II through ACE2. ANG II can activate either the $AT_1$ subtype of angiotensin receptor or the $AT_2$ ANG II subtype receptor. ANG 1-7 activates the Mas receptor.

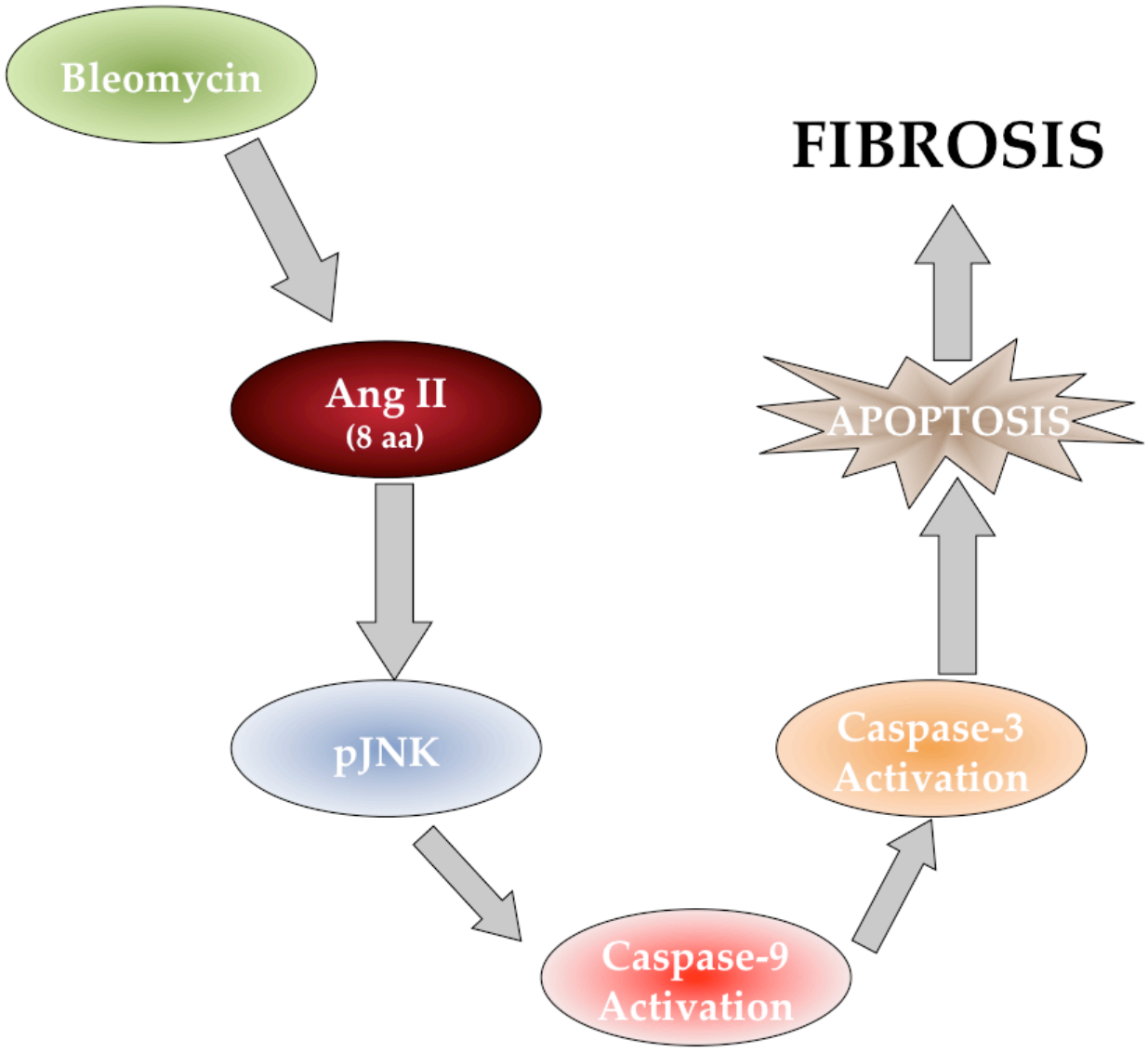


**Figure 2.7**.

**Summary of Earlier Published Data.** For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

## METHODS

### Cell culture

The mouse lung epithelial MLE-12 cell line was a gift from the laboratory of Dr. Jeffrey Whitsett, University of Cincinnati, OH, and was grown in complete HITES medium for MLE12 supplemented with 2% fetal bovine serum according to ATCC's guidelines. All cells were grown in 12-, 24-, or 6-well chambers and were analyzed at subconfluent densities of 50-80%. 12 hours before treatment, cells were switched from growth medium containing fetal bovine serum to serum-free medium. All subsequent incubations with bleomycin and/or other test agents were performed in serum free medium. In all experiments, cells were treated with inhibitors or antagonists 30 minutes prior to treatment with bleomycin or ANGII. For exposure of extended periods, cells were exposed to test agents as described and after one hour, culture media were replaced with new media containing fresh A779 and ANG1-7 but not bleomycin. Due to the low biological half-lives of the A779 and ANG1-7 peptides, they were replaced every 2 hours until cells were harvested. Primary human alveolar epithelial cells isolated from human lung tissue were obtained from ScienCell Research Laboratories. They were cultured in Alveolar Epithelial Cell Medium according to ScienCell Research Laboratories guidelines.

### Detection of Apoptosis

Apoptotic cells were detected by nuclear fragmentation with propidium iodide (PI) after enzymatic digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5μg/ml PI. During fixation with 70% ethanol, detached cells were retained by centrifugation of the 24-well or 12-well culture plates. Cells with discrete nuclear

fragmentation containing condensed chromatin were scored as apoptotic. Apoptotic cells were scored over four separate microscopic fields from each of four culture wells per treatment group. The active forms of caspase-3 and caspase-9 were detected by Western blotting using antibodies specific for the active cleaved forms.

**Western Blotting**

For caspase-3 and caspase-9 detections, cells were harvested and lysed in an NP40-based lysis buffer containing protease inhibitors. For detection of phosphor-proteins, cells were harvested and lysed with a modified lysis buffer containing 50mM HEPES, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1uM EGTA, 1.5mM MgCl2, phosphatase inhibitor cocktail (Phospho Stop, Roche, Nutley, NJ) and the protease inhibitor cocktail (Complete Mini, Roche, Nutley, NJ). Proteins were separated on polyacrylamide gels and transferred to PVDF membranes. Immunoreactive bands were visualized by ECL detection systems (Thermo Scientific, Rockford, IL).

**RESULTS**

Earlier results from this lab, as summarized in figure 2.7, proposes that pulmonary fibrosis induced by bleomycin begins with an increased production of ANG II. ANG II then induces JNK phosphorylation and downstream activation of caspase 9 and 3. The signaling eventually leads to apoptosis of the alveolar epithelial cells followed by fibrosis. In this study, we wanted to investigate how Angiotensin 1-7 fits in to this pathway and what role it plays in the regulation of AEC apoptosis.

To begin examining the hypothesis that ANG1-7 acts to oppose the actions of ANG II, we assessed the effect of ANG II on JNK phosphorylation in the presence and absence of purified ANG1-7 in serum-free media. JNK phosphorylation has previously been shown to be required for apoptosis in alveolar epithelial cells (Lee et al., 2005). Figure 2.8 shows that 5 minutes of exposure to ANG II ($10^{-7}$ M) also induces JNK phosphorylation. 30 minute pre-incubation with ANG1-7 ($10^{-7}$ M) prevented the ANG II-induced increase in JNK phosphorylation, indicating that ANG1-7 is able to block the actions of ANG II at equimolar concentrations. Moreover, incubation with the specific *mas* receptor blocker A779 blocked the effects of ANG1-7. This indicates that ANG1-7 is able to signal through its receptor *mas* to prevent ANGII induced JNK phosphorylation.

In a previous study, it was shown that bleomycin-induced apoptosis of AECs requires autocrine synthesis of ANGII and its subsequent binding to ANG receptor $AT_1$ **(**Li et al., 2003). Given that ANG1-7 inhibited ANG II induced JNK phosphorylation (Figure 2.8), we theorized that it may also block the JNK phosphorylation that is required in bleomycin-stimulated JNK phosphorylation. A 30-minute pretreatment with ANG1-7 **(**$10^{-7}$ M) prevented the increase in p-JNK observed 5 minutes after exposure of MLE-12 cells to

purified bleomycin ($10^{-7}$ M) (Figure 2.8). We then tested bleomycin-induced JNK phosphorylation again with a 30-minute pretreatment of the *mas* antagonist A-779 (D-Ala$^7$-Ang1-7, $10^{-7}$ M). The A779 pretreatment prevented the pJNK inhibition by ANG1-7 (Figure 2.9), suggesting that ANG1-7 prevents bleomycin-induced JNK phosphorylation through the receptor *mas.*

Since ANG1-7 prevented both ANG II- and bleomycin-induced JNK phosphorylation, and JNK phosphorylation is a required event in bleomycin-induced apoptosis, we then examined if ANG1-7 might also prevent AEC apoptosis. In Figure 2.10, preincubation with ANG1-7 blocked the bleomycin-induced activation of caspase-3 in MLE-12 cells. Figure 2.11 shows that ANG1-7 could also prevent bleomycin-induced caspase-9 activation in primary cultures of human alveolar epithelial cells and that the *mas* antagonist A779 prevents the action of ANG1-7, confirming that bleomycin induces apoptosis through the mitochondrial pathway as previously established. Furthermore, Figure 2.12 shows that ANG1-7 prevented the bleomycin-induced increase in nuclear fragmentation detected in MLE-12 cells after 12 hours exposure to bleomycin ($p<0.01$). Preincubation with the *mas* antagonist A779 prevented the effect exerted by ANG1-7. It was previously demonstrated that bleomycin-induced AEC apoptosis involves the release of cytochrome c, indicating that apoptosis is occurring versus necrosis. In Figure 2.13A, MLE-12 cells were treated with 25mU/ml bleomycin for 20 hours and stained with active caspase-3 antibodies. The results further confirm the activities of apoptosis. Figure 2.13 also shows that ANG1-7 could also prevent bleomycin-induced nuclear fragmentation in primary cultures of human alveolar epithelial cells ($p<0.01$). As seen in MLE-12 cells, the blockade of nuclear fragmentation by ANG1-7 was prevented by A779. Both nuclear fragmentation and

caspase-3 activation are markers of apoptosis. Figure 2.14 provides a brief summary of the results of this study and related earlier works, along with topics for future research.

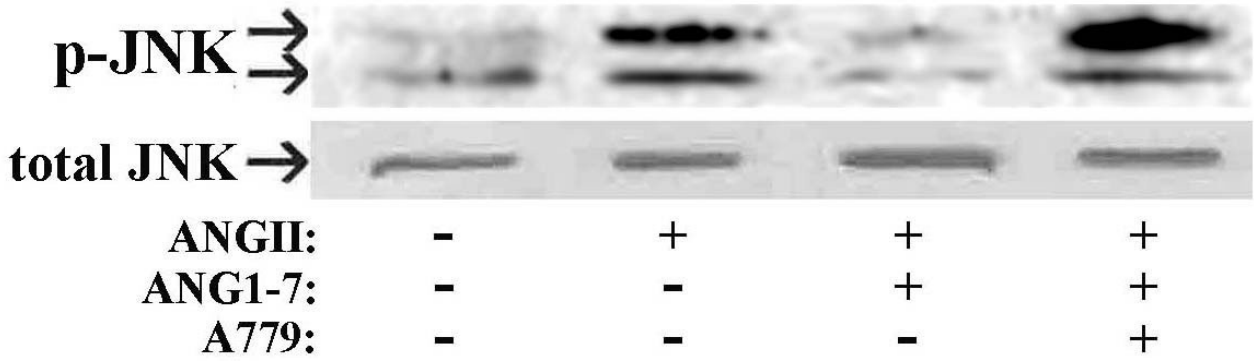


**Figure 2.8**.

**ANG1-7 inhibits ANG II induced JNK phosphorylation through the *mas* receptor**. MLE-12 cells in serum free conditions were preincubated with specific *mas* antagonist A779 $10^{-7}$ M for 30 minutes followed by incubation in $10^{-7}$ M ANG1-7 for an additional 30 minutes. Afterwards, cells were treated with 5mU/ml ANG II for 10 minutes and harvested for Western blotting.

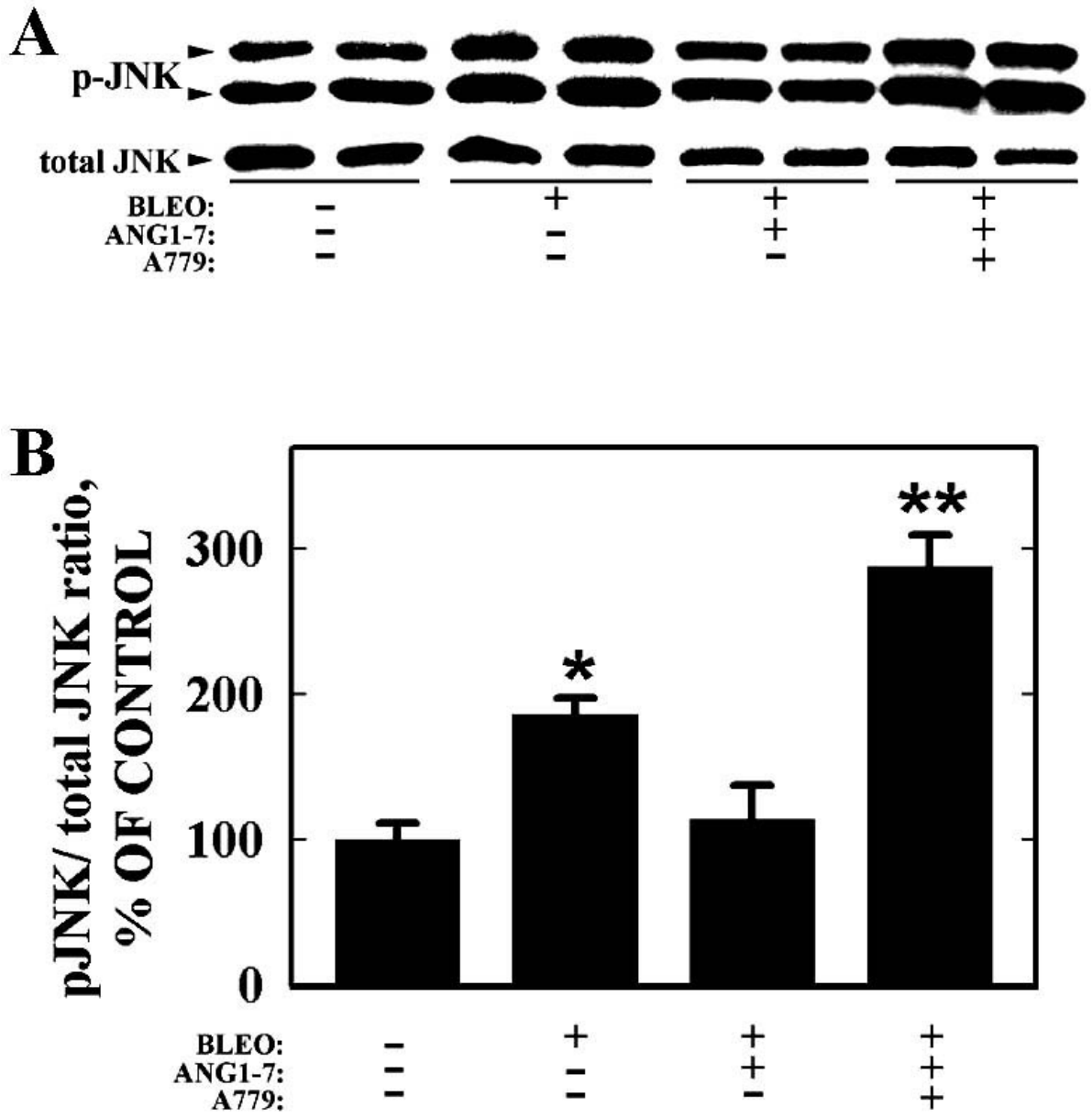


**Figure 2.9**.

**ANG1-7 inhibits BLEO induced JNK phosphorylation through the *mas* receptor**. A: MLE-12 cells in serum free conditions were preincubated with specific *mas* antagonist A779 $10^{-7}$ M for 30 minutes followed by incubation in $10^{-7}$ M ANG1-7 for an additional 30 minutes. Afterwards, cells were treated with 5mU/ml bleomycin (BLEO) for 5 minutes and harvested for Western blotting. B: Ratio of the densitometric quantification of the Western blot performed in Panel A and the Western blot of total JNK of the same conditions (data not shown). Bars are the means. *: $p<0.05$ vs. CTRL and **: $p<0.01$ vs CTRL by ANOVA and Student-Newman-Keul's post-hoc analysis.

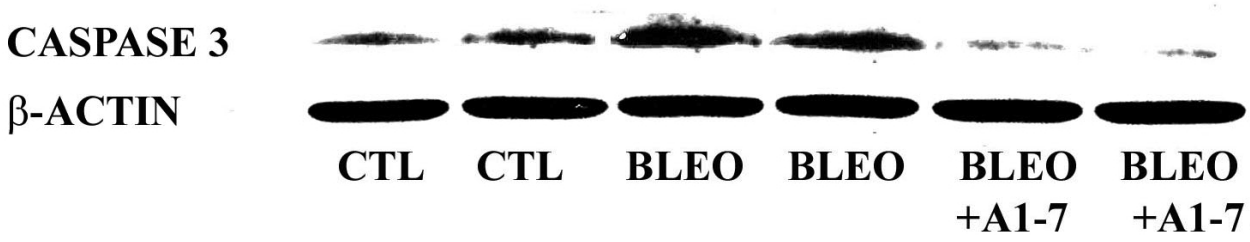


**Figure 2.10**.

**ANG1-7 inhibits BLEO induced caspase-3 activation of MLE-12 cells**. MLE-12 cells in serum free conditions were preincubated in $10^{-7}$ M ANG1-7 for 30 minutes. Afterwards, cells were treated with 5mU/ml bleomycin (BLEO) for 2 hours and harvested for Western blotting.

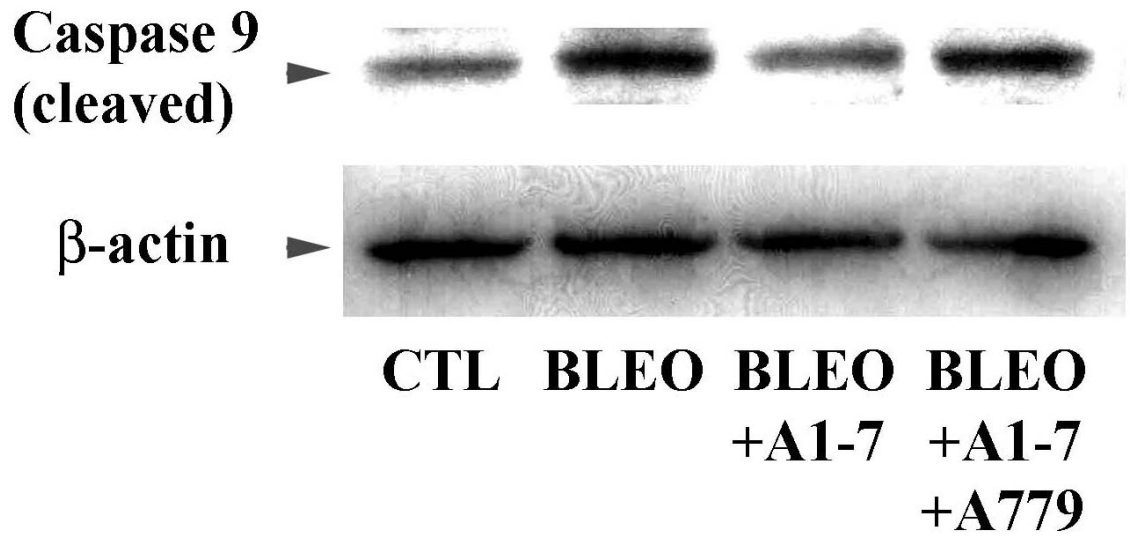


**Figure 2.11**.

**ANG1-7 inhibits BLEO induced caspase-9 activation of primary AECs through the *mas* receptor**. Primary cultures of human alveolar epithelial cells were preincubated with the specific *mas* antagonist A779 ($10^{-7}$ M) for 30 minutes followed by ANG1-7 ($10^{-7}$ M) for an additional 30 minutes. Thereafter the cells were exposed to bleomycin (BLEO, 5mU/ml) for an additional 6 hours.

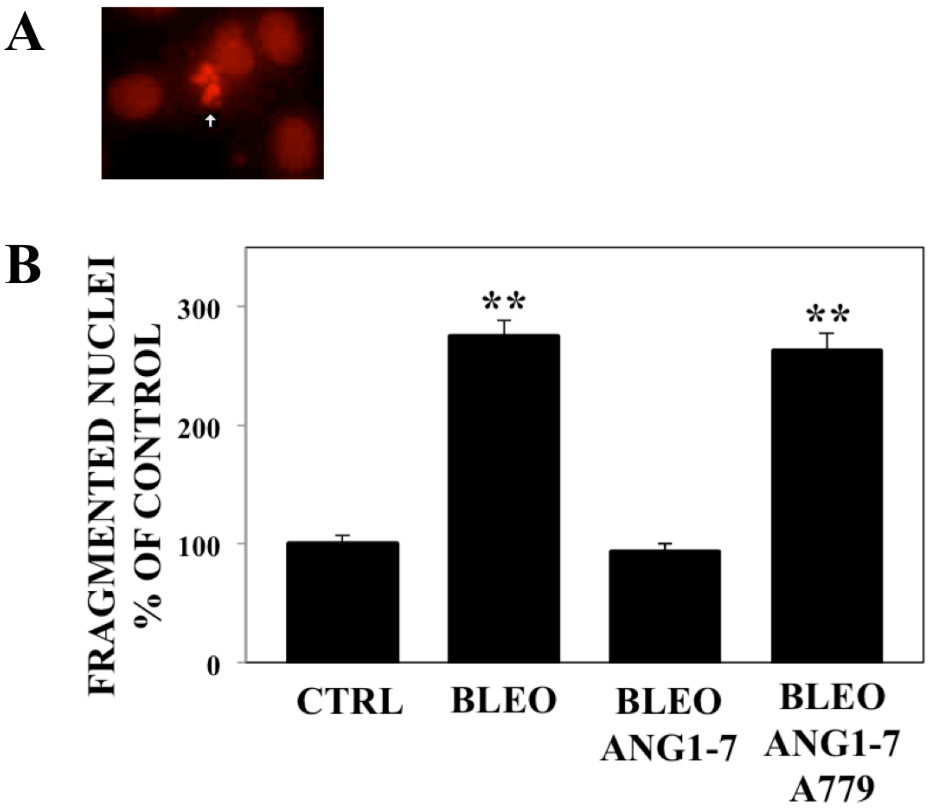


**Figure 2.12**.

**ANG1-7 inhibits bleomycin-induced nuclear fragmentation through the *mas* receptor in MLE-12 cells**. A: Primary cultures of AECs fixed in 70% ethanol and stained with propidium iodide. Arrow points to cells that exhibit chromatin condensation and nuclear fragmentation. B. MLE-12 cells in serum free conditions were preincubated with specific *mas* antagonist A779 $10^{-7}$ M for 30 minutes followed by incubation in $10^{-7}$ M ANG1-7 for an additional 30 minutes. Afterwards, cells were treated with 5mU/ml bleomycin (BLEO) for 12 hours. A779 and ANG1-7 were replaced every 2 hours. Cells were then harvested for detection of nuclear fragmentation. Absolute value for basal (CTRL) nuclear fragmentation was 3.30 ± 0.25%. Bars represent the means ± S.E.M. of four separate cell cultures. **: $p<0.001$ vs. CTRL by ANOVA and Student-Newman-Keul's post-hoc analysis.

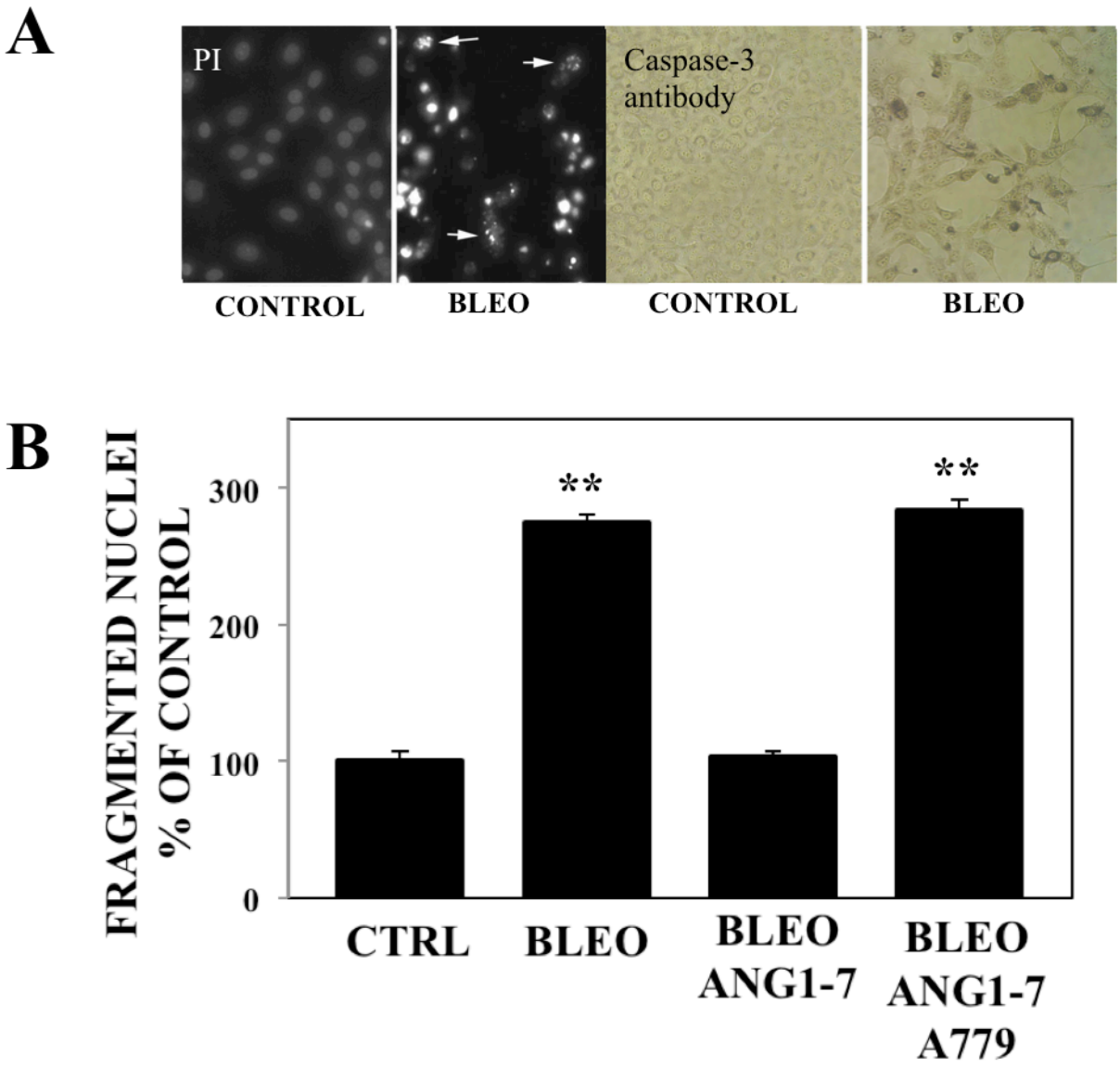


**Figure 2.13**.
**ANG1-7 inhibits bleomycin-induced nuclear fragmentation through the *mas* receptor in primary human AECs**. A. Cells treated with 25 mU/ml bleomycin for 20 hrs stained with PI or with active caspase-3 antibody. Arrows indicate fragmented nuclei with condensed chromatin (bright fluorescence). B. Primary human alveolar epithelial cells in serum free conditions were preincubated with specific *mas* antagonist A779 $10^{-7}$ M for 30 minutes followed by incubation in $10^{-7}$ M ANG1-7 for an additional 30 minutes. Afterwards, cells were treated $10^{-7}$with 5mU/ml bleomycin (BLEO) for 12 hours. A779 and ANG1-7 were replaced every 2 hours. Cells were then harvested for detection of nuclear fragmentation. Absolute value for basal (CTRL) nuclear fragmentation was 3.11 ± 0.21%. Bars represent the means ± S.E.M. of four separate cell cultures. **: $p<0.001$ vs. CTRL by ANOVA and Student-Newman-Keul's post-hoc analysis.

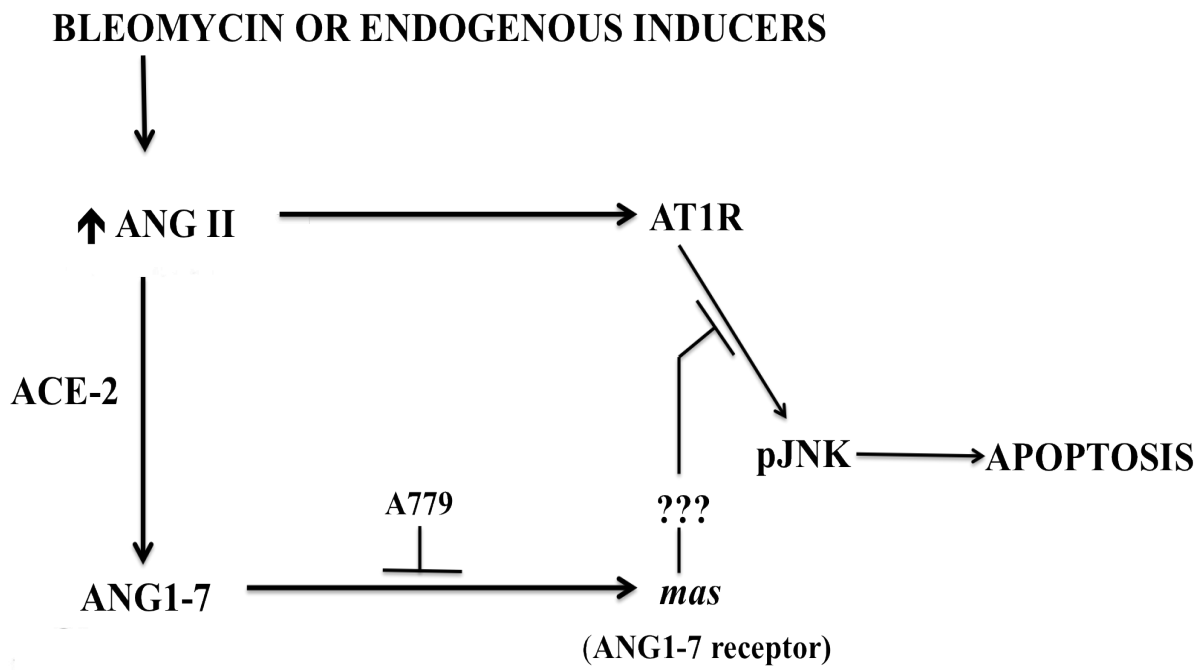


**Figure 2.14**.

**Summary**. The autocrine production of ANGII is required for AEC apoptosis and can be stimulated by bleomycin or the endogenous proapoptotic inducers Fas ligand or TNF-α. Apoptosis signaling proceeds through the ANG receptor $AT_1$ and JNK phosphorylation (pJNK). ANG 1-7 inhibits JNK phosphorylation and apoptosis through binding to the specific ANG 1-7 receptor *mas*. The mechanisms of how activation of *mas* leads to inhibition of JNK phosphorylation are currently unknown and may be topics for future inquiry.

## DISCUSSION

Recent studies have established a new regulatory axis in the renin-angiotensin system (RAS). Much attention has been paid to studying the role of the ACE-2/ANG1-7/mas axis in organ injury and fibrogenesis. The heptapeptide ANG1-7, has been shown to oppose the vasoconstrictive, proliferative, and profibrotic actions of ANG II in a number of tissues (Ferrario et al., 2005; Ferrario 2010). ANG1-7 can be generated through cleavage by a variety of enzymes including neprilysin, prolyl-endopeptidase, and thimet oliogopeptidase (Gallager and Tallant, 2004). The primary pathway of ANG 1-7 synthesis is through ACE2 degradation of ANG II, in which ACE2 removes the ANG II C-terminal phenylalanine to produce ANG1-7 (Li et al., 2008). Figure 2.6 illustrates the cleavage pathway of AGT into ANG 1-7. Studies of the adult mouse lung indicate that the type II pneumocytes are the only cells to express ACE2 in the lungs (Wiener et al., 2007).

The heptapeptide ANG1-7 can bind to the receptor *mas*, a seven trans-membrane protein with domains containing sequences characteristic of G protein-coupled receptors encoded by the oncogene of the same name (Young et al., 1988). Some suggested that ANG1-7 may also bind to the $AT_1$ receptor (Zisman, 2005). Binding data, however, reveal low affinity of ANG1-7 for $AT_1$ or $AT_2$ receptors (Fontes et al., 1994). Currently there is very little data regarding the functions of ANG1-7 in apoptosis and its role in the regulation of organ systems. In the liver, ACE-2 and ANG1-7 may be protective against the development of liver fibrosis (Warner et al., 2007), the molecular and cellular mechanisms of which are still unclear.

Evidence from numerous cell systems demonstrates the ability of ANG1-7 to inhibit the effects of ANG II. For example, in the heart, ANG 1-7 prevents the development of

cardiac fibrosis induced by ANG II infusion (Grobe et al., 2007), and attenuates the ANG II-stimulated production of endothelin-1 and leukaemia inhibitory factor in cardiac fibroblasts (Iwata et al., 2005). In the rat proximal tubular cells, ANG 1-7 inhibited ANG II-induced phosphorylation of MAPKs including p38, ERK1/2, and JNK while also partially blocking activation of TGF-β1 promoted by ANG II (Su et al., 2006). Also in the kidney, ANG1-7 was found to reverse ANG II-induced stimulated $Na^+$-ATPase (Lara et al., 2010). In all of these systems, ANG 1-7 blockade of ANG II actions was inhibited by a knockdown of *mas* or the application of the specific *mas* receptor blocker A779.

Consistent with these results from other experimental systems, Figure 2.8 demonstrates the ability of ANG 1-7 to prevent JNK phosphorylation stimulated by ANG II in alveolar epithelial cells. In addition, Figure 2.12 indicates that ANG 1-7 also prevents apoptosis induced by bleomycin. The finding that bleomycin activates caspase-9 (Figure 2.11) confirms that bleomycin also induces apoptosis through the mitochondrial pathway and that this induction is prevented by A1-7, whose effects, in turn, were blocked by A779. Together, our data reinforces the previously established concept that autocrine production of ANG II and its binding to the $AT_1$ receptor is required in bleomycin-induced apoptosis of alveolar epithelial cells. As seen in Figure 2.12 and 2.13, the *mas* antagonist A779 was able to block the actions of ANG1-7 in both the MLE cell line and primary human AECs. This suggests that in alveolar epithelial cells, the ability of ANG1-7 to inhibit ANG II actions is mediated through the *mas* receptor, just as in other cell types studied thus far.

It is still unclear as to how intracellular signaling through the *mas* receptor can counteract the effects of ANG II. Evidence indicate that factors such as Akt phosphorylation, protein kinase C (PKC) activation and MAPK inhibition may play a role

in this pathway. In adult ventricular myocytes and endothelial cells, ANG 1-7 causes NO synthase phosphorylation and release through the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt)– dependent pathway (Sampaio et al., 2007; Dias-Peixoto et al., 2008). This was effectively prevented by the *mas* antagonist A779 or knockout of the *mas* gene. Moreover, it was found in rat extracardiac tissues that ANG 1-7 induces the *in vivo* phosphorylation of Akt by phosphorylating at threonine 308 and serine 473 (Muñoz et al., 2010). Studies with isolated perfused rat atria also showed ANG 1-7 to be exerting its effects through the mas/ PI3K/Akt pathway (Shah et al., 2010). In addition, they also found ANG 1-7 to activate the $Na^+/H^+$ exchanger-1 and CaMKII through the *mas* receptor. However, little is known about how these mechanisms interact to antagonize the signaling of ANG II in the cell. It was previously shown that PKC expression is required for ANG II-stimulated AEC apoptosis in primary cultures (Papp et al., 2002). Recent evidence reveals that in renal tubule cells, ANG1-7 signals via the *mas* receptor to inhibit ANG II-stimulated $Na^+$-ATPase activity by reversing PKC activation through a Gs/PKA pathway (Lara et al., 2010). Future studies focusing on the cAMP/PKA pathways in AECs might further elucidate the mechanism by which ANG1-7 inhibits ANG II signaling.

In summary, this study demonstrated that ANG1-7 acts through the receptor *mas* to block phosphorylation of JNK stimulated by either ANG II or bleomycin. ANG 1-7 treatment also inhibited bleomycin-induced apoptosis of alveolar epithelial cells, in agreement with the previously established requirement of JNK phosphorylation in AEC apoptosis. Together, the data demonstrates that ANG 1-7, produced from ANG II by ACE2, acts through the *mas* receptor to play a key role in the regulation of alveolar epithelial cell survival by preventing both JNK phosphorylation and subsequent apoptosis.

# REFERENCES

American Thoracic Society Idiopathic pulmonary fibrosis: diagnosis and treatment International consensus statement American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med. 2000 Feb; 161 (2 Pt 1) :646-64.

Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell. 2002 Feb; 9 (2) :423-32.

Adamson IY, Young L, Bowden DH. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. Am J Pathol. 1988 Feb; 130 (2) :377-83.

American Thoracic Society, European Respiratory Society. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. Am J Respir Crit Care Med. 2002 Jan 15; 165 (2) :277-304.

Antoniades HN, Bravo MA, Avila RE, Galanopoulos T, Neville-Golden J, Maxwell M, Selman M. Platelet-derived growth factor in idiopathic pulmonary fibrosis. J Clin Invest. 1990 Oct; 86 (4) :1055-64.

Antoniou KM, Pataka A, Bouros D, Siafakas NM. Pathogenetic pathways and novel pharmacotherapeutic targets in idiopathic pulmonary fibrosis. Pulm Pharmacol Ther. 2007; 20 (5) :453-61

Bajwa EK, Ayas NT, Schulzer M, Mak E, Ryu JH, Malhotra A. Interferon-gamma1b therapy in idiopathic pulmonary fibrosis: a metaanalysis. Chest. 2005 Jul; 128 (1) :203-6.

Barbas-Filho JV, Ferreira MA, Sesso A, Kairalla RA, Carvalho CR, Capelozzi VL. Evidence of type II pneumocyte apoptosis in the pathogenesis of idiopathic pulmonary fibrosis (IFP)/usual interstitial pneumonia (UIP). J Clin Pathol. 2001 Feb; 54 (2) :132-8.

Bargout R, Jankov A, Dincer E, Wang R, Komodromos T, Ibarra-Sunga O, Filippatos G, Uhal BD. Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol. 2000 May; 278 (5) :L1039-44.

Battegay EJ, Raines EW, Colbert T, Ross R. TNF-alpha stimulation of fibroblast proliferation Dependence on platelet-derived growth factor (PDGF) secretion and alteration of PDGF receptor expression. J Immunol. 1995 Jun 1; 154 (11) :6040-7.

Blum RH, Carter SK, Agre K. A clinical review of bleomycin--a new antineoplastic agent. Cancer. 1973 Apr; 31 (4) :903-14.

Boggs SS, Sartiano GP, DeMezza A. Minimal bone marrow damage in mice given bleomycin. Cancer Res. 1974 Aug; 34 (8) :1938-42.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol. 1999; 15:269-90.

Budinger GR, Mutlu GM, Eisenbart J, Fuller AC, Bellmeyer AA, Baker CM, Wilson M, Ridge K, Barrett TA, Lee VY, Chandel NS. Proapoptotic Bid is required for pulmonary fibrosis. Proc Natl Acad Sci U S A. 2006 Mar 21; 103 (12) :4604-9.

Bullock GR, Steyaert I, Bilbe G, Carey RM, Kips J, De Paepe B, Pauwels R, Praet M, Siragy HM, de Gasparo M. Distribution of type-1 and type-2 angiotensin receptors in the normal human lung and in lungs from patients with chronic obstructive pulmonary disease. Histochem Cell Biol. 2001 Feb; 115 (2) :117-24.

Burkhardt A. Alveolitis and collapse in the pathogenesis of pulmonary fibrosis. Am Rev Respir Dis. 1989 Aug; 140 (2) :513-24.

Cantin AM, North SL, Fells GA, Hubbard RC, Crystal RG. Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis. J Clin Invest. 1987 Jun; 79 (6) :1665-73.

Chapman JT, Farver CF. (2004). Idiopathic interstitial lung disease: a review of recent classifications. Clin Pulm Med. 11: 17-24.

Cherniack RM, Colby TV, Flint A, Thurlbeck WM, Waldron J, Ackerson L, King TE Jr. Quantitative assessment of lung pathology in idiopathic pulmonary fibrosis The BAL Cooperative Group Steering Committee. Am Rev Respir Dis. 1991 Oct; 144 (4) :892-900.

Coultas DB, Zumwalt RE, Black WC, Sobonya RE. The epidemiology of interstitial lung diseases. Am J Respir Crit Care Med. 1994 Oct; 150 (4) :967-72.

Cryns V, Yuan J. Proteases to die for. Genes Dev. 1998 Jun 1; 12 (11) :1551-70.

de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology XXIII The angiotensin II receptors. Pharmacol Rev. 2000 Sep; 52 (3) :415-72.

Desmoulière A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol. 1995 Jan; 146 (1) :56-66.

Dias-Peixoto MF, Santos RA, Gomes ER, Alves MN, Almeida PW, Greco L, Rosa M, Fauler B, Bader M, Alenina N, Guatimosim S. Molecular mechanisms involved in the angiotensin-(1-7)/Mas signaling pathway in cardiomyocytes. Hypertension. 2008 Sep; 52 (3) :542-8.

Dlugi AM, Robie KM, Mitchell MS. Failure of bleomycin to affect humoral or cell-mediated immunity in the mouse. Cancer Res. 1974 Oct; 34 (10) :2504-7.

Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting

enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res. 2000 Sep 1; 87 (5) :E1-9.

Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 1999; 68:383-424.

Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J Clin Invest. 1996 Jan 1; 97 (1) :232-7.

Fan JM, Ng YY, Hill PA, Nikolic-Paterson DJ, Mu W, Atkins RC, Lan HY. Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. Kidney Int. 1999 Oct; 56 (4) :1455-67.

Ferrario CM. Angiotensin-converting enzyme 2 and angiotensin-(1-7): an evolving story in cardiovascular regulation. Hypertension. 2006 Mar; 47 (3) :515-21.

Ferrario CM. New physiological concepts of the renin-angiotensin system from the investigation of precursors and products of angiotensin I metabolism. Hypertension. 2010 Feb; 55 (2) :445-52.

Ferrario CM, Trask AJ, Jessup JA. Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. Am J Physiol Heart Circ Physiol. 2005 Dec; 289 (6) :H2281-90.

Finlay GA, Thannickal VJ, Fanburg BL, Paulson KE. Transforming growth factor-beta 1-induced activation of the ERK pathway/activator protein-1 in human lung fibroblasts requires the autocrine induction of basic fibroblast growth factor. J Biol Chem. 2000 Sep 8; 275 (36) :27650-6.

Fonseca C, Abraham D, Black CM. Lung fibrosis. Springer Semin Immunopathol. 1999; 21 (4) :453-74.

Fontes MA, Silva LC, Campagnole-Santos MJ, Khosla MC, Guertzenstein PG, Santos RA. Evidence that angiotensin-(1-7) plays a role in the central control of blood pressure at the ventro-lateral medulla acting through specific receptors. Brain Res. 1994 Nov 28; 665 (1) :175-80.

Fukuda Y, Ishizaki M, Kudoh S, Kitaichi M, Yamanaka N. Localization of matrix metalloproteinases-1, -2, and -9 and tissue inhibitor of metalloproteinase-2 in interstitial lung diseases. Lab Invest. 1998 Jun; 78 (6) :687-98.

Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia. 1971 May 15; 27 (5) :549-50.

Gallagher PE, Tallant EA. Inhibition of human lung cancer cell growth by angiotensin-(1-7). Carcinogenesis. 2004 Nov; 25 (11) :2045-52.

Gallin JI, Goldstein IM, et al. (1992) Inflammation: Basic principles and clinical correlates, Second edition, Raven press, Chapter 5: 991-997

Gava E, Samad-Zadeh A, Zimpelmann J, Bahramifarid N, Kitten GT, Santos RA, Touyz RM, Burns KD. Angiotensin-(1-7) activates a tyrosine phosphatase and inhibits glucose-induced signalling in proximal tubular cells. Nephrol Dial Transplant. 2009 Jun; 24 (6) :1766-73.

Giaid A, Michel RP, Stewart DJ, Sheppard M, Corrin B, Hamid Q. Expression of endothelin-1 in lungs of patients with cryptogenic fibrosing alveolitis. Lancet. 1993 Jun 19; 341 (8860) :1550-4.

Grobe JL, Mecca AP, Lingis M, Shenoy V, Bolton TA, Machado JM, Speth RC, Raizada MK, Katovich MJ. Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). Am J Physiol Heart Circ Physiol. 2007 Feb; 292 (2) :H736-42.

Gross TJ, Simon RH, Kelly CJ, Sitrin RG. Rat alveolar epithelial cells concomitantly express plasminogen activator inhibitor-1 and urokinase. Am J Physiol. 1991 Apr; 260 (4 Pt 1) :L286-95.

Grünert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol. 2003 Aug; 4 (8) :657-65.

Günther A, Mosavi P, Ruppert C, Heinemann S, Temmesfeld B, Velcovsky HG, Morr H, Grimminger F, Walmrath D, Seeger W. Enhanced tissue factor pathway activity and fibrin turnover in the alveolar compartment of patients with interstitial lung disease. Thromb Haemost. 2000 Jun; 83 (6) :853-60.

Hagimoto N, Kuwano K, Inoshima I, Yoshimi M, Nakamura N, Fujita M, Maeyama T, Hara N. TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. J Immunol. 2002 Jun 15; 168 (12) :6470-8.

Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, Hara N. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. Am J Respir Cell Mol Biol. 1997 Sep; 17 (3) :272-8.

Harmer D, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. FEBS Lett. 2002 Dec 4; 532 (1-2) :107-10.

Haschek WM, Witschi H. Pulmonary fibrosis--a possible mechanism. Toxicol Appl Pharmacol. 1979 Dec; 51 (3) :475-87.

Hasegawa T, Sorensen L, Dohi M, Rao NV, Hoidal JR, Marshall BC. Induction of urokinase-type plasminogen activator receptor by IL-1 beta. Am J Respir Cell Mol Biol. 1997 Jun; 16 (6) :683-92.

Hattori N, Sisson TH, Xu Y, Desai TJ, Simon RH. Participation of urokinase-type plasminogen activator receptor in the clearance of fibrin from the lung. Am J Physiol. 1999 Sep; 277 (3 Pt 1) :L573-9.

Hayashi T, Stetler-Stevenson WG, Fleming MV, Fishback N, Koss MN, Liotta LA, Ferrans VJ, Travis WD. Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis. Am J Pathol. 1996 Oct; 149 (4) :1241-56.

Heino J, Ignotz RA, Hemler ME, Crouse C, Massagué J. Regulation of cell adhesion receptors by transforming growth factor-beta Concomitant regulation of integrins that share a common beta 1 subunit. J Biol Chem. 1989 Jan 5; 264 (1) :380-8.

Huang B, Eberstadt M, Olejniczak ET, Meadows RP, Fesik SW. NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. Nature. 1996 Dec 19-26; 384 (6610) :638-41.

Hubbard R, Lewis S, Richards K, Johnston I, Britton J. Occupational exposure to metal or wood dust and aetiology of cryptogenic fibrosing alveolitis. Lancet. 1996 Feb 3; 347 (8997) :284-9.

Ichikawa T, Matsuda A, Miyamoto K, Tsubosaki M, Umezawa H. Biological studies on bleomycin A. J Antibiot (Tokyo). 1967 Jul; 20 (3) :149-55.

Ichikawa T, Nakano I, Hirokawa I. Bleomycin treatment of the tumors of penis and scrotum. J Urol. 1969 Dec; 102 (6) :699-707.

Ignotz RA, Massagué J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem. 1986 Mar 25; 261 (9) :4337-45.

Iwai K, Mori T, Yamada N, Yamaguchi M, Hosoda Y. Idiopathic pulmonary fibrosis Epidemiologic approaches to occupational exposure. Am J Respir Crit Care Med. 1994 Sep; 150 (3) :670-5.

Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest. 2002 Aug; 110 (3) :341-50.

Iwata M, Cowling RT, Gurantz D, Moore C, Zhang S, Yuan JX, Greenberg BH. Angiotensin-(1-7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. Am J Physiol Heart Circ Physiol. 2005 Dec; 289 (6) :H2356-63.

Johnston ID, Prescott RJ, Chalmers JC, Rudd RM. British Thoracic Society study of cryptogenic fibrosing alveolitis: current presentation and initial management Fibrosing Alveolitis Subcommittee of the Research Committee of the British Thoracic Society. Thorax. 1997 Jan; 52 (1) :38-44.

Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest. 2003 Dec; 112 (12) :1776-84.

Kang HR, Cho SJ, Lee CG, Homer RJ, Elias JA. Transforming growth factor (TGF)-beta1 stimulates pulmonary fibrosis and inflammation via a Bax-dependent, bid-activated pathway that involves matrix metalloproteinase-12. J Biol Chem. 2007 Mar 9; 282 (10) :7723-32.

Kapanci Y, Desmouliere A, Pache JC, Redard M, Gabbiani G. Cytoskeletal protein modulation in pulmonary alveolar myofibroblasts during idiopathic pulmonary fibrosis Possible role of transforming growth factor beta and tumor necrosis factor alpha. Am J Respir Crit Care Med. 1995 Dec; 152 (6 Pt 1) :2163-9.

Kazerooni EA, Martinez FJ, Flint A, Jamadar DA, Gross BH, Spizarny DL, Cascade PN, Whyte RI, Lynch JP 3rd, Toews G. Thin-section CT obtained at 10-mm increments versus limited three-level thin-section CT for idiopathic pulmonary fibrosis: correlation with pathologic scoring. AJR Am J Roentgenol. 1997 Oct; 169 (4) :977-83.

Khalil N, O'Connor RN, Flanders KC, Unruh H. TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. Am J Respir Cell Mol Biol. 1996 Feb; 14 (2) :131-8.

Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci U S A. 2006 Aug 29; 103 (35) :13180-5.

Kimura I, Onoshi T, Kunimasa I, Takano J. Treatment of malignant lymphomas with bleomycin. Cancer. 1972 Jan; 29 (1) :58-60.

King TE Jr, Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron JA Jr, Flint A, Thurlbeck W, Cherniack RM. Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. Am J Respir Crit Care Med. 2001 Sep 15; 164 (6) :1025-32.

Kotani I, Sato A, Hayakawa H, Urano T, Takada Y, Takada A. Increased procoagulant and antifibrinolytic activities in the lungs with idiopathic pulmonary fibrosis. Thromb Res. 1995 Mar 15; 77 (6) :493-504.

Kuhn C, McDonald JA. The roles of the myofibroblast in idiopathic pulmonary fibrosis Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. Am J Pathol. 1991 May; 138 (5) :1257-65.

Kuhn C 3rd, Boldt J, King TE Jr, Crouch E, Vartio T, McDonald JA. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis. 1989 Dec; 140 (6) :1693-703.

Kuwano K, Kunitake R, Kawasaki M, Nomoto Y, Hagimoto N, Nakanishi Y, Hara N. P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1996 Aug; 154 (2 Pt 1) :477-83.

Kuwano K, Nakashima N, Inoshima I, Hagimoto N, Fujita M, Yoshimi M, Maeyama T, Hamada N, Watanabe K, Hara N. Oxidative stress in lung epithelial cells from patients with idiopathic interstitial pneumonias. Eur Respir J. 2003 Feb; 21 (2) :232-40.

Kuwano K, Kunitake R, Maeyama T, Hagimoto N, Kawasaki M, Matsuba T, Yoshimi M, Inoshima I, Yoshida K, Hara N. Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor. Am J Physiol Lung Cell Mol Physiol. 2001 Feb; 280 (2) :L316-25.

Kuwano K, Hagimoto N, Nakanishi Y. The role of apoptosis in pulmonary fibrosis. Histol Histopathol. 2004 Jul; 19 (3) :867-81.

Kyriakis JM, Avruch J. Sounding the alarm: protein kinase cascades activated by stress and inflammation. J Biol Chem. 1996 Oct 4; 271 (40) :24313-6.

Lara LS, Vives D, Correa JS, Cardozo FP, Marques-Fernades MF, Lopes AG, Caruso-Neves C. PKA-mediated effect of MAS receptor in counteracting angiotensin II-stimulated renal Na+-ATPase. Arch Biochem Biophys. 2010 Apr 15; 496 (2) :117-22.

Lawson WE, Loyd JE. The genetic approach in pulmonary fibrosis: can it provide clues to this complex disease?. Proc Am Thorac Soc. 2006 Jun; 3 (4) :345-9.

Lee CG, Cho SJ, Kang MJ, Chapoval SP, Lee PJ, Noble PW, Yehualaeshet T, Lu B, Flavell RA, Milbrandt J, Homer RJ, Elias JA. Early growth response gene 1-mediated apoptosis is essential for transforming growth factor beta1-induced pulmonary fibrosis. J Exp Med. 2004 Aug 2; 200 (3) :377-89.

Lee VY, Schroedl C, Brunelle JK, Buccellato LJ, Akinci OI, Kaneto H, Snyder C, Eisenbart J, Budinger GR, Chandel NS. Bleomycin induces alveolar epithelial cell death through JNK-dependent activation of the mitochondrial death pathway. Am J Physiol Lung Cell Mol Physiol. 2005 Oct; 289 (4) :L521-8.

Lehane DE, Hurd E, Lane M. The effects of bleomycin on immunocompetence in man. Cancer Res. 1975 Oct; 35 (10) :2724-8.

Leung PS. The physiology of a local renin-angiotensin system in the pancreas. J Physiol. 2007 Apr 1; 580 (Pt 1) :31-7.

Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998 Aug 21; 94 (4) :491-501.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997 Nov 14; 91 (4) :479-89.

Li X, Rayford H, Uhal BD. Essential roles for angiotensin receptor AT1a in bleomycin-induced apoptosis and lung fibrosis in mice. Am J Pathol. 2003 Dec; 163 (6) :2523-30.

Li X, Zhang H, Soledad-Conrad V, Zhuang J, Uhal BD. Bleomycin-induced apoptosis of alveolar epithelial cells requires angiotensin synthesis de novo. Am J Physiol Lung Cell Mol Physiol. 2003 Mar; 284 (3) :L501-7.

Li X, Molina-Molina M, Abdul-Hafez A, Uhal V, Xaubet A, Uhal BD. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. Am J Physiol Lung Cell Mol Physiol. 2008 Jul; 295 (1) :L178-85.

Li X, Zhuang J, Rayford H, Zhang H, Shu R, Uhal BD. Attenuation of bleomycin-induced pulmonary fibrosis by intratracheal administration of antisense oligonucleotides against angiotensinogen mRNA. Curr Pharm Des. 2007; 13 (12) :1257-68.

Lorena D, Uchio K, Costa AM, Desmoulière A. Normal scarring: importance of myofibroblasts. Wound Repair Regen. 2002 Mar-Apr; 10 (2) :86-92.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 1998 Aug 21; 94 (4) :481-90.

Marshall RP. The pulmonary renin-angiotensin system. Curr Pharm Des. 2003; 9 (9) :715-22.

Marshall RP, McAnulty RJ, Laurent GJ. Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor. Am J Respir Crit Care Med. 2000 Jun; 161 (6) :1999-2004.

Marshall RP, Gohlke P, Chambers RC, Howell DC, Bottoms SE, Unger T, McAnulty RJ, Laurent GJ. Angiotensin II and the fibroproliferative response to acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2004 Jan; 286 (1) :L156-64.

Martinez FJ, Safrin S, Weycker D, Starko KM, Bradford WZ, King TE Jr, Flaherty KR, Schwartz DA, Noble PW, Raghu G, Brown KK, IPF Study Group. The clinical course of patients with idiopathic pulmonary fibrosis. Ann Intern Med. 2005 Jun 21; 142 (12 Pt 1) :963-7.

Mason RJ, Shannon JM. Alveolar type II cells. In: The lung: Scientific Foundations. Crystal RG, Ed. Lippincot-Raven Publishers, Philadelphia, 1997.

Matsuno H, Yudoh K, Watanabe Y, Nakazawa F, Aono H, Kimura T. Stromelysin-1 (MMP-3) in synovial fluid of patients with rheumatoid arthritis has potential to cleave membrane bound Fas ligand. J Rheumatol. 2001 Jan; 28 (1) :22-8.

McCormack FX, King TE Jr, Voelker DR, Robinson PC, Mason RJ. Idiopathic pulmonary fibrosis Abnormalities in the bronchoalveolar lavage content of surfactant protein A. Am Rev Respir Dis. 1991 Jul; 144 (1) :160-6.

Molina-Molina M, Serrano-Mollar A, Bulbena O, Fernandez-Zabalegui L, Closa D, Marin-Arguedas A, Torrego A, Mullol J, Picado C, Xaubet A. Losartan attenuates bleomycin induced lung fibrosis by increasing prostaglandin E2 synthesis. Thorax. 2006 Jul; 61 (7) :604-10.

Molina-Molina M, Xaubet A, Li X, Abdul-Hafez A, Friderici K, Jernigan K, Fu W, Ding Q, Pereda J, Serrano-Mollar A, Casanova A, Rodríguez-Becerra E, Morell F, Ancochea J, Picado C, Uhal BD. Angiotensinogen gene G-6A polymorphism influences idiopathic pulmonary fibrosis disease progression. Eur Respir J. 2008 Oct; 32 (4) :1004-8.

Molteni A, Moulder JE, Cohen EF, Ward WF, Fish BL, Taylor JM, Wolfe LF, Brizio-Molteni L, Veno P. Control of radiation-induced pneumopathy and lung fibrosis by angiotensin-converting enzyme inhibitors and an angiotensin II type 1 receptor blocker. Int J Radiat Biol. 2000 Apr; 76 (4) :523-32.

Molteni A, Ward WF, Ts'ao CH, Solliday NH, Dunne M. Monocrotaline-induced pulmonary fibrosis in rats: amelioration by captopril and penicillamine. Proc Soc Exp Biol Med. 1985 Oct; 180 (1) :112-20.

Montani JP, Van Vliet BN. (2004) General Physiology and Pathophysiology of the Renin – Angiotensin System. From Handb Exp Pharmacol; Angiotensin (Eds. Unger T, Schölkens BA). 163(I):3-29. Springer – New York.

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM. An induced proximity model for caspase-8 activation. J Biol Chem. 1998 Jan 30; 273 (5) :2926-30.

Muñoz MC, Giani JF, Dominici FP. Angiotensin-(1-7) stimulates the phosphorylation of Akt in rat extracardiac tissues in vivo via receptor Mas. Regul Pept. 2010 Apr 9; 161 (1-3) :1-7.

Nagase H, Woessner JF Jr. Matrix metalloproteinases. J Biol Chem. 1999 Jul 30; 274 (31) :21491-4.

Nguyen L, Ward WF, Ts'ao CH, Molteni A. Captopril inhibits proliferation of human lung fibroblasts in culture: a potential antifibrotic mechanism. Proc Soc Exp Biol Med. 1994 Jan; 205 (1) :80-4.

Nicholson AG, Fulford LG, Colby TV, du Bois RM, Hansell DM, Wells AU. The relationship between individual histologic features and disease progression in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2002 Jul 15; 166 (2) :173-7.

Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci. 1997 Aug; 22 (8) :299-306.

Oparil S, Tregear GW, Koerner T, Barnes BA, Haber E. Mechanism of pulmonary conversion of angiotensin I to angiotensin II in the dog. Circ Res. 1971 Dec; 29 (6) :682-90.

Otsuka M, Takahashi H, Shiratori M, Chiba H, Abe S. Reduction of bleomycin induced lung fibrosis by candesartan cilexetil, an angiotensin II type 1 receptor antagonist. Thorax. 2004 Jan; 59 (1) :31-8.

Panos RJ, Mortenson RL, Niccoli SA, King TE Jr. Clinical deterioration in patients with idiopathic pulmonary fibrosis: causes and assessment. Am J Med. 1990 Apr; 88 (4) :396-404.

Papp M, Li X, Zhuang J, Wang R, Uhal BD. Angiotensin receptor subtype AT(1) mediates alveolar epithelial cell apoptosis in response to ANG II. Am J Physiol Lung Cell Mol Physiol. 2002 Apr; 282 (4) :L713-8.

Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physiol Rev. 2006 Jul; 86 (3) :747-803.

Plataki M, Koutsopoulos AV, Darivianaki K, Delides G, Siafakas NM, Bouros D. Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. Chest. 2005 Jan; 127 (1) :266-74.

Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol. 1999 Dec 16-30; 9 (24) :1441-7

Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA, King TE Jr, Idiopathic Pulmonary Fibrosis Study Group. A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. N Engl J Med. 2004 Jan 8; 350 (2) :125-33.

Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2006 Oct 1; 174 (7) :810-6.

Ramos C, Montaño M, García-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol. 2001 May; 24 (5) :591-8.

Ravagnan L, Roumier T, Kroemer G. Mitochondria, the killer organelles and their weapons. J Cell Physiol. 2002 Aug; 192 (2) :131-7.

Re RN. Tissue renin angiotensin systems. Med Clin North Am. 2004 Jan; 88 (1) :19-38.

Renatus M, Stennicke HR, Scott FL, Liddington RC, Salvesen GS. Dimer formation drives the activation of the cell death protease caspase 9. Proc Natl Acad Sci U S A. 2001 Dec 4; 98 (25) :14250-5.

Rodriguez A, Oliver H, Zou H, Chen P, Wang X, Abrams JM. Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nat Cell Biol. 1999 Sep; 1 (5) :272-9.

Ruiz-Ortega M, Esteban V, Egido J. The regulation of the inflammatory response through nuclear factor-kappab pathway by angiotensin IV extends the role of the renin angiotensin system in cardiovascular diseases. Trends Cardiovasc Med. 2007 Jan; 17 (1) :19-25.

Saika S, Kono-Saika S, Ohnishi Y, Sato M, Muragaki Y, Ooshima A, Flanders KC, Yoo J, Anzano M, Liu CY, Kao WW, Roberts AB. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. Am J Pathol. 2004 Feb; 164 (2) :651-63.

Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. Proc Natl Acad Sci U S A. 1999 Sep 28; 96 (20) :10964-7.

Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell. 1997 Nov 14; 91 (4) :443-6.

Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM. Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. Hypertension. 2007 Jan; 49 (1) :185-92.

Sausville EA, Stein RW, Peisach J, Horwitz SB. Properties and products of the degradation of DNA by bleomycin and iron(II). Biochemistry. 1978 Jul 11; 17 (14) :2746-54.

Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays. 2001 Oct; 23 (10) :912-23.

Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis: the myofibroblast in focus. Chest. 2007 Oct; 132 (4) :1311-21.

Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, Selman M. Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. Chest. 2000 Mar; 117 (3) :684-94.

Selman M, King TE, Pardo A, American Thoracic Society, European Respiratory Society, American College of Chest Physicians. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med. 2001 Jan 16; 134 (2) :136-51.

Selman M, Montaño M, Ramos C, Chapela R. Concentration, biosynthesis and degradation of collagen in idiopathic pulmonary fibrosis. Thorax. 1986 May; 41 (5) :355-9.

Selman M, Thannickal VJ, Pardo A, Zisman DA, Martinez FJ, Lynch JP 3rd. Idiopathic pulmonary fibrosis: pathogenesis and therapeutic approaches. Drugs. 2004; 64 (4) :405-30.

Selman M, Ruiz V, Cabrera S, Segura L, Ramírez R, Barrios R, Pardo A. TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis A prevailing nondegradative lung microenvironment?. Am J Physiol Lung Cell Mol Physiol. 2000 Sep; 279 (3) :L562-74.

Shah A, Gul R, Yuan K, Gao S, Oh YB, Kim UH, Kim SH. Angiotensin-(1-7) stimulates high atrial pacing-induced ANP secretion via Mas/PI3-kinase/Akt axis and Na+/H+ exchanger. Am J Physiol Heart Circ Physiol. 2010 May; 298 (5) :H1365-74.

Shahar I, Fireman E, Topilsky M, Grief J, Schwarz Y, Kivity S, Ben-Efraim S, Spirer Z. Effect of endothelin-1 on alpha-smooth muscle actin expression and on alveolar fibroblasts proliferation in interstitial lung diseases. Int J Immunopharmacol. 1999 Nov; 21 (11) :759-75.

Siegel RM, Lenardo MJ. Apoptosis signaling pathways. Curr Protoc Cytom. 2002 Aug; Chapter 7:Unit 7.18.

Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest. 1997 Aug 15; 100 (4) :768-76.

Simon RH, Gross TJ, Edwards JA, Sitrin RG. Fibrin degradation by rat pulmonary alveolar epithelial cells. Am J Physiol. 1992 Apr; 262 (4 Pt 1) :L482-8.

Sisson TH, Hanson KE, Subbotina N, Patwardhan A, Hattori N, Simon RH. Inducible lung-specific urokinase expression reduces fibrosis and mortality after lung injury in mice. Am J Physiol Lung Cell Mol Physiol. 2002 Nov; 283 (5) :L1023-32.

Steele MP, Speer MC, Loyd JE, Brown KK, Herron A, Slifer SH, Burch LH, Wahidi MM, Phillips JA 3rd, Sporn TA, McAdams HP, Schwarz MI, Schwartz DA. Clinical and pathologic features of familial interstitial pneumonia. Am J Respir Crit Care Med. 2005 Nov 1; 172 (9) :1146-52.

Strausz J, Müller-Quernheim J, Steppling H, Ferlinz R. Oxygen radical production by alveolar inflammatory cells in idiopathic pulmonary fibrosis. Am Rev Respir Dis. 1990 Jan; 141 (1) :124-8.

Su Z, Zimpelmann J, Burns KD. Angiotensin-(1-7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. Kidney Int. 2006 Jun; 69 (12) :2212-8.

Tallant EA, Ferrario CM, Gallagher PE. Angiotensin-(1-7) inhibits growth of cardiac myocytes through activation of the mas receptor. Am J Physiol Heart Circ Physiol. 2005 Oct; 289 (4) :H1560-6.

Thabut G, Mal H, Castier Y, Groussard O, Brugière O, Marrash-Chahla R, Lesèche G, Fournier M. Survival benefit of lung transplantation for patients with idiopathic pulmonary fibrosis. J Thorac Cardiovasc Surg. 2003 Aug; 126 (2) :469-75.

Thannickal VJ, Aldweib KD, Rajan T, Fanburg BL. Upregulated expression of fibroblast growth factor (FGF) receptors by transforming growth factor-beta1 (TGF-beta1) mediates enhanced mitogenic responses to FGFs in cultured human lung fibroblasts. Biochem Biophys Res Commun. 1998 Oct 20; 251 (2) :437-41.

Thannickal VJ, Fanburg BL. Activation of an H2O2-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. J Biol Chem. 1995 Dec 22; 270 (51) :30334-8.

The American Society for transplant physicians (ASTP)/ American Thoracic Society (ATS)/European Respiratory Society (ERS)/ International Society for Heart and Lung Transplantation (ISHLT). International guidelines for the selection of lung transplant candidates. Am. J. Respir. Crit. Care Med. 1998; 158: 335–9.

Thomas AQ, Lane K, Phillips J 3rd, Prince M, Markin C, Speer M, Schwartz DA, Gaddipati R, Marney A, Johnson J, Roberts R, Haines J, Stahlman M, Loyd JE. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. Am J Respir Crit Care Med. 2002 May 1; 165 (9) :1322-8.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. A combinatorial approach defines specificities of members of the caspase family and granzyme B Functional relationships established for key mediators of apoptosis. J Biol Chem. 1997 Jul 18; 272 (29) :17907-11.

Thrall RS and Scalise PJ. (1995). Bleomycin In: Phan SH and Thrall RS, ed. Pulmonary fibrosis. New York: Marcel Dekker, Inc., 231-264

Uhal BD. Cell cycle kinetics in the alveolar epithelium. Am J Physiol. 1997 Jun; 272 (6 Pt 1) :L1031-45.

Uhal BD, Gidea C, Bargout R, Bifero A, Ibarra-Sunga O, Papp M, Flynn K, Filippatos G. Captopril inhibits apoptosis in human lung epithelial cells: a potential antifibrotic mechanism. Am J Physiol. 1998 Nov; 275 (5 Pt 1) :L1013-7.

Uhal BD, Joshi I, True AL, Mundle S, Raza A, Pardo A, Selman M. Fibroblasts isolated after fibrotic lung injury induce apoptosis of alveolar epithelial cells in vitro. Am J Physiol. 1995 Dec; 269 (6 Pt 1) :L819-28.

Uhal BD, Joshi I, Hughes WF, Ramos C, Pardo A, Selman M. Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. Am J Physiol. 1998 Dec; 275 (6 Pt 1) :L1192-9.

Uhal BD, Kim JK, Li X, Molina-Molina M. Angiotensin-TGF-beta 1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. Curr Pharm Des. 2007; 13 (12) :1247-56.

Umezawa H. [Bleomycin]. Gan No Rinsho. 1967 Oct; 13 (10) :735.

Umezawa H. Studies on bleomycin: chemistry and the biological action. Biomedicine. 1973 Nov; 18 (6) :459-75.

Umezawa H. Chemistry and mechanism of action of bleomycin. Fed Proc. 1974 Nov; 33 (11) :2296-302.

Umezawa H, Maeda K, Takeuchi T, Okami Y. New antibiotics, bleomycin A and B. J Antibiot (Tokyo). 1966 Sep; 19 (5) :200-9.

Umezawa H, Takeuchi T, Hori S, Sawa T, Ishizuka M. Studies on the mechanism of antitumor effect of bleomycin of squamous cell carcinoma. J Antibiot (Tokyo). 1972 Jul; 25 (7) :409-20.

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem. 2002 Apr 26; 277 (17) :14838-43.

Wallach-Dayan SB, Izbicki G, Cohen PY, Gerstl-Golan R, Fine A, Breuer R. Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway. Am J Physiol Lung Cell Mol Physiol. 2006 Apr; 290 (4) :L790-L796.

Wang R, Alam G, Zagariya A, Gidea C, Pinillos H, Lalude O, Choudhary G, Oezatalay D, Uhal BD. Apoptosis of lung epithelial cells in response to TNF-alpha requires angiotensin II generation de novo. J Cell Physiol. 2000 Nov; 185 (2) :253-9.

Wang R, Ibarra-Sunga O, Verlinski L, Pick R, Uhal BD. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. Am J Physiol Lung Cell Mol Physiol. 2000 Jul; 279 (1) :L143-51.

Ward WF, Molteni A, Ts'ao CH, Hinz JM. Captopril reduces collagen and mast cell accumulation in irradiated rat lung. Int J Radiat Oncol Biol Phys. 1990 Dec; 19 (6) :1405-9.

Wells AU, Hansell DM, Rubens MB, Cullinan P, Black CM, du Bois RM. The predictive value of appearances on thin-section computed tomography in fibrosing alveolitis. Am Rev Respir Dis. 1993 Oct; 148 (4 Pt 1) :1076-82.

Wells C, Mannino DM. Pulmonary fibrosis and lung cancer in the United States: analysis of the multiple cause of death mortality data, 1979 through 1991. South Med J. 1996 May; 89 (5) :505-10.

Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999 Jan; 79 (1) :143-80.

Wiener RS, Cao YX, Hinds A, Ramirez MI, Williams MC. Angiotensin converting enzyme 2 is primarily epithelial and is developmentally regulated in the mouse lung. J Cell Biochem. 2007 Aug 1; 101 (5) :1278-91.

Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol. 2005 May; 166 (5) :1321-32.

Yagoda A, Mukherji B, Young C, Etcubanas E, Lamonte C, Smith JR, Tan CT, Krakoff IH. Bleomycin, an antitumor antibiotic Clinical experience in 274 patients. Ann Intern Med. 1972 Dec; 77 (6) :861-70.

Young D, O'Neill K, Jessell T, Wigler M. Characterization of the rat mas oncogene and its high-level expression in the hippocampus and cerebral cortex of rat brain. Proc Natl Acad Sci U S A. 1988 Jul; 85 (14) :5339-42.

Yu H, Königshoff M, Jayachandran A, Handley D, Seeger W, Kaminski N, Eickelberg O. Transgelin is a direct target of TGF-beta/Smad3-dependent epithelial cell migration in lung fibrosis. FASEB J. 2008 Jun; 22 (6) :1778-89.

Yurchenco PD, Schittny JC. Molecular architecture of basement membranes. FASEB J. 1990 Apr 1; 4 (6) :1577-90.

Zavadil J, Böttinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene. 2005 Aug 29; 24 (37) :5764-74.

Zisman DA, Keane MP, Belperio JA, Strieter RM, Lynch JP 3rd. Pulmonary fibrosis. Methods Mol Med. 2005; 117:3-44.

Zisman LS. ACE and ACE2: a tale of two enzymes. Eur Heart J. 2005 Feb; 26 (4) :322-4.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 1997 Aug 8; 90 (3) :405-13.

Zou H, Li Y, Liu X, Wang X. An APAF-1cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem. 1999 Apr 23; 274 (17) :11549-56.