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REGULATION OF ANGIOTENSINOGEN GENE EXPRESSION BY TRANSFORMING GROWTH FACTOR-BETA1 IN LUNG FIBROBLASTS

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REGULATION OF ANGIOTENSINOGEN GENE EXPRESSION BY TRANSFORMING GROWTH FACTOR-BETA1 IN LUNG FIBROBLASTS

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ABSTRACT

REGULATION OF ANGIOTENSINOGEN GENE EXPRESSION BY TRANSFORMING GROWTH FACTOR-BETA1 IN LUNG FIBROBLASTS

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Idiopathic Pulmonary Fibrosis (IPF) is a progressive and usually fatal lung disease leading to decreased lung volume with distorted architecture and thick walled airspaces. Local activation of renin angiotensin system (RAS) plays a key role in the fibrogenic response of the lung tissue. Several studies showed that the octapeptide angiotensin II (Ang II), the active peptide of RAS, plays an important role in alveolar epithelial cell apoptosis and hence contributes to fibrosis of the lung. Angiotensinogen (AGT) is the only known precursor to Ang II, while angiotensin converting enzyme-2 (ACE-2) acts on Ang II peptide to produce the opposing action heptapeptide angiotensin 1-7 (Ang 1-7).

In this study, expression of these two genes (AGT and ACE-2) is investigated in IPF with focus on AGT gene expression in human lung fibroblasts. Increased AGT gene expression is detected in the IPF lung tissue and found to co-localize to apoptotic alveolar epithelial cells and to myofibroblast foci, these are histologic features of IPF with myofibroblast foci an indicator of worsening of fibrosis. Myofibroblasts originate from fibroblasts under the influence of the profibrotic cytokine transforming growth factor-beta1 (TGF-β1). This transition from fibroblasts to myofibroblasts is found to be accompanied by an increase in AGT gene expression. In the study presented here, molecular mechanisms by which TGF-β1 induces AGT gene expression are investigated.

The data show that TGF-β1 stimulates AGT gene expression in human lung fibroblasts by increasing the binding of two transcription factors, JunD and hypoxia inducible factor (HIF)-1α, to an AGT promoter domain close to the transcription start site, suggesting a molecular mechanism linking hypoxia signaling and fibrogenic stimuli in the lungs. This TGF-β1 responsive domain in AGT promoter contains three single nucleotide polymorphisms (SNPs). These SNPs are shown here to alter transcription factor binding to AGT promoter in response to TGF-β1 in human lung fibroblasts. This suggests that AGT expression in response to TGF-β1 may be dependent on the individual's haplotype. On the other hand, gene expression of ACE-2, the Ang II degrading enzyme, is found to be down-regulated in IPF. Mechanisms for this down-regulation involve the ACE-2 product Ang 1-7 and the angiotensin receptor AT_1. This suggests impairment of balance between Ang II production and degradation in conditions promoting pulmonary fibrosis.

In conclusion, this study implies that the haplotype of the individual contributes to the imbalance between Ang II production and degradation by affecting AGT gene expression in response to the profibrotic factor TGF-β1.

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KEY TO ABBREVIATIONS

ACE	Angiotensin-Converting Enzyme
ACE-2	Angiotensin Converting Enzyme-2
ACEi	Angiotensin-Converting Enzyme inhibitor
AEC	alveolar epithelial cell
AGCE1	Human Angiotensinogen Core Promoter Element 1
AGCF1	Human Angiotensinogen Core Promoter Binding Factor 1
AGT	Angiotensinogen
AIP	acute interstitial pneumonia
ALE	ATF-Like Element
Ang 1-7	Angiotensin 1-7
Ang II	Angiotensin II
Ang III	Angiotensin III
Ang IV	Angiotensin IV
AP-1	activating protein-1
APRE	Acute Phase Response Element
ARA	Angiotensin Receptor Antagonist
ATS/ERS	American Thoracic Society/European Respiratory Society
BMP	bone morphogenetic protein
C/EBP	CAAT/enhancer-binging protein
ChIP	Chromatin Immunoprecipitation
COP	cryptogenic organizing pneumonia

CRE	Cyclic AMP Responsive Element
DIP	desquamative interstitial pneumonia
DPLD	Diffuse Parenchymal Lung Disease
DR	Direct Repeat Sequence
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
Erk	extracellular signal-regulated protein kinase
GDF	growth and differentiation factor
GR	Glucocorticoid Receptor
HIF-1	Hypoxia Inducible Factor -1
HNF	Hepatocyte Nuclear Factor
HRE	Hypoxia responsive element
HX	histiocytosis/histiocytosis X
IFN-γ1b	interferon-Gamma1b
IIPs	Idiopathic Interstitial Pneumonias
IL	Interleukin
ILD	Interstitial Lung Disease
IPF	Idiopathic Pulmonary Fibrosis
JNK	c-Jun N-terminal kinase
LAM	lymphangioleiomyomatosis
LIP	lymphoid interstitial pneumonia
MAPK	mitogen activated protein kinase
MAPKKK	MAPK kinase kinase

MEKK1	MAP/ERK kinase kinase 1
MIF	Mullerian inhibitory substance
NFκB	nuclear factor-kappa B
NSIP	nonspecific interstitial pneumonia
PI3K	Phosphoinositide 3-kinase
RAS	Renin Angiotensin System
RB-ILD	respiratory bronchiolitis-associated interstitial lung disease
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SARS	severe acute respiratory syndrome
SHP	Small Heterodimer Partner
shRNA	short hairpin RNA
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphism
SP-C	surfactant protein C
STAT	Signal Transducers and Activators of Transcription
TAK1	TGF-β-activated kinase 1
TGF-β1	Transforming Growth Factor-beta 1
TNF-α	tumor necrosis factor-alpha
TβRI	Type I TGFβ receptor
TβRII	Type II TGFβ receptor
UIP	usual interstitial pneumonia
αSMA	alpha-smooth muscle actin

CHAPTER 1:

GENERAL INTRODUCTION

RENIN ANGIOTENSIN SYSTEM

Components of Renin Angiotensin System

The renin angiotensin system (RAS) (Figure 1.1) has been traditionally viewed as an endocrine system "Endocrine RAS" playing a significant role in blood pressure regulation. The active player of the renin angiotensin system is known as the octapeptide angiotensin II (Ang II). Angiotensinogen (AGT) is the only known precursor to Ang II. AGT is produced and secreted into the circulation by liver hepatocytes. It may also be expressed locally within tissues. In the endocrine RAS, the kidney produced enzyme "renin" acts on circulating AGT protein. Renin cleaves AGT to produce a fragment of 10 amino acids known as angiotensin I (Ang I) that is converted by angiotensin-converting enzyme (ACE) to the active octapeptide Ang II. Alternate enzymatic pathways to generate Ang I and Ang II exist as well, where enzymes other than renin and ACE, such as tonin or Cathepsin D and trypsin, Cathepsin G or chymase, contribute to the production of Ang I and Ang II.

Ang II exerts its actions through binding to specific cell surface angiotensin receptors. Two main receptors to Ang II have been identified; AT_1 and AT_2, both belong to superfamily of seven transmembrane G-protein coupled receptors. The AT_1 receptor mediates all of the classical actions of Ang II (vasoconstriction, sodium retention, cell growth and proliferation), while AT_2 receptor promotes vasodilatation, cell differentiation, inhibition of cell growth and apoptosis and may play a counterbalancing role to the effects of Ang II on AT_1 receptor (de Gasparo *et al.* 2000).

In addition to Ang I and Ang II peptides, a number of bioactive peptides, such as angiotensin III (Ang III), angiotensin IV (Ang IV) and angiotensin 1-7 (Ang 1-7) can be generated by alternative enzymes including angiotensin converting enzyme 2 (ACE-2), aminopeptidase A, endopeptidase and aminopeptidase B/N. Ang III and Ang IV are thought to play a major role in the brain and blood pressure control. Ang 1-7, also a bioactive peptide in RAS, is considered to play a role of opposing effects to Ang II and is thought to represent a negative feedback mechanism controlling Ang II actions in tissue and cardiovascular functions. (Filippatos *et al.* 2001, Montani & Van Vliet 2004, Ferrario *et al.* 2004, Chansel & Ardaillou 1998, Banegas *et al.* 2006, Pan *et al.* 2008).

Local Renin Angiotensin System (Local RAS)

More recently, RAS components expression has been detected in variety of tissues such as heart, kidney, liver, lung, brain, pancreas and adipose tissue as well as nervous, reproductive and digestive systems, introducing the concept of "Local RAS" (Filippatos & Uhal 2003, Strazzullo & Galletti 2004, Leung & Carlsson 2005, Paul *et al.* 2006). This local system can operate in an autocrine, paracrine and/or intracrine manner in response to various physiological or pathophysiological stimuli. The local RAS exerts both hemodynamic functions as well as multiple and novel functions where brain and intrarenal RAS are thought to contribute to salt balance and blood pressure control; heart and vascular RAS are involved in cardiovascular pathology; while novel actions of locally generated angiotensin peptides include regulation of cell growth, differentiation, proliferation and apoptosis, reactive oxygen species (ROS) generation, hormonal

secretion, tissue inflammation and fibrosis (Leung 2004, 2007, Montani & Van Vliet 2004, Li *et al.* 2004a).

Local RAS and Fibrosis

Local RAS is believed to play a key role in fibrogenesis. Fibrogenesis studies have utilized inhibitors of the RAS, angiotensin converting enzyme inhibitors (ACEi's) and angiotensin receptor antagonists (ARAs), to show the role of RAS in the initiation and progression of fibrous tissue accumulation. These RAS inhibitors have shown to prevent fibrogenesis of the heart (Weber & Sun 2000), liver (Yoshiji *et al.* 2001), kidney (Klahr & Morrissey 1997), pancreas (Ko *et al.* 2006) and lung (Wang *et al.* 2000a, Marshall *et al.* 2004, Uhal *et al.* 2007a). This suggests that the local activation of RAS plays a key role in fibrogenic response of these tissues by mechanisms independent of the blood-derived RAS as shown by *in vitro* and tissue explants studies (Li *et al.* 2003b, 2004a) for pulmonary fibrosis.

ANGIOTENSINOGEN

Angiotensinogen Gene

The human angiotensinogen (AGT) gene is located on chromosome 1q42-q43 and contains 5 exons/4 introns showing organization similar to other serine protease inhibitors (serpins) superfamily to which AGT belongs. The human AGT gene encodes the human angiotensinogen molecule, an α_2-globulin protein (485 amino acid), with a molecular weight of about 61 kDa. Exon 1 of the human AGT gene encodes for a short

37bp 5′ untranslated tract, with the second exon encoding the initiation methionine, signal peptide, and the majority of the mature protein (Jeunemaitre 2004, Brasier *et al.* 1999).

A large number of single nucleotide polymorphisms (SNPs), over 140 SNPs, have been described in the 5′ flanking region, exons, introns, and 3′ region of the AGT gene (www.ncbi.nlm.nih.gov/SNP, AGT gene ID:183). Among these SNPs, two polymorphisms; the coding SNP (M235T) in exon 2 and the 5′ flanking region SNP G-6A, occurring almost at the same frequency and are in complete linkage disequilibrium, have been most frequently studied (Jeunemaitre *et al.* 1997). The M235T polymorphism has been associated with increased plasma angiotensinogen levels, while the G-6A polymorphism has been associated with increased expression of the AGT gene in vitro using luciferase assays suggesting transcriptional regulation as a possible explanation for increased plasma angiotensinogen with the M235T polymorphisms (Inoue *et al.* 1997). However, the effects of polymorphisms on AGT gene expression may be more complex, since other polymorphisms, C-532T, A-217G, C-18T, A-20C, T+31C, are also in linkage disequilibrium with G-6A and M235T, and may affect AGT transcription (Jeunemaitre 2004).

Angiotensinogen Gene Expression

AGT abundance is regulated at the transcriptional level through hormonal and cell-type specific regulators. Constitutive human AGT gene transcriptional control has been studied extensively in liver (Brasier *et al.* 1999). The human angiotensinogen core promoter element 1 (AGCE1) site, located between the TATA box and transcription initiation site, was shown to be a critical regulator of AGT transcription by binding to

human angiotensinogen core promoter binding factor 1(AGCF1) in the human hepatoma cell line HepG2 cells (Yanai *et al.* 1996). USF1 has been identified as a component of AGCF1 (Yanai *et al.* 1997a). AGCE1 was also shown to be required for the activity of an upstream AGT enhancer, ATF-like element (ALE) (Yanai *et al.* 1997b). In addition to AGCE1, Yanai also describes two AGCE2 sites, located upstream and downstream of the transcription initiation site and show a possible cell type-dependent function (Yanai *et al.* 1997c). Other cell type-dependent elements controlling AGT transcription are; direct repeat sequences (DR) in AGT promoter that contribute 50 or >95% to AGT transcription in liver or kidneys respectively, whereas same sequences are not required in the heart and brain (Shimizu *et al.* 2005), and a 3'-downstream enhancer that binds an AP-3-related factor and human angiotensinogen enhancer factor-1 (hAEF-1) (Nibu *et al.* 1994).

Inducible human AGT transcription has been studied in response to several factors. In HepG2 cells, interleukin (IL)-6 induces AGT transcription via STAT3 binding to one of three acute phase response elements (APREs) present between -350 to –122 upstream of transcription start site (Ray *et al.* 2005). Recently, Jain *et al.* suggested that three transcription factors glucocorticoid receptor (GR), STAT-3, and HNF-1α bind to the APREs region of the hAGT gene promoter and are responsible for IL-6 induced promoter activity of this gene in liver cells (Jain *et al.* 2007). Upregulation of human AGT gene transcription in Hep3B hepatocytes by interferon-gamma was shown to involve STAT1-binding motif in the AGT promoter between -271 and -279 in a mechanism separate from IL-6 upregulation of AGT by STAT3 (Jain *et al.* 2006).

Other inducers of AGT transcription include estrogen, through an estrogen responsive element near TATA box of the promoter (Morgan *et al.* 2000). In a study

suggesting relation between adipose AGT secretion in obesity and elevated blood pressure, AGT gene expression and secretion in adipose tissue were found to be stimulated by cyclic AMP via increased DNA cyclic AMP responsive element (CRE) binding activity (Serazin *et al.* 2004).

Repression of AGT gene transcription was described by Date *et al.*, where Finb, a multiple zinc finger protein, was reported to repress transcription of the human angiotensinogen gene via binding of two elements in the 5′ flanking region of the human AGT promoter (Date *et al.* 2004). Expression of AGT gene was also shown to be repressed in HepG2 cells in response to bile acids via the small heterodimer partner (SHP) acting on the binding site of hepatocyte nuclear factor-4 (HNF-4) on the AGT gene promoter (Shimamoto *et al.* 2004).

AGT gene expression control in the lung has been studied in pulmonary epithelial cells, where the cardiovascular drug "amiodarone" induced AGT transcription through the AP-1 site present between the TATA box and transcription start site (Uhal *et al.* 2007b). Data in support of these findings will be discussed in detail in chapter 2 and appendix B.

Polymorphisms and Angiotensinogen Expression

The AGT promoter region between TATA box and transcription start site has three identified SNPs, G-6A, C-18T and A-20C. These SNPs have shown variations in the AGT promoter – driven transcription and in transcription factor binding to variant DNA sequences (Inoue *et al.* 1997, Yanai *et al.* 1997a) indicating the role of this promoter region in controlling the level of AGT expression. Among the SNPs that affect

transcription factors binding is the A-20C, where estrogen receptor and the orphan receptor Arp-1, were shown to bind the AGT promoter in HepG2 cells when nucleotide A is present (Zhao *et al.* 1999, Narayanan *et al.* 1999). The G-6A SNP showed specific interaction with the nuclear factor YB1 where co-transfection experiments of YB1 reduced basal AGT promoter activity in a dose-dependent manner. Although these observations suggested a possible role for YB1 in modulating AGT expression, this function is likely to occur in the context of complex interactions involving other nuclear factors (Nakajima *et al.* 2002).

The AGT gene promoter SNP A-217G, was also reported to play a role in AGT gene expression. The nucleotide sequence of this region of the AGT gene promoter was shown to bind strongly to CAAT/enhancer-binding protein (C/EBP) family transcription factors and glucocorticoid receptor (GR) when nucleotide A was present at -217. In addition, reporter constructs containing the human AGT gene promoter with nucleotide A at -217 had increased basal and interleukin-6 stimulated transcriptional activity compared with nucleotide G at -217 (Jain *et al.* 2002, 2005).

In a recent study comparing the expression driven by eight haplotypes of the human AGT promoter in human astrocyte, proximal tubule, and hepatocyte cell lines, showed that the -20 and -217 polymorphisms have the largest influence on angiotensinogen transcription with other polymorphisms having a smaller impact on angiotensinogen transcription, and the transcriptional influence of the promoter polymorphisms may act cell specifically (Dickson *et al.* 2007).

Angiotensinogen and Disease

Hypertension and Cardiovascular Disease

Due to the key role that RAS plays in blood pressure regulation, many studies investigate AGT polymorphisms association with essential hypertension. A large number of case-control studies have tested the association of M235T polymorphism and hypertension. Meta-analyses of white Caucasian case-control studies showed significant association of the 235T allele with hypertension and the risk of elevated blood pressure (Kunz *et al.* 1997, Staessen *et al.* 1999). In a study conducted on Japanease population, analysis of the AGT polymorphisms confirmed that the G-6A, T+31C, and M235T polymorphisms are in absolute linkage disequilibrium and that the -6A, +31C and 235T haplotype is associated with hypertension (Sato *et al.* 2000). In addition, several studies demonstrated association of AGT gene G-6A, T174M, and M235T polymorphisms with increased angiotensinogen plasma levels with -6A, 174T or 235T alleles presence or homozygosity (Jeunemaitre *et al.* 1992, Bloem *et al.* 1995, 1997, Schunkert *et al.* 1997, Azizi *et al.* 2000, Sethi *et al.* 2001, 2003). Recently, meta-analysis of the AGT polymorphisms association with hypertension showed dual role for the A-20C SNP, where the -20C allele, which was associated with a decreased risk of hypertension in populations of mixed and European ancestries, but with a 24% increase in the odds of hypertension in Asian subjects (Pereira *et al.* 2008). In African-American population study A-217G polymorphism of the AGT gene was associated with hypertension, where the frequency of allele A was significantly increased in the genomic DNA of African-American hypertensive patients (Kumar *et al.* 2005). Consistent with its association with hypertension, the TT genotype of the AGT gene M235T polymorphism was associated

with an increased risk of coronary heart disease and myocardial infarction in several studies (Ludwig *et al.* 1997, Rodríguez-Pérez *et al.* 2001, Buraczyńska *et al.* 2003).

Fibrotic Disease

Studies of AGT gene polymorphisms in fibrotic diseases of certain tissues revealed association of these SNPs with development and/or progression of fibrosis. Liver fibrosis caused by chronic viral hepatitis or fatty liver showed association with AGT polymorphisms. In chronic hepatitis B significant relationship was seen between polymorphisms of the core promoter region of the AGT gene (-20 and -6) and liver cirrhosis (Xiao *et al.* 2006). In patients with chronic hepatitis C, statistically significant relationship was also seen between AGT G-6A SNP and the stage of hepatic fibrosis Individuals with the A/A homozygous genotype were more likely to have high severity of hepatic fibrosis compared with individuals inheriting the A/G or the G/G homozygous genotype. In the same study, transforming growth factor-beta1 (TGF-β1) gene (codon 25: Arg/Pro) polymorphism showed relationship with the stage of fibrosis with Arg/Arg genotype being profibrotic. The patients who inherited both of the profibrotic genotypes for AGT and TGF-β1 genes correlated with highest progression of fibrosis (Powell *et al.* 2000). Similar results were obtained when the same profibrotic genotypes (AGT -6 A/A and TGF-β1 gene 25 Arg/Arg) were investigated in advanced hepatic fibrosis obese patients with non alcoholic fatty liver disease, where patients who inherited both profibrotic genotypes appeared to be at greater risk of advanced fibrosis (Dixon *et al.* 2003). Similarly, AGT and TGF-β1 gene polymorphisms were associated with stricturing

Crohn's disease, a complication of Crohn's disease that involves development of a fibrotic intestinal stricture (Hume *et al.* 2006).

Kidney Disease

Polymorphisms of the AGT gene are involved in renal pathophysiology and progression of renal disease. Many studies showed the association between M235T (T allele) or G-6A (A allele) and renal diseases, such as; end-stage renal disease, development of interstitial nephritis (Buraczyńska *et al.* 2002, 2006), and chronic kidney disease progression through induction of tissue growth and fibrosis (Hsu *et al.* 2006).

Figure 1.1.

Schematic diagram of the Renin Angiotensin System (RAS). The diagram illustrates enzymatic cleavage of angiotensinogen protein to yield angiotensin I (Ang I), angiotensin II (Ang II), angiotensin 1-9 (Ang 1-9), angiotensin 1-7 (Ang 1-7), angiotensin III (Ang III, Ang 2-8), and angiotensin IV (Ang IV, Ang 3-8) peptides. Shown are known receptors for angiotensin peptides (de Gasparo *et al.* 2000). AT_1, AT_2, AT_4: angiotensin receptors 1, 2, 4; Mas: Mas proto-oncogene a G-protein coupled receptor for Ang 1-7; ACE: angiotensin converting enzyme; ACE-2: angiotensin converting enzyme-2; aa: amino acid. "Images in this dissertation are presented in color."

Angiotensinogen (485 aa)
Renin/Tonin/CathepsinD
Ang I
(10 aa)
ACE-2
Ang 1-9
(9 aa)
Endopeptidases
ACE/Trypsin/CathepsinG
/Chymase
Ang II
(8 aa)
ACE-2
Ang 1-7
(7 aa)
Amino-
peptidase A
Ang III
(Ang 2-8)
(7 aa)
Amino-
peptidase B
Ang IV
(Ang 3-8)
(6 aa)
AT4
AT2
Receptors
AT1
Mas

IDIOPATHIC PULMONARY FIBROSIS

Interstitial Lung Diseases

The interstitial lung diseases (ILDs), also termed diffuse parenchymal lung disease (DPLD), are a diverse group of lung diseases that can be classified according to the combination of clinical, radiologic, physiologic and pathologic criteria. The interstitium includes the space between the epithelial and endothelial basement membranes and it is the primary site of injury in the ILDs/DPLDs. However, these disorders frequently affect not only the interstitium, but also the airspaces, peripheral airways, and vessels along with their respective epithelial and endothelial linings. While the underlying pathogenic mechanisms are known or inferred in some of the ILDs/DPLDs (for example hypersensitivity pneumonitis), the pathogenesis of the majority of these entities, particularly those characterized by the development of progressive lung fibrosis, is poorly understood (Steele & Brown 2007). These ILDs/DPLDs consist of disorders of known causes (collagen vascular disease, environmental or drug related) as well as disorders of unknown cause. The latter include idiopathic interstitial pneumonias (IIPs), granulomatous lung disorders (e.g., sarcoidosis), and other forms of interstitial lung disease including lymphangioleiomyomatosis (LAM), pulmonary Langerhans' cell histiocytosis/histiocytosis X (HX), and eosinophilic pneumonia (King & Selman 2006).

Idiopathic Interstitial Pneumonias

The idiopathic interstitial pneumonias (IIPs) are a group of ILDs/DPLDs. The IIPs are a heterogeneous group of nonneoplastic disorders resulting from damage to the

lung parenchyma by varying patterns of inflammation and fibrosis. The American Thoracic Society/European Respiratory Society (ATS/ERS) classifies IIPs into seven clinico-radiologic-pathologic entities: idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (AIP), respiratory bronchiolitis-associated interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), and lymphoid interstitial pneumonia (LIP). Idiopathic pulmonary fibrosis (IPF) is the most common of the IIPs and carries the worst prognosis (American Thoracic Society & European Respiratory Society 2002, King & Selman 2006).

Idiopathic Pulmonary Fibrosis (IPF)

Definition

Pulmonary fibrosis results from injury to the lung and an ensuing fibrotic response that leads to thickening of alveolar walls and obliteration of alveolar space. If the etiology is unknown the condition is designated as idiopathic pulmonary fibrosis (IPF) (Li *et al.* 2004b). The ATS/ERS classification defines IPF as "a specific form of chronic fibrosing interstitial pneumonia of unknown etiology, limited to the lung and associated with the histological entity of usual interstitial pneumonia" (American Thoracic Society & European Respiratory Society 2002).

Epidemiology

Significant differences in the incidence, prevalence, diagnostic approach, therapies and survival for patients with idiopathic pulmonary fibrosis (IPF) exist in

epidemiological studies. The prevalence of IPF, for example, varies widely depending on location, identifying criteria and year of study, ranging from 3–6 per 100,000 in the UK up to 16–18 per 100,000 in Finland (Wilson *et al.* 2008). In the United States, prevalence was estimated to range from 14.0 to 42.7 per 100,000 and incidence from 6.8 to 16.3 per 100,000 according to criteria used for estimation (Raghu *et al.* 2006), suggesting that the prevalence is increasing for IPF than previously reported (Zisman *et al.* 2005). Median survival among persons with IPF is believed to be from 3 to 5 yr with average age- and sex-adjusted mortality rate of 50.8 per 1,000,000 people. Respiratory failure is the most frequent cause of death, and has been reported to account for over 80% of all fatalities (Raghu *et al.* 2006, Olson *et al.* 2007).

Most studies suggest IPF is more common in men, and is a disease predominantly of the elderly, with a mean age of onset typically 67–69 years (Coultas *et al.* 1994, Johnston *et al.* 1997, Hubbard *et al.* 1996, Dempsey 2006). Risk factors associated with pulmonary fibrosis include smoking, environmental exposures, gastroesophageal reflux disease, commonly prescribed drugs, diabetes mellitus, infectious agents, and genetic factors (Zisman *et al.* 2005).

Clinical Features

Symptoms of IPF depend on the extent of the lung damage, the rate at which the disease progresses, and the development of complications, such as lung infections and cor pulmonale. The clinical manifestations of IPF include dyspnea on exertion, nonproductive cough, and inspiratory crackles. Common symptoms include weight loss and fatigue. Late in the disease, as the level of oxygen in the blood decreases, cyanosis

develops with or without digital clubbing where the ends of the fingers may become thick or club-shaped. Chest radiography and high-resolution computed tomography typically show patchy, predominantly peripheral, subpleural, lower lung zone reticular opacities. High-resolution computed tomography also shows variable but limited ground-glass opacity (usually associated with traction bronchiectasis/bronchial dilation) and subpleural honeycombing. Pulmonary function tests show that the amount of air the lungs can hold is below normal revealing restrictive impairment, reduced diffusing capacity for carbon monoxide, and arterial hypoxemia exaggerated or elicited by exercise. The definite diagnosis of idiopathic pulmonary fibrosis requires a compatible clinical history, the exclusion of other known causes of interstitial lung disease (such as drug injuries, environmental exposures, or collagen vascular disease), and a surgical lung biopsy showing usual interstitial pneumonia (Selman *et al.* 2001, American Thoracic Society & European Respiratory Society 2002).

Histologic Features

IPF is a progressive and usually fatal lung disease leading to small lung with distorted architecture and thick walled airspaces (Selman *et al.* 2001). The main histologic features of the fibrotic lung are described as usual interstitial pneumonia (UIP) showing persistent and unrepaired alveolar epithelial cells (AECs) damage, proliferation and patchy accumulation of fibroblasts and myofibroblasts (fibroblast foci), increased extracellular matrix (ECM) production and collagen deposition (Pardo & Selman 2002). Morphologic change in the lung associated with subsequent progression to dense fibrosis is related to the presence and extent of fibroblastic foci in the injured lung (Kuhn *et al.*

1989, Katzenstein & Myers 1998, Selman *et al.* 2001). Fibroblastic foci are areas rich in extracellular matrix and mesenchymal cells with cell phenotypes ranging from proliferating fibrobasts to fully differentiated smooth muscle cells. However, the predominant cell type in the fibroblast foci is the myofibroblasts aligned parallel to one another and probably contribute to active contraction, distorted architecture and excess collagen deposition (Kuhn & McDonald 1991).

Myofibroblasts and Alveolar Epithelial Cells in IPF

Myofibroblasts are considered key mediators of fibrosis in the lung as they are believed to play a central role in the pathogenesis of IPF. Increased numbers of fibroblastic foci are associated with disease progression and worse prognosis in IPF (King *et al.* 2001). These cells are characterized by a spindle or stellate morphology with intracytoplasmic stress fibers, a contractile phenotype, expression of α-smooth muscle actin (αSMA) immunocytochemical marker, and collagen production. Myofibroblasts increase extracellular matrix deposition and remodeling through expression of procollagens and pro-fibrotic cytokines, including transforming growth factor-β1 (TGF-β1) (Schurch *et al.* 1998, Kuhn & McDonald 1991). The myofibroblasts, surrounded by altered extracellular matrix, account for the hypercontractile properties and low compliance of fibrotic lung (Schissel & Layne 2006, Willis *et al.* 2006).

Many studies support that the destruction of the healthy alveolar epithelium, i.e. injury alone, is capable of creating a profibrotic microenvironment that initiates fibrosis (Hagimoto *et al.* 1997a, 1997b, Uhal 2003). Studies of lung biopsies from patients with IPF showed that fibrotic foci colocalize with apoptotic or absent alveolar epithelium

(Uhal *et al.* 1998, Li *et al.* 2006). Myofibroblasts also contribute to alveolar epithelial cells (AECs) damage. Myofibroblasts' contribution to AECs damage was demonstrated *in vitro* (Uhal *et al.* 1995) and *in vivo* (Uhal *et al.* 1998) by production of a soluble inducer of apoptosis identified as angiotensin peptide, Ang II. In addition, constitutive expression of AGT by myofibroblasts has been demonstrated *in vitro* after isolation of these cells from the lungs of patients with IPF (Wang *et al.* 1999a). Production of AGT mRNA and protein in the IPF lung was found to increase relative to normal lung and co-localize to both apoptotic epithelia and myofibroblast foci (Li *et al.* 2006).

Source/Origin of Myofibroblasts in IPF

Several cell types present are potential candidates as precursors of myofibroblasts, which appear to originate from fibroblasts under the influence of various factors (Phan 1996, Serini & Gabbiani 1999). Among these factors, transforming growth factor (TGF)-β is well known to induce the phenotypic modulation of fibroblasts to myofibroblasts (Desmouliere *et al.* 1993, Yokozeki *et al.* 1997), characterized by expression of the myofibroblast marker α-smooth muscle actin (αSMA) (Sappino *et al.* 1990, Roy *et al.* 2001). Uncertainty still exists regarding myofibroblasts' cellular origin(s) and the molecular mechanisms regulating their differentiation, proliferation, and death in IPF. Several studies have addressed the source of myofibroblasts whether it is a result of fibroblasts differentiation, smooth muscle cells de-differentiation, from circulating fibrocytes (bone marrow derived fibroblast precursor sells), or alveolar epithelial cells origin through a process termed "epithelial-mesenchymal transition" (EMT) (Wynn 2008, Schissel & Layne 2006).

Data from several studies support exclusion of smooth muscle cells de-differentiation as the origin of myofibroblasts. Immunohistological studies of human and animal model of IPF revealed that myofibroblasts express α-SMA, vimentin, and desmin but they do not typically express smooth muscle myosin (Mitchell *et al.* 1989, Kuhn & McDonald 1991). In addition, the type of procollagen expressed and electron microscopy features of myofibroblasts are more similar to those of fibroblasts than smooth muscle cells (Oda *et al.* 1988). Finally, TGF-β1 and interleukin 4 induced α-SMA expression and myofibroblast-like differentiation in tissue-derived fibroblasts *in vitro* (Mattey *et al.* 1997).

Many studies in mice suggested that fibroblasts recruited to the lung after injury are derived from fibrocytes. However, the role of fibrocytes in the pathogenesis of human pulmonary fibroses is controversial since some of these studies showed that very few αSMA – expressing cells were of bone marrow origin, and that recruited fibroblasts were resistant to conversion to myofibroblasts by TGF-β1 (Hashimoto *et al.* 2004, Gomperts & Strieter 2007, Schissel & Layne 2006).

In addition to resident mesenchymal cells, myofibroblasts are now thought to be derived from epithelial cells in the process termed epithelial-mesenchymal transition (EMT). In lung cells, chronic exposure to TGF-β1 on the AECs resulted in increased expression of α-SMA, type I collagen, vimentin, and desmin, with concurrent transition to a fibroblast-like morphology. In addition, cells co-expressing epithelial markers and α-SMA were detected in lung tissue from IPF patients. However, the identification of specific pathways involved in EMT during fibrosis in the lung still needs further studies (Willis *et al.* 2005, 2006).

Therapy Options in IPF

Many of the treatments advocated in current guidelines are largely ineffective, with limited evidence to support their continued use. In particular, convincing evidence to support using conventional 'anti-inflammatory' therapy (oral corticosteroids and azathioprine) does not exist, and treatment-related toxicity is common. If patients are clinically stable, with limited disease progression, many clinicians would suggest to wait, assessing patients frequently over the next 6–12 months and only considering potentially toxic drug therapy if there was evidence of clinical, radiological or physiological deterioration. If so, empirical conventional treatment can be considered (Dempsey *et al.* 2006, Orens *et al.* 2006, Egan *et al.* 2005). Current recommendations for treatment of IPF include treatment with combination immunosuppressant therapy (prednisolone plus azathioprine) +/− anti-oxidant therapy i.e. *N*-acetylcysteine (Davies *et al.* 2003, Flaherty *et al.* 2001). Although controversy exists regarding the potential role of interferon-γ1b (IFN-γ1b) in the treatment of IPF (Teirstein 2004), a meta-analysis of the combined results of multiple studies showed that IFN-γ1b therapy may be efficacious in improving survival and is associated with reduced mortality in patients with IPF (Bajwa *et al.* 2005). However, patients with more severe disease do not seem to benefit from treatment with IFN-γ1b (Martinez *et al.* 2005).

Where appropriate, the patient should be considered for lung transplantation. Single-lung transplantation is generally the procedure of choice, although as in most transplant procedures, the availability of donor organs is the limiting factor. Other factors that determine surgical feasibility include patient's age, other major organ dysfunction,

nutritional state, previous thoracic surgery, presence of osteoporosis, and psychological and social factors (Dempsey *et al.* 2006, Nathan 2005).

Due to its role in the pathogenesis of pulmonary fibrosis, studies were conducted to test the therapeutic potential of antagonizing Ang II in IPF. Many studies showed that inhibitors of the RAS such as angiotensin converting enzyme inhibitors (ACEi's), angiotensin receptor blockers or receptor deletion blocked experimental lung fibrosis (Wang *et al.* 2000b, Marshall *et al.* 2004, Uhal *et al.* 2007a, Molteni *et al.* 2000, Molina-Molina *et al.* 2006) as well as improved lung functions in IPF patients (Woo *et al.* 2003).

Other areas of management include oxygen therapy (domiciliary and ambulatory), pulmonary rehabilitation, nutrition, osteoporosis prophylaxis, smoking cessation, assessment and treatment of common co-morbidities such as cardiovascular disease, psychological support for impaired quality of life and ultimately palliative care (Dempsey 2006).

Genetics of IPF

Familial Pulmonary Fibrosis

The familial clustering of idiopathic pulmonary fibrosis, familial pulmonary fibrosis or familial IIP, is identified by confirming IIP in two or more members of the same family (Grutters & du Bois 2005). This 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). In familial lung fibrosis, genetic studies have demonstrated an association with two mutations of surfactant protein C (SP-C) gene

(Thomas *et al.* 2002, Nogee *et al.* 2001). These have been found to result in protein misfolding and causing type-II alveolar epithelial cell injury. Different histological patterns were observed in the affected subjects (UIP and NSIP), suggesting that the pleiotropic effects of the SP-C gene mutation is under the influence of modifier genes and/or environmental factors (Thomas *et al.* 2002). However, SP-C gene variations have not been associated with sporadic cases of IPF (Grutters & du Bois 2005).

Genes Contributing to IPF Susceptibility

Development of and susceptibility to IPF probably involves multiple genetics factors and a combination of polymorphisms related to epithelial cell injury and abnormal wound healing. The genetic associations of many genes and gene polymorphisms with IPF susceptibility and progression have been evaluated in case/control studies. Candidate genes that have been analyzed for their association include pro-/anti-inflammation genes (IL-1 cluster, tumor necrosis factor-alpha (TNF-α), chemokine-related genes, Th1/Th2 response genes, and Complement receptor genes), pulmonary surfactant protein genes, coagulation cascade (PAI genes), fibroblast-related pathways (TGF-β genes, RAS-related genes) (Grutters & du Bois 2005). However, only a limited number of gene polymorphisms demonstrated confirmed association with disease susceptibility including interleukin-1RN (IL1RN gene) (+2018T) (Whyte *et al.* 2000), TNF-α gene (-308A) (Whyte *et al.* 2000, Riha *et al.* 2004), complement receptor (CR1 gene) (+5507G) (Zorzetto *et al.* 2003), surfactant protein A1 (SFTPA1 gene) ($6A^4$ in nonsmokers), surfactant protein B (SFTPB gene) (1580C in smokers) (Selman *et al.* 2003), and angiotensin converting enzyme (ACE gene) D allele (Morrison *et al.* 2001).

TRANSFORMING GROWTH FACTOR-BETA

Characteristics

The Transforming growth factor-beta (TGF-β) superfamily is composed of the three TGF- β isoforms (β1, β2, and β3), Activins, Nodals, bone morphogenetic proteins (BMPs), growth and differentiation factor (GDF), and Mullerian inhibitory substance (MIF). TGF-β is a multifunctional cytokine regulating a variety of important biological responses including cell growth and differentiation, apoptosis, cell migration, immune cell function and ECM production (Sporn & Roberts 1992, Massague 1998). It also plays a key role in mediating lung remodeling and fibrosis via several effects (Bartram & Speer 2004). The active form of TGF-β is a 25 kDa dimer (a homodimer of two 12.5 kDa polypeptides) in which the two polypeptides interact via a disulfide bond and hydrophobic interactions. TGF-β is initially synthesized as a precursor protein which is proteolytically processed into an inactive (latent) form composed of mature TGF-β non-covalently bound to the amino-terminal precursor remnant termed latency-associated peptide (LAP) (Munger *et al.* 1997). After being secreted by the cell, latent TGF-β can be activated via transient acidification, alkanization, proteases (*e.g.* plasmin or cathepsin) or substances that induce conformational rearrangement, such as thrombospondin-1 (Grande 1997, Murphy-Ullrich & Poczatek 2000, Yang *et al.* 2007).

Signaling Pathways

Intracellular signaling of TGF-β occurs via two receptor serine/threonine kinases. The active form of TGF-β binds and brings together type I (TβRI) and type II (TβRII)

receptors on the cell surface to form tetrameric complexes (Shi & Massague 2003). Receptor activation occurs through phosphorylation of the tetrameric receptor complex (Piek *et al.* 1999). Many of the biological effects induced by TGF-β are associated with activation of diverse transcription factor systems, including Smad molecules, activating protein-1 (AP-1) and mitogen activated protein kinase (MAPK) family members (Derynck & Zhang 2003).

Binding of TGF-β ligand to TβRII initiates TGF-β signal transduction. TβRI is then recruited into the receptor complex, followed by stabilization of TβRI/TβRII heterotetrameric complexes, and phosphorylation of the kinase domain of TβRI by the constitutively autophosphorylated TβRII kinase. Phosphorylated TβRI then phosphorylates downstream signaling molecules (Eickelberg 2001).

The Smads are the mainly established intracellular effectors of TGF-β signaling. There are eight vertebrate Smads: Smad 1 to Smad 8. Smads are classified into three subgroups; receptor regulated Smads (R-Smads: Smad2 and 3 for TGF-β ligands and Smad1, 5, 8 for BMP ligands), which directly function as substrate molecules for phosphorylated type I receptors, the common mediator Smad (Co-Smad: Smad4), which is thought to be the binding partner for all receptor-regulated Smads, facilitating their translocation into the nucleus, and inhibitory Smads (I-Smads: Smad6 and 7), which inhibit the association of receptor-regulated Smads with the type I receptors and thereby antagonize TGF-β-induced effects (Massague 1998, Piek *et al.* 1999, Rahimi & Leof 2007). In Smad-dependent TGF-β signaling pathway, phosphorylated TβRI recruits and phosphorylates Smad2/3 molecules, which dissociate from TβRI, form dimers with

Smad4, translocate into the nucleus, and regulate transcription of target genes (Eickelberg 2001).

Besides Smad-mediated signaling, TGF-β activates other signaling cascades. TGF-β is also known to activate MAPK pathways including the extracellular signal-regulated protein kinase (Erk), c-Jun N-terminal kinase (JNK) and p38 MAPK pathways and this can occur independent of Smad phosphorylation (Engel *et al.* 1999, Yu *et al.* 2002, Derynck & Zhang 2003). Both TGF-β and BMP-4 can activate TGF-β-activated kinase 1 (TAK1), a MAPK kinase kinases (MAPKKK) family member (Yamaguchi *et al.* 1999). MAP/ERK kinase kinase 1 (MEKK1) may also function in the TGF- β -mediated activation of MAPKKs; thus, MEKK1 and TAK1 could activate JNK through MAPK kinase 4 (MKK4), and p38 MAPK through MKK3 or MKK6, in response to TGF- β. TGF- β /BMP signaling may also induce NF-κB signaling by activating TAK1 that can phosphorylate and activate IκB kinase and consequently stimulating NF-κB signaling (Derynck & Zhang 2003).

Many of the biological effects induced by TGF-β are associated with activation of diverse transcription factor systems, including activating protein-1 (AP-1) and MAPK family members. The cellular response to TGF-β can be extremely variable depending on the cell type and stimulation context. The preferential activation of one TGF-β signaling pathway vs. another (e.g. Smad2 vs. Smad3, or AP-1 vs. Smads) through biochemical or pharmaceutical modulation can therefore potentially modify the cellular response to TGF-β.

Role of TGF-β in IPF

While the responses elicited by TGF-β play a role in the normal physiology of tissue repair following injury, too often this process does not properly resolve and chronic pathological fibrotic conditions result. TGF-β1 is the isoform thought to play the most significant role in wound healing and possible subsequent fibrosis (Flanders 2004). In addition, TGF-β1 isoform was expressed in epithelial cells adjacent to fibroblastic foci of fibrotic lungs (Khalil *et al.* 1996). Source of TGF-β in fibrotic lung include several cell types including epithelial cells and macrophages of the terminal airways and alveoli as well as the subepithelial regions of dense fibroconnective tissue deposistion (Bartram & Speer 2004).

The conversion of inactive latent TGF-β to an active signaling molecule in the context of pulmonary fibrosis involves the epithelial integrin, αvβ6. This integrin is up-regulated in pulmonary fibrosis. αvß6 integrin knockout mice that cannot activate transforming growth factor (TGF)-β1 develop an exaggerated inflammatory response to bleomycin as a model of IPF, yet these knockout mice were protected from pulmonary fibrosis, indicating the critical role that TGF-β1 plays in the development of pulmonary fibrosis (Munger *et al.* 1999).

TGF-β has been referred to as a "master switch" in the induction of fibrosis in many organs, including the lung (Willis *et al.* 2006). TGF-β1 plays an essential role in the induction of EMT. EMT in many tissues, including retina, lens, and kidney, is induced via TGF-β signaling pathways (Saika *et al.* 2004, Roberts *et al.* 2006). Stimulation of Smad-independent TGF-β signaling pathways allows for induction of EMT within particular tissues. Cross-talk between classical TGF-β Smad-dependent

pathways and other modulatory signaling molecules, including Rho, Ras, ERK, p38 MAPK, Notch, Wnt proteins, NFκB, and PI3K, have all been demonstrated to affect the extent and reversibility of EMT (Masszi *et al.* 2003, Zavadil & Bottinger 2005).

Remodeling of lung tissue with deposition of connective tissue can be mediated by TGF-β via several effects. TGF-β1 can stimulate fibroblast differentiation to the myofibroblast phenotype and suppress myofibroblast apoptosis (Zhang & Phan 1999). TGF-β is also known as the most potent direct stimulator of collagen production in its three isoforms. In addition, TGF-β induces synthesis of other components of the ECM by pulmonary fibroblasts, such as fibronectin, glycosaminoglycans, and proteoglycans (Ignotz & Massague 1986, Raghu *et al.* 1989, Broekelmann *et al.* 1991, Grande 1997). TGF-β also induces chemotaxis of macrophages and monocytes and increases its own production in these cells (Border & Ruoslahti 1992), as well as the release of matrix glycoproteins such as fibronectin and various other mesenchymal growth factors (Lacronique *et al.* 1984). Inhibition of matrix degradation by changing gene transcription and modulation of cell-matrix interactions by modifying cell-matrix adhesion protein expression by TGF-β also play important role in tissue remodeling after injury and fibrosis (Grande 1997).

Seven polymorphisms in the human TGF-β1 gene have been identified. Three of the seven allelic variations are located in the 5' flanking region of the TGF-β1 gene, three are located in the coding region, and a C insertion is located in the 5' untranslated region (Cambien *et al.* 1996). The circulating concentrations of TGF-β1 varies between individuals and partly depends on two of the polymorphisms (SNPs) in the coding region affecting the amino acid-coding sequence of TGF-β1 gene; codon 10 (T or C: leucine or

proline), and codon 25 (G or C: arginine or proline) (Grainger *et al.* 1999). This effect of genetic variation on TGF-β1 production can influence diseases where TGF-β1 plays role, including IPF. A study of the TGF-β1 gene polymorphisms association with IPF showed that polymorphisms in codons 10 and 25 do not predispose to IPF development. However, codon 10 polymorphism affected disease progression in IPF patients, where the presence of proline allele in codon 10 of the TGF-β1 gene was associated with increased deterioration in gas exchange in patients with IPF (Xaubet *et al.* 2003).

TGF-β Signaling in Pulmonary Fibrosis

TGF-β activity via Smads has been implicated in the pathophysiology of pulmonary fibrosis (Nakao *et al.* 1999, Flanders 2004). However, stimulation of collagen and ECM deposition in lung fibroblasts is mediated by JunD, a member of AP-1 family of transcription factors (Eickelberg *et al.* 2001). JunD is also known as an antiapoptotic factor in fibroblasts (Hess *et al.* 2004).

AP-1 transcription factors are activated by MAPKs. JNK, one of the MAPKs, leads to induction of AP-1-dependent target genes (Karin & Gallagher 2005). Studies have suggested role of JNK in pulmonary fibrosis (Yoshida *et al.* 2002, Khalil *et al.* 2005, Shi-Wen *et al.* 2006). JNK was shown to mediate TGF-β – induced myofibroblast generation in human lung fibroblasts (Hashimoto *et al.* 2001). Activation of JNK by induction of its phosphorylation occurs in primary pulmonary fibroblast cultures treated with TGF-β (Khalil *et al.* 2005) and leads to induction of AP-1 dependent transcription (Shi-Wen *et al.* 2006).

Regulation of Angiotensinogen by TGF-β1

Angiotensin – TGF-β1 crosstalk in human IPF has been recently investigated. Studies by Uhal *et al.* showed that myofibroblast autocrine expression of AGT drives autocrine expression of TGF-β1, which in turn drives AGT constitutive expression to form an "autocrine loop". It is speculated that the autocrine loop recruits additional normal fibroblasts into the transition to myofibroblasts and forms an autonomous persistent profibrotic microenvironment as in human IPF (Uhal *et al.* 2007a). In that study, induction of the myofibroblast transition in the IMR90 human lung fibroblast cell line with TGF-β1 increased AGT mRNA levels. TGF-β1 treatment of the transfected cells increased luciferase reporter expression driven by AGT promoter to the similar increase in the endogenous AGT mRNA levels suggesting a transcriptional mechanism.

In a study on rat kidney proximal tubular cells, TGF-β1 was found to stimulate rat angiotensinogen gene expression as well. Data from that study supported that this action involves ROS generation, p38 MAPK activation, and p53 expression (Brezniceanu *et al.* 2006). Local intrarenal RAS activation was previously shown to be essential for TGF-β1 gene expression in renal proximal tubular cells (Zhang *et al.* 2002). Taken together, these also suggest that angiotensin and TGF-β1 may form a positive autocrine feedback loop to enhance their respective gene expression in other tissues.

CHAPTER 2:

INVESTIGATION OF GENES LOCALLY PRODUCING AND DEGRADING ANGIOTENSIN II PEPTIDE IN PULMONARY FIBROSIS

ABSTRACT

Several studies showed that the octapeptide angiotensin II (Ang II) plays an important role in alveolar epithelial cell apoptosis and hence contributes to fibrosis of the lung. Local production is believed to be the main source of Ang II in tissue fibrogenesis. Angiotensinogen (AGT) is the only known precursor to Ang II, while angiotensin converting enzyme-2 (ACE-2) acts on Ang II peptide to produce the opposing action heptapeptide angiotensin 1-7 (Ang 1-7). In this chapter, expression of these two genes (AGT and ACE-2) is investigated in idiopathic pulmonary fibrosis (IPF). In IPF patient lung tissue AGT mRNA and protein were increased (21-fold and 3.6-fold respectively, $p<0.05$) relative to normal lung tissues from normal subjects. In response to the profibrotic drug amiodarone, AGT mRNA level was increased in A549 human alveolar epithelial cells (350%, $p<0.01$) compared to cells without amiodarone treatment. This increase was completely blocked by actinomycin-D with no effect of amiodarone on AGT mRNA half-life. ACE-2 mRNA was markedly decreased in IPF patients (92%, $p<0.01$) relative to normal lung tissues from normal subject controls. Treatment of MLE12 mouse lung epithelial cells with the profibrotic peptide Ang II resulted in decreased ACE-2 mRNA level (45% reduction, $p<0.05$) that was reversed when cells were treated with the angiotensin receptor AT_1 antagonist losartan or with Ang 1-7 peptide. For future *in vivo* testing of ACE-2 roles in experimental pulmonary fibrosis models, alveolar epithelial cell-specific short hairpin RNA (shRNA) expression plasmid was designed and constructed to express ACE-2 shRNA for ACE-2 gene silencing. This

plasmid showed successful knockdown of ACE-2 in alveolar epithelial cells but not in lung fibroblasts indicating specificity of shRNA expression. Overall, data from this chapter suggest impairment of balance between Ang II production and degradation in conditions promoting pulmonary fibrosis.

EXTRAVASCULAR SOURCES OF LUNG ANGIOTENSIN PEPTIDE SYNTHESIS IN IDIOPATHIC PULMONARY FIBROSIS

INTRODUCTION

Angiotensinogen (AGT) is the only known precursor to angiotensin II (Ang II), the active peptide of the renin angiotensin system (RAS). Local activation of RAS plays a key role in fibrogenic response of the lung tissue by mechanisms independent of the blood-derived RAS. Several lines of evidence suggest that the source(s) of precursor for the Ang II synthesis that drives fibrogenesis in the lung is generated locally, i.e., within the lung tissue itself. Cultured AEC of either human or rat origin synthesize angiotensinogen (AGT) mRNA and secrete Ang peptides on exposure to proapoptotic stimuli such as Fas ligand (Wang *et al.* 1999b), TNF-α (Wang *et al.* 2000a), or bleomycin (Li *et al.* 2003b). Ang II itself also induces apoptosis of AEC through angiotensin receptor AT_1 (Wang *et al.* 1999c). In addition, myofibroblasts isolated from the lungs of patients with idiopathic pulmonary fibrosis (IPF) also synthesize AGT mRNA constitutively and secrete Ang peptides (Wang *et al.* 1999a). The notion that local pulmonary synthesis of Ang II *de novo*, i.e., from the precursor AGT within the lung, is required for lung fibrogenesis is supported by the recent demonstration that intratracheal administration of antisense oligonucleotides against AGT mRNA could prevent bleomycin-induced lung fibrosis in rats without affecting circulating levels of AGT protein (Li *et al.* 2007).

Prior to this study, the evidence in support of local pulmonary synthesis of AGT protein *de novo* is derived from either animal models (Li *et al.* 2003a, 2007,) or indirect studies of primary cells isolated from fibrotic human lung (Wang *et al.* 1999a). In the present study it was therefore of interest to directly examine fibrotic human lung tissue in an attempt to identify local tissue sources of Ang peptide generation. The findings of Ang peptide expression in at least two cell types, apoptotic AEC and myofibroblasts, in lung tissue from patients with IPF are reported here.

METHODS

Tissue Samples

Human lung tissue was obtained by open lung biopsy or video-assisted thoracoscopic surgery performed at Instituto del Tórax, Hospital Clínic de Barcelona from patients with idiopathic pulmonary fibrosis (IPF) or normal subjects (CTL). Biopsy tissues were used for total RNA isolation, protein extraction and formalin fixation-paraffin embedding for sections (details in appendix A).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from biopsies with TRI reagent (Molecular Research Center) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA with Superscript II reverse transcriptase (Invitrogen) and oligo $(dT)_{12-18}$. Real-time RT-PCR was performed with cDNA synthesized from 50 ng of total RNA, SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA) according

to the manufacturer's instructions, and 0.2 μM specific primers for human AGT (forward 5'-GAG CAA TGA CCG CAT CAG-3' and reverse 5'-CAC AGC AAA CAG GAA TGG-3') and β-actin (forward 5'-AGG CCA ACC GCG AGA AGA TGA CC-3' and reverse 5'-GAA GTC CAG GGC GAC GTA GC-3'), which produce PCR products of 151 and 332 bp, respectively. The PCR thermal profile started with 10-minute activation of *Taq* polymerase at 95°C followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 37 seconds, and extension at 72°C for 37 seconds, ending with dissociation curve analysis to validate the specificity of the PCR products. Reactions were performed in Mx3000P machine (Stratagene, La Jolla, CA) and threshold cycle (CT) data were collected with MxPro-Mx3000P software version 3.0. The relative AGT expression was normalized to β-actin and calculated with the comparative CT method of $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{AGT} - CT_{\beta\text{-actin}}$ and $\Delta\Delta CT = \Delta CT_{IPF} - \Delta CT_{Control}$.

Cell Culture

The human lung cell line A549 was cultured as described previously (Wang *et al.* 1999a, 1999b). The primary human lung fibroblast strain N13, isolated from normal human lung, was recovered from cryostorage and cultured as described previously (Wang *et al.* 1999a). Before analysis, cells were switched from growth medium containing fetal bovine serum to serum-free medium (Ham's F-12) for at least 2 days before harvesting. Immunoreactive AGT was detected by Western blotting of cells lysed in buffer containing NP-40 detergent and a commercially available protease inhibitor cocktail.

Western Blotting

Protein was extracted from biopsy samples by tissue homogenization in ice-cold Tris-buffered saline pH 8.0, supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and tributylphosphine. Soluble protein extracts of tissues (10 μg) were diluted 1:2 in Laemmli sample buffer (Bio-Rad, Hercules, CA), loaded on 10% Tris·HCl polyacrylamide gels, separated by SDS-PAGE, and then transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) in Towbin buffer containing 25mM Tris, 192 mM glycine, 20% methanol. Blotting membrane was blocked by 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. Western blot analysis of AGT was performed with anti-Ang peptide antibody (1:400 dilution; Santa Cruz Biotechnology). Bands were visualized by HRP-conjugated donkey anti-goat secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology) and the chemiluminescent substrate Super Signal West Femto Maximum Sensitivity (Pierce, Rockford, IL). Images of the chemiluminescence-exposed film were analyzed for band intensity with Scion Image software (release beta 4.0.2) and normalized to total protein band intensities obtained by silver staining of SDS-polyacrylamide gels of replicate biopsy extracts. Silver staining was performed with a commercially available kit (Silver Stain Plus, Bio-Rad) according to the manufacturer's instructions.

Histology and Labeling

In situ end labeling of fragmented DNA, immunohistochemistry (IHC) and in situ hybridization (ISH) were performed to localize apoptotic cells, AGT mRNA and Ang

peptides in normal and IPF lung tissue formalin fixed – paraffin embedded sections (details in appendix A).

RESULTS

AGT mRNA is More Abundant in IPF Tissue

To obtain a quantitative assessment of Ang peptide expression in normal versus fibrotic human lung, real-time RT-PCR for AGT and β-actin mRNAs was performed on total RNA isolated from lung biopsies from five IPF patients and four normal subjects without fibrotic lung disease. In Figure 2.1, AGT mRNA was found to be 21-fold more abundant in IPF lung tissue relative to the control specimens of human lung ($P < 0.05$).

AGT Protein is More Abundant in IPF Tissue

By western blotting with the same antibodies used for Immunohistochemistry, two bands of differing molecular mass, which represent the two isoforms of human AGT, were detected in a commercially available purified AGT standard isolated from human serum (Figure 2.2, *lane 1*) and in IPF lung (Figure 2.2, *lanes 8–12*). In human serum, the higher (~61 kDa)- and lower (~58 kDa)-molecular-mass isoforms of AGT were detected in apparently similar abundance. Cultured human lung epithelial cells (A549; Figure 2.2, *lane 2*) and primary human lung fibroblasts (N13; Figure 2.2, *lane 3*) expressed exclusively the 61-kDa isoform of AGT. Lung biopsies from both normal lung (Figure 2.2, *lanes 4–7*) and IPF lung (Figure 2.2, *lanes 8–12*) contained primarily the higher-molecular-mass isoform of AGT expressed by the isolated lung cells, although IPF

biopsies did contain some of the low-molecular-mass isoform. Figure 2.3 shows quantitation of immunoreactive AGT in total protein extracts from a panel of lung biopsy specimens and purified human lung cells. Densitometric quantitation of the higher-molecular-mass (~61 kDa) isoform of AGT only relative to total protein revealed 3.6-fold higher levels of the 61-kDa isoform of AGT in IPF lung biopsies compared to nonfibrotic (CTL) lung ($P < 0.05$; Figure 2.2).

Localization of AGT mRNA and Protein

Both AGT mRNA and protein in the IPF lung co-localized to both apoptotic epithelia and myofibroblastic foci suggesting that apoptotic alveolar epithelial cells (AECs) and myofibroblasts constitute key sources of locally derived ANG peptides in the IPF lung (details in appendix A).

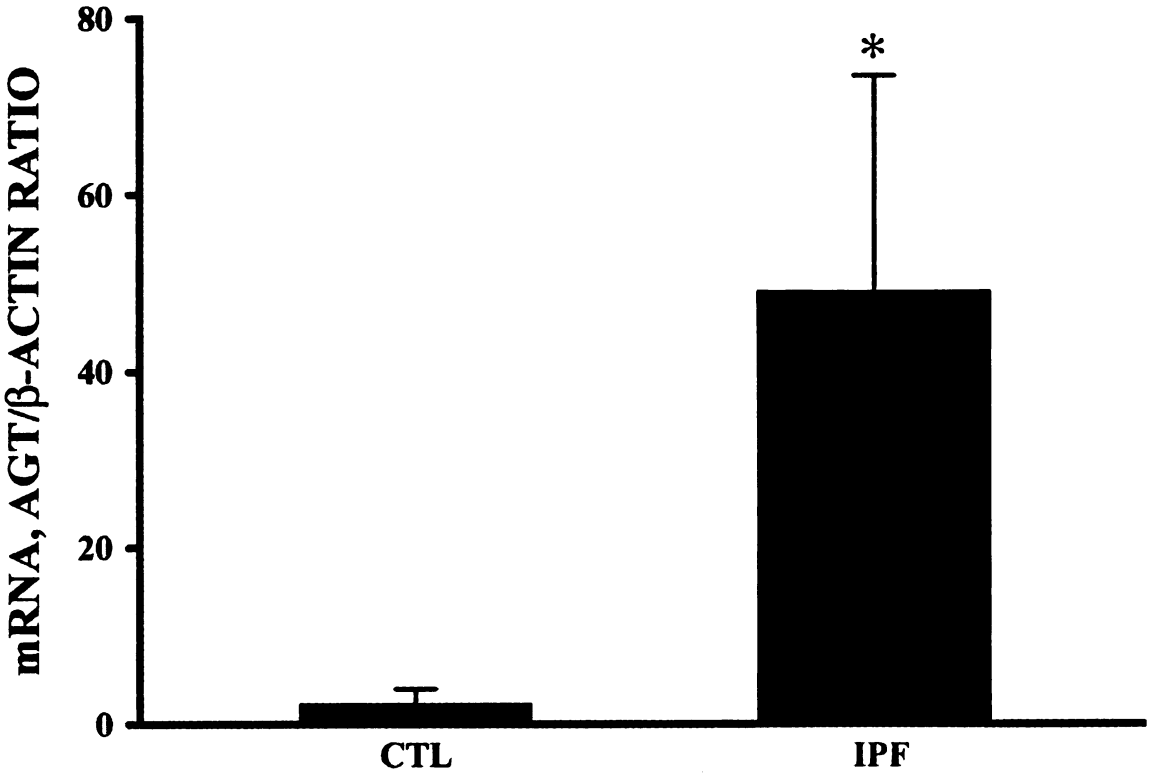


Figure 2.1.

Quantitative RT-PCR of AGT mRNA in normal and fibrotic human lung. Fresh IPF or normal control (CTL) lung tissue was obtained by biopsy and immediately prepared for isolation of total RNA (see METHODS). Real-time RT-PCR was performed for both AGT and β-actin mRNAs as described in METHODS. Bars are means + SE of data collected from the biopsies of 4 (CTL) and 5 (IPF) separate patients; $*P < 0.05$ by Mann-Whitney test.

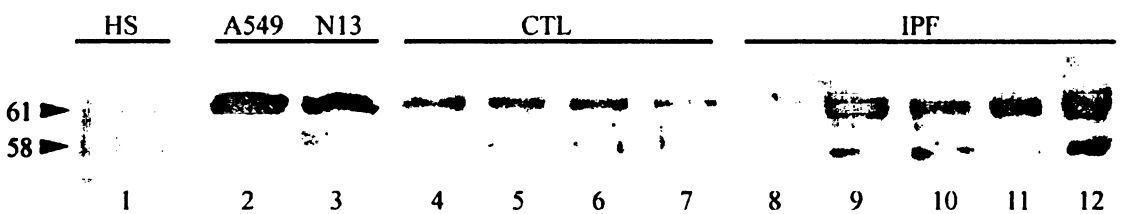


Figure 2.2.

Western blotting of AGT protein in normal and fibrotic human lung and isolated lung cells. Fresh IPF or normal control lung tissue was obtained by biopsy and immediately flash frozen for isolation of total protein (see METHODS). Western blotting was performed as described in METHODS; equal amounts of total protein per lane were loaded for *lanes 4–12*. *Lane 1*, positive control of AGT protein purified from human serum (HS); note 2 isoforms of AGT at ~58 and 61 kDa in similar abundance. *Lanes 2* and *3*, positive AGT protein controls of A549 cells (*lane 2*) or N13 primary human lung fibroblasts isolated from normal lung (*lane 3*). Note expression of high-molecular-mass isoform (top band, ~61 kDa) of AGT by isolated lung cells. *Lanes 4–12*, immunoreactive AGT in lung biopsies from normal (CTL, *lanes 4–7*) or IPF (*lanes 8–12*) patients.

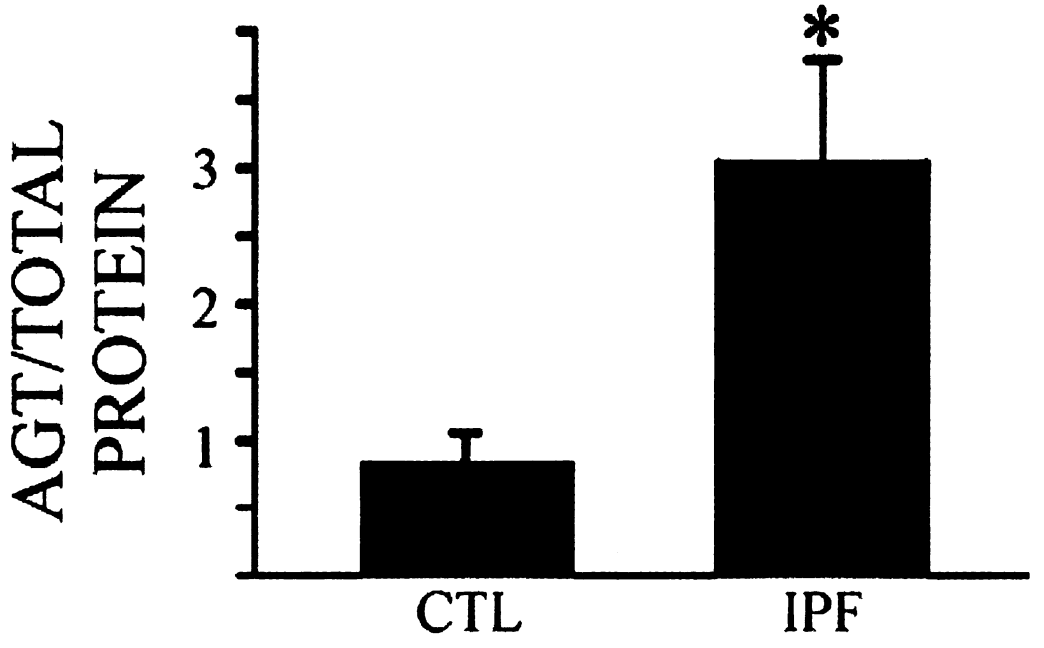


Figure 2.3.

Quantitation of AGT protein in normal and fibrotic human lung and isolated lung cells. Densitometry of AGT 61-kDa bands (from Figure 2.2) was determined as described in METHODS and expressed as AGT – to – total protein ratio. Bars are means + SE of data collected from biopsies of 4 (CTL) and 5 (IPF) separate patients. $*P < 0.05$ by Student's *t*-test.

DISCUSSION

In the study presented herein we show that local synthesis of AGT is upregulated in the human IPF lung tissue. Here we present evidence of increased AGT gene expression in the fibrotic lung tissue compared with normal lung tissue (Figure 2.1). This increased AGT mRNA is consistent with earlier *in vitro* cell culture studies on human or animal AECs and human IPF myofibroblasts, where AGT mRNA was upregulated in AECs exposed to proapoptotic factors and in IPF myofibroblasts isolates (Wang *et al.* 1999a, 1999b, 1999c, 2000a, Li *et al.* 2003b). The AGT mRNA in IPF tissue localization to both cuboidal epithelia and α-actin-positive foci by ISH (appendix A), supports the contention that the Ang peptides are being synthesized *de novo* in AEC and myofibroblasts rather than being sequestered, for example, from other sources such as the blood.

Earlier studies on the fibrotic human lung showing that myofibroblasts and apoptotic AECs cell types were capable of synthesizing Ang peptides *de novo* were performed *in vitro* in cell culture (Wang *et al.* 1999a, 1999b). The study presented here provides evidence that these same cell types also synthesize Ang peptides *in situ* in the intact fibrotic human lung by IHC (appendix A). The Ang peptide antibody used in this study, which was derived against the peptide Ang I, recognizes Ang I, Ang II, and the two isoforms of AGT protein found in serum (Figure 2.2). Given that the ~58- and ~61-kDa isoforms of AGT are both found in human serum, whereas isolated lung cells express only the 61-kDa isoform (Figure 2.2), the finding of both isoforms in IPF lung biopsies suggests that some of the increase in lung tissue AGT in the IPF specimens may be due to serum-derived AGT. On the other hand, the observation that the 61-kDa isoform

expressed by isolated lung cells was more highly abundant than the 58-kDa form in both normal and IPF lung supports the theory that most of the increase in lung tissue AGT protein (Figure 2.3, Figure 2.4) in IPF occurs through *de novo* synthesis of AGT within the lung. Given the roles of Ang II in stimulating collagen deposition in the lungs (Li *et al.* 2003a, Marshall *et al.* 2004) and other organs (Klahr & Morrissey 1997, Weber & Sun 2000, Yoshiji *et al.* 2001), it is theorized that the Ang peptides produced by apoptotic AEC and myofibroblasts contribute to the fibrogenic response in IPF lung.

In summary, both AGT mRNA and protein were upregulated in the IPF lung and co-localized to both apoptotic epithelia and myofibroblast foci, suggesting that apoptotic alveolar epithelial cells (AECs) and myofibroblasts constitute key sources of locally derived Ang peptides in the IPF lung. Determinations of the mechanisms by which AGT expression is regulated in epithelial and myofibroblast lung cells are discussed in this chapter and next chapter.

AMIODARONE INDUCES ANGIOTENSINOGEN GENE EXPRESSION IN LUNG ALVEOLAR EPITHELIAL CELLS THROUGH ACTIVATION PROTEIN-1

INTRODUCTION

Amiodarone is a widely used and very effective drug for the treatment of cardiac arrhythmias (Wilson & Lippmann 1990, Piepoli *et al.* 1998). Despite its effectiveness and general safety, amiodarone has several side effects that limit the maximal dose (Kennedy *et al.* 1987); the most severe of these is pulmonary toxicity that includes a fibrotic response. This fibrosis is reproducible in animal models subjected to long-term administration of amiodarone (Carvalho *et al.* 1996, Uhal *et al.* 2003).

Several lines of evidence suggest that direct cytotoxicity of amiodarone to various lung cell types could contribute to the lung toxicity. This direct cytotoxicity was shown in cell cultures of alveolar macrophages (Ogle *et al.* 1990), lung fibroblasts (Martin *et al.* 1985), human pulmonary artery endothelial cells (Powis *et al.* 1990) and lung alveolar epithelial cells (Bargout *et al.* 2000). Induction of alveolar type II pneumocytes apoptosis by amiodarone was inhibited by angiotensin-converting enzyme inhibitors, or the angiotensin receptor (AT_1)-selective antagonist losartan (Bargout *et al.* 2000). In addition, systemic administration of either an angiotensin-converting enzyme inhibitor or losartan prevents amiodarone – induced lung fibrosis in rats (Uhal *et al.* 2003).

Work prior to this study showed up-regulation of angiotensinogen expression in AECs by other apoptosis inducers, including Fas ligand (Wang *et al.* 1999b, 1999c), tumour necrosis factor-α (Wang *et al.* 2000a) and the antineoplastic agent bleomycin (Li *et al.* 2003b). Accordingly, this study addressed the possibility that amiodarone might also induce angiotensinogen expression in AECs, and begins to examine the mechanisms controlling angiotensinogen gene expression in these cells. This study reports here that amiodarone up-regulates angiotensinogen gene expression in human alveolar epithelial cells through a mechanism mediated by activation protein-1 (AP-1) family transcription factors and that requires an AP-1 binding site close to the transcription start site.

METHODS

Cell Culture and Treatment

Human lung adenocarcinoma cell line A549 was obtained from the American Type Cell Culture Collection and cultured in complete growth medium; Ham's F12 medium supplemented with 10% fetal bovine serum. Cells were grown in 6-well culture plates and were analyzed at subconfluent densities of ~80%. All subsequent incubations with amiodarone and/or actinomycin-D were performed in serum-free Ham's F12 medium after 24 hours of serum starvation.

RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from the human A549 cells using Trizol Reagent® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First strand cDNA

was synthesized from total RNA using Superscript II Reverse Transcriptase and oligo $(dT)_{12\text{-}18}$ (Invitrogen). Real-time RT–PCR was performed as described on page (35) on 50ng cDNA from human A549 cells, using the same specific primers for human AGT, and β-actin that yield a final PCR products of 151 bp and 332 bp respectively. The relative AGT expression was normalized to β-actin and calculated with the comparative CT method of $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{AGT} - CT_{\beta\text{-actin}}$ and $\Delta\Delta CT = \Delta CT_{Treatment} - \Delta CT_{Control}$.

Half-life determinations of angiotensinogen mRNA

To determine relative AGT mRNA stability, a method modified from Chen *et al.* was adopted (Chen *et al.* 2005). A549 cells were serum-starved for 24 hr, were treated with or without amiodarone, and 20 hr later actinomycin-D was added at 2 μg/ml. Cells were then harvested for total RNA extraction at 0, 3, 6 and 9 hr after actinomycin-D addition. Triplicate treatments for each time point were used in real-time RT–PCR measures of angiotensinogen relative to β-actin.

Mechanism of Amiodarone – Induced AGT Gene Transcription

The mechanism of amiodarone – induced AGT gene transcription was studied with a reporter construct containing the firefly luciferase gene immediately downstream of a portion of the human angiotensinogen gene, specifically 1013 bp (-991 to +22 bp) of the angiotensinogen promoter. Deletion mutants of the angiotensinogen/luciferase (AGT-LUC) promoter-reporter were constructed and were tested for the ability to eliminate responsiveness to amiodarone as well as signal transducer activator of transcription

(STAT) sites deletions and activation protein (AP-1) sites mutant reporters. DNA/protein array was used for screening of transcription factor binding activity in response to amiodarone and confirmed with electrophoretic mobility shift assay (as described in appendix B).

RESULTS

Amiodarone Induces AGT Gene Transcription

Figure 2.4 shows that amiodarone (AMIO, 3 μg/ml) increased steady state AGT mRNA in A549 cells by 350% in 20 hours compared with AGT mRNA level in cells that had no amiodarone treatment. AGT mRNA level was measured relative to the housekeeping gene β-actin mRNA level by real-time RT-PCR. To test whether amiodarone increases AGT mRNA via transcription, the RNA polymerase inhibitor actinomycin-D (ACT-D, 2 μg/ml) was added to A549 cells for 30 minutes prior to amiodarone treatment. Amiodarone – treated A549 cells that had actinomycin-D treatment showed complete blocking of relative AGT mRNA levels up-regulation due to inhibition of transcription initiation by actinomycin-D.

Amiodarone Does Not Affect the Decay Rate of AGT mRNA

Sequential real-time RT-PCR measurements of AGT mRNA levels relative to β-actin after the addition of actinomycin-D were normalized to zero time-point level of each treatment group (with or without prior amiodarone addition) being 100%.

Normalized levels were plotted against time of incubation with actinomycin-D to reflect AGT mRNA stability relative to the stable housekeeping gene β-actin mRNA (Figure 2.5). Figure 2.5 shows that the relative AGT mRNA stability is similar in both amiodarone – treated or untreated cells. These results revealed no effect of amiodarone on the half-life of the mRNA.

Amiodarone Induces AGT Expression Through AP-1

AGT-LUC was induced by amiodarone, with two amiodarone-responsive domains in the angiotensinogen promoter between -350 to -260 bp and -203 to -46 bp. DNA/Protein array and electrophoretic mobility shift assays showed that amiodarone increases DNA binding of both AP-1 and STAT-5 transcription factors. Mutation of STAT binding sites within the amiodarone-response domains had no effect on amiodarone-induced AGT-LUC while mutagenesis of the AP-1 binding site at -13 bp completely eliminated the response to amiodarone indicating its role on amiodarone response (see appendix B).

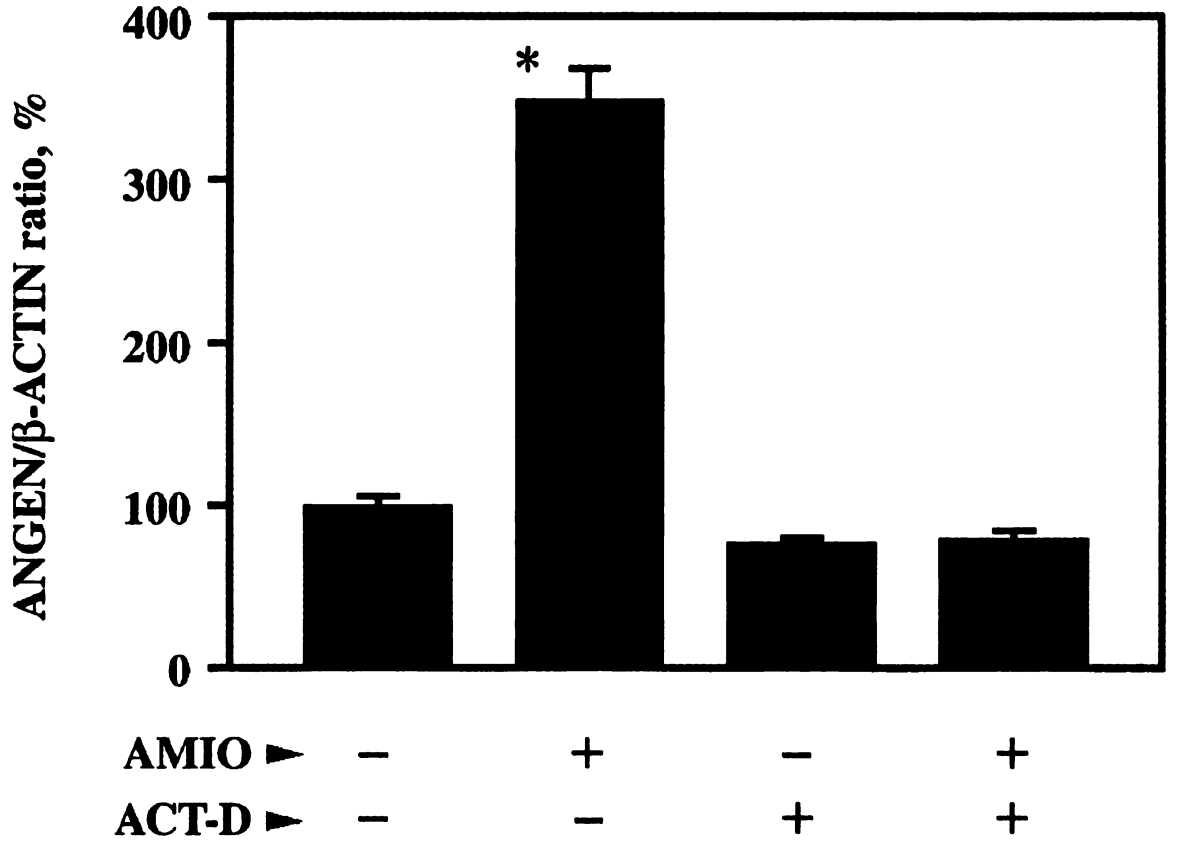


Figure 2.4.

Amiodarone up-regulates angiotensinogen gene expression in the A549 adenocarcinoma cell line. A549 cells were exposed to amiodarone (AMIO, 3 μg/ml) in serum-free medium for 20 hr, in the presence and absence of actinomycin–D (ACT-D, 2 μg/ml). Data represent real-time RT–PCR measurements of angiotensinogen mRNA (ANGEN), relative to β-actin. Bars are the means + S.E.M. of n = 6; *P < 0.01 by analysis of variance and Student–Newman–Keul's test.

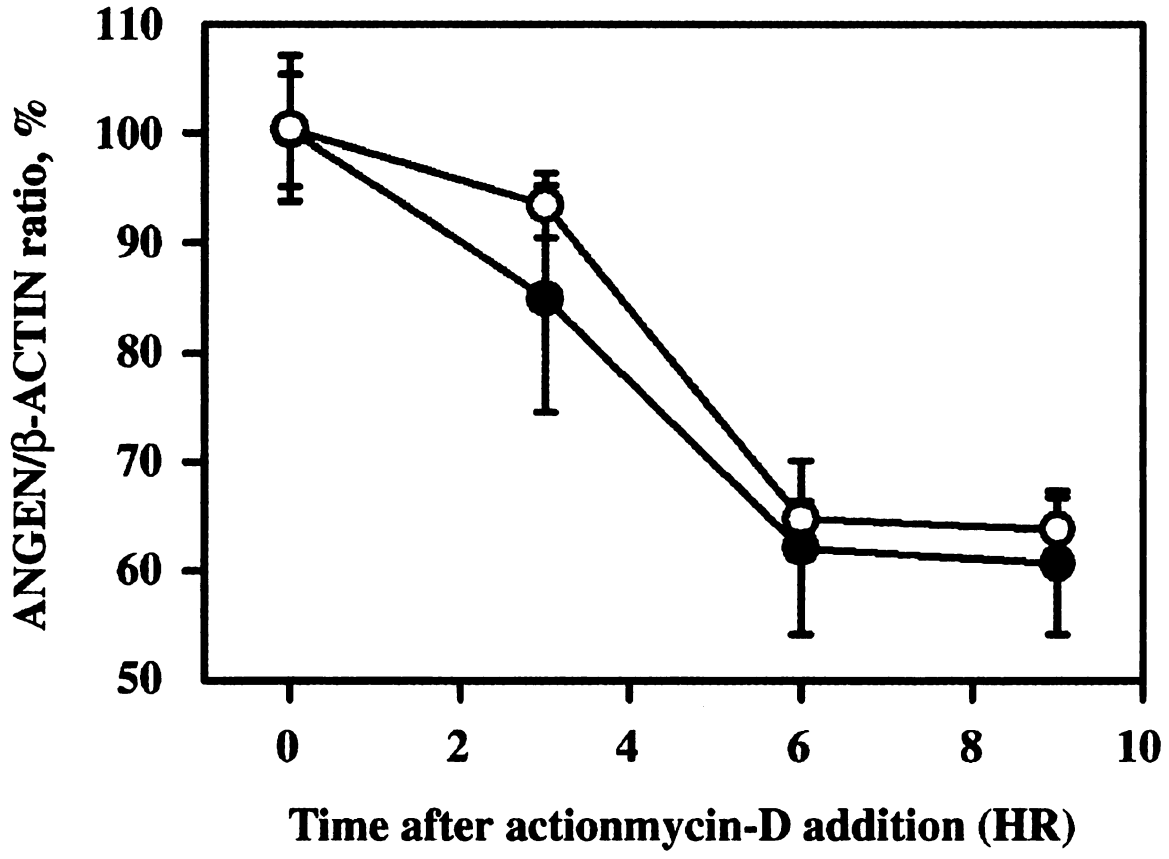


Figure 2.5.

Amiodarone does not affect the decay rate of angiotensinogen mRNA. A549 cells were treated with (○) or without (●) amiodarone (AMIO, 3 μg/ml) in serum-free media. Twenty hours later, actinomycin-D was added at 2 μg/ml. Cells were harvested for total RNA extraction at 0, 3, 6 and 9 hr after actinomycin-D addition. Data represent real-time RT–PCR measurements of angiotensinogen mRNA (ANGEN), relative to β-actin. Bars are the means ± S.E.M. of n = 3.

DISCUSSION

Previous work from this laboratory demonstrated that exposure of alveolar epithelial cells to the apoptosis inducers Fas ligand, tumour necrosis factor-α or bleomycin resulted in up-regulation of AGT gene expression (Wang *et al.* 1999b, 1999c, 2000b, Li *et al.* 2003b). In each of those prior studies, the proteolytic processing of angiotensinogen to the peptide Ang II was found to be required for the apoptotic response. An earlier study of alveolar epithelial cells exposed to amiodarone, demonstrated blockade of amiodarone-induced apoptosis by the angiotensin-converting enzyme inhibitor captopril or the angiotensin receptor antagonist saralasin (Bargout *et al.* 2000), suggesting that amiodarone might also up-regulate AGT gene expression in alveolar epithelial cells.

The experiments reported here provide direct evidence in support of this theory by demonstrating that amiodarone up-regulates AGT mRNA in either primary rat alveolar epithelial cells (appendix B) or in the human alveolar epithelial cells-derived A549 cell line (Figure 2.4). The notion that the up-regulation occurs primarily at the transcriptional level is supported by the ability of actinomycin-D to prevent the amiodarone-induced increase in angiotensinogen mRNA (Figure 2.4), by the failure of amiodarone to affect the decay rate of angiotensinogen mRNA (Figure 2.5) and by the quantitative similarity between inducible angiotensinogen mRNA levels measured by real-time PCR (~3.2 times, Figure 2.4) and amiodarone-inducible luciferase activity measured through the angiotensinogen/luciferase reporter system (~2.9 times, appendix B).

In the liver, the human AGT promoter is well documented to be regulated by interleukin-6 via three STAT binding sites (Sherman & Brasier 2001, Brasier *et al.* 1994).

However, mutation of all three STAT sites, either individually or together, failed to eliminate the AGT promoter response to amiodarone, excluding the possibility of AGT regulation by STAT in response to amiodarone (appendix B).

On the other hand, AP-1 family transcription factors have been implicated in the induction of apoptosis in a variety of cell types including AECs of rat and mouse as well as other epithelial cell types such as keratinocytes and hepatocytes (Hess *et al.* 2004, Janssen *et al.* 1997, Timblin *et al.* 2002). Considering that amiodarone causes both AECs apoptosis (Bargout *et al.* 2000), and induces AGT expression (Figure 2.4), it was hypothesized that AP-1 mediates AGT expression. In support of this hypothesis, the data obtained on mechanisms of AGT gene transcription induction by amiodarone (Appendix B); ability of amiodarone to increase AP-1 binding to DNA, the sufficiency of AP-1 expression to induce AGT expression and the abrogation of amiodarone-induced AGT expression in A549 cells by a-Fos (the AP-1 dominant negative) and AP-1 binding site mutation, suggest that amiodarone induces AGT transcription via AP-1 family of transcription factors action on AP-1 binding site between TATA box and transcription start site. This is consistent with the critical role played by this *cis*-regulatory sequence in AGT transcription as shown previously in the human hepatoma cell line HepG2 cells (Yanai *et al.* 1996).

In summary, this report is the first study of the molecular mechanisms regulating AGT expression by pulmonary alveolar epithelial cells. The data presented here show that AP-1 family transcription factors mediate amiodarone-induced AGT expression in human alveolar epithelial cells and identify an AP-1 site, located between the TATA box and the transcription initiation site, which is required for the response.

ANGIOTENSIN CONVERTING ENZYME-2 IS DOWNREGULATED IN HUMAN AND EXPERIMENTAL LUNG FIBROSIS

INTRODUCTION

The biological half-life of the peptide Ang II is very short, on the order of thirty seconds to fifteen minutes in the serum and local tissue compartments, respectively (Filippatos *et al.* 2001, Van Kats *et al.* 1997). One reason for its short half-life is the abundant protease activities capable of degrading the peptide. One of these is the carboxypeptidase angiotensin converting enzyme-2 (ACE-2), which cleaves a single amino acid from the C-terminal end of Ang II to yield the heptapeptide Ang 1-7 (Imai *et al.* 2005, Figure 2.6). ACE-2 is now known to be the receptor for binding and entry of the severe acute respiratory syndrome (SARS) coronavirus (Li *et al.* 2003c), which causes severe acute lung injury and death of many infected patients. Earlier studies of knockout mice have shown a clear protective effect of ACE-2 on experimental acute lung injury in response to acid aspiration or sepsis (Imai *et al.* 2005); the protective effect of ACE-2 was associated with reduced levels of Ang II after experimental lung injury. A study on rat brain astrocytes showed that Ang II downregulates ACE-2 mRNA through AT_1 receptor in a positive feed-forward system that favors Ang II – mediated responses (Gallagher *et al.* 2006).

On this basis we hypothesized that the pathogenesis of lung fibrosis might involve the downregulation of ACE-2 that can be mediated by Ang II and antagonized by Ang 1-7. We report here the findings that ACE-2 mRNA is severely decreased in human lung fibrosis. Moreover, we show that Ang II causes AT_1 – dependent decrease of ACE-2

mRNA that is reversed by Ang 1-7 in mouse AECs. Finally, in this chapter we present the construction of cell-specific ACE-2 gene silencing plasmid utilizing short hairpin RNA (shRNA) silencing that can be used as a tool for future investigations of ACE-2 roles in IPF.

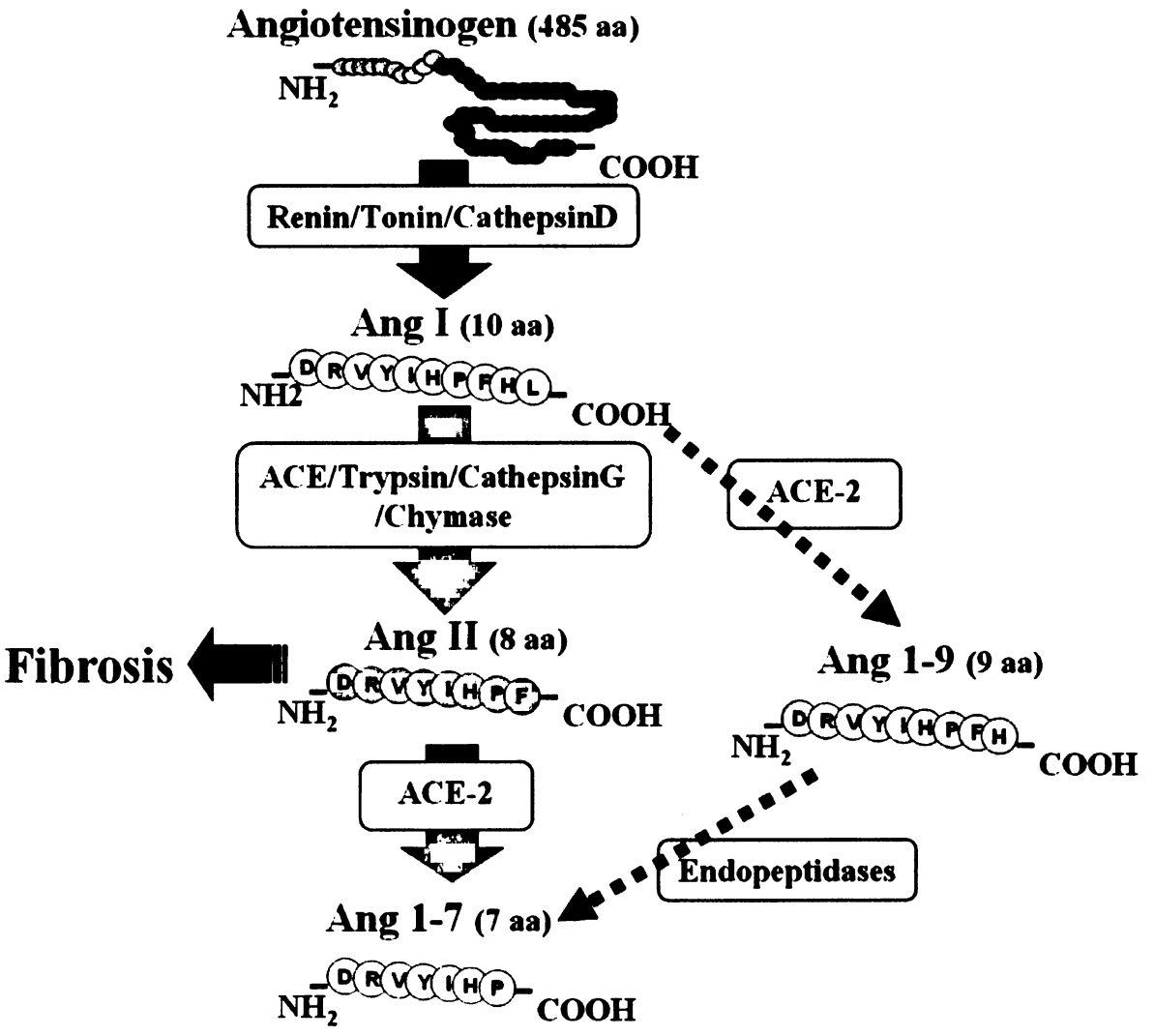


Figure 2.6.

Angiotensin (1-7) production by angiotensin converting enzyme-2. The diagram shows production of the angiotensin peptide angiotensin 1-7 (Ang 1-7) by sequential enzymatic cleavage of angiotensinogen protein. Alternative pathway for Ang 1-7 production is indicated by dashed arrow lines Angiotensin I: Ang I, angiotensin II: Ang II, angiotensin 1-9: Ang 1-9, ACE: angiotensin converting enzyme, ACE-2: angiotensin converting enzyme-2, aa: amino acid.

METHODS

Tissue Samples

Human lung tissue was obtained by open lung biopsy or video-assisted thoracoscopic surgery performed at Instituto del Tórax, Hospital Clínic de Barcelona from patients with idiopathic pulmonary fibrosis (IPF) or normal subjects. Biopsy tissues were used for total RNA isolation as mentioned previously (page 35).

Cell Culture and Transfection

The mouse lung epithelial (MLE12) and mouse lung fibroblast (MLg) cell lines was obtained from the American Type Culture Collection (ATCC) and cultured in HITES medium for MLE12 or Eagle's Minimum Essential Medium (EMEM) for MLg supplemented with 2% or 10% fetal bovine serum respectively according to ATCC's guidelines. Before analysis, cells were switched from growth medium containing fetal bovine serum to serum-free medium for at least 1 day before harvesting in Trizol reagent (Invitrogen) for RNA extraction or in NP-40 lysis buffer for protein extraction. For plasmid transfections, cells were transfected with plasmid DNA using Lipofectamine2000 transfection reagent (Invitrogen) in serum-free conditions.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from biopsies with TRI reagent (Molecular Research Center) and from cell cultures with Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized and real-time RT-PCR was

performed as mentioned previously (page 35) using the appropriate specific primers for human ACE-2 (forward: 5'- CAT TGG AGC AAG TGT TGG ATC TT -3', reverse: 5'- GAG CTA ATG CAT GCC ATT CTC A -3') and human β-actin (forward: 5'- AGG CCA ACC GCG AGA AGA TGA CC -3', reverse 5'- GAA GTC CAG GGC GAC GTA GC -3'), or mouse ACE-2 (forward: 5'- GGA TAC CTA CCC TTC CTA CAT CAG C -3', reverse: 5'- CTA CCC CAC ATA TCA CCA AGC A -3') and mouse β-actin (forward: 5'- TCC TGT GGC ATC CAT GAA ACT -3', reverse 5'- CTT CGT GAA CGC CAC GTG CTA -3'). The PCR thermal profile started with 10 minutes activation of *Taq* polymerase at 95°C followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, ending with dissociation curve analysis to validate the specificity of the PCR products. Data were collected and analyzed as mentioned in page (35).

Western Blotting

Protein was extracted from cells by harvesting in NP-40 lysis buffer supplemented with protease inhibitor cocktail (Roche). Equal amounts of protein (45 μg) were separated by SDS-PAGE, transferred to membrane and western blotting was performed as mentioned earlier on page 37 using antibodies against mouse ACE-2, and β-actin proteins (Santa Cruz Biotechnology).

Construction of Cell – Specific ACE-2 shRNA Plasmids

ACE-2 shRNA sequences were cloned downstream of human surfactant protein-C (hSP-C) promoter in pUC18 plasmid. Two clones were constructed; each clone has

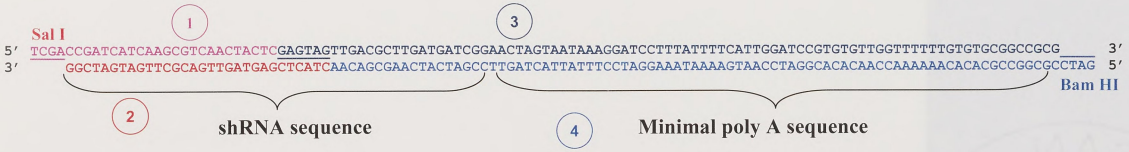
different shRNA target sequence for mouse ACE-2 (mACE-2). For these clones, two shRNA sequences were selected from MISSION® shRNA sequences for mACE-2 (Sigma-Aldrich); TRCN0000031146 and TRCN0000031147 to make plasmids si-1 and si-2 respectively. This selection is based upon the ability of lentiviral transduction particles expressing these shRNA sequences driven by U6 promoter to knockdown mACE-2 in MLE12 cells (data not shown). Four single strand oligonucleotides were synthesized for each shRNA (IDT DNA Technologies); sequences of these oligos are shown in Figure 2.7. For each shRNA, pairs of oligos (1+2) and (3+4) were annealed in equimolar ratio in STE buffer (10 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA). These oligos code for mACE-2 shRNA followed by minimal polyadenylation signaling sequence (Figure 2.8). The addition of the minimal poly A sequence is recommended for shRNAs transcribed by the RNA polymerase Pol II (Xia *et al.* 2002). The human surfactant protein-C (hSP-C) promoter, a Pol II promoter, was utilized to induce alveolar epithelial cell-specific expression of ACE-2 shRNA. Figure 2.9 shows the steps of shRNA plasmid construction from pGP22 plasmid, a kind gift from Dr. Michael O'Reilly, University of Rochester. The pGP22 plasmid contains hSP-C promoter and SV40 small-t intron and poly A signal in pUC18 vector (Roper *et al.* 2003). The 0.4kb SV40 small-t intron and poly A signal were excised from pGP22 by digestion with Sal I and Bam HI restriction endonucleases. The linear pUC18 plasmid containing hSP-C promoter was ligated with the double stranded synthetic oligonucleotides with complementary (sticky) ends for Sal I and Bam HI (Figure 2.9) to make plasmid for mACE-2 shRNA expression driven by hSP-C promoter. The ligated construct was transformed and replicated in DH5α competent bacterial cells (Invitrogen). The shRNA

plasmid constructs were checked by DNA sequencing using the pUC18 sequencing primer M13-reverse in Michigan State University's Research Technology Support Facility.

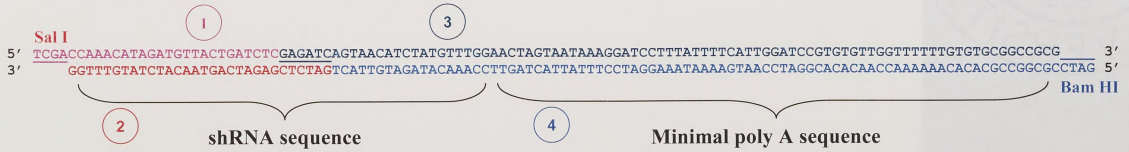
Figure 2.7.

Synthetic oligonucleotide sequence used for ACE-2 shRNA construct. For each shRNA clone (si-1 or si-2), four oligonucleotides (1: red, 2: pink, 3: dark blue, 4: blue) were synthesized. The diagram shows position and sequence of Sal I and Bam HI sticky ends, sequence coding for shRNA, and minimal poly A sequence.

Oligonucleotide sequence for shRNA si-1:



Oligonucleotide sequence for shRNA si-2:



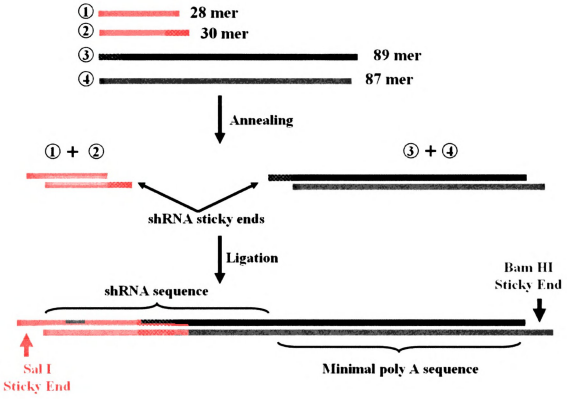


Figure 2.8.

Schematic diagram for construction mACE-2 shRNA synthetic oligos containing the minimal poly A sequence.

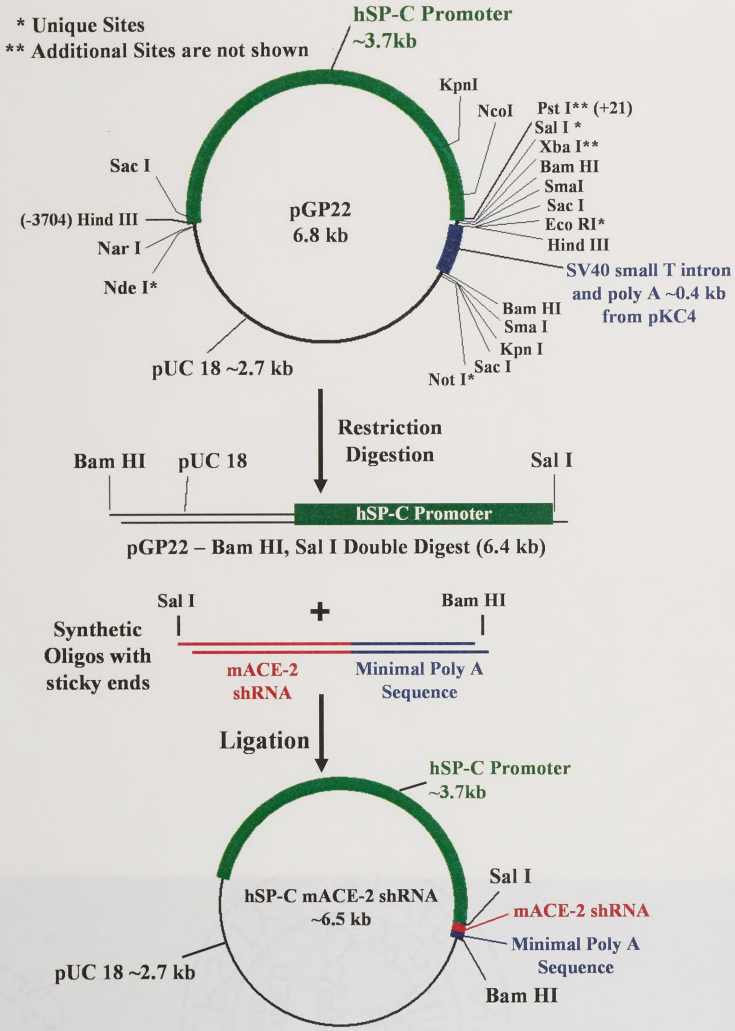


Figure 2.9.
Schematic diagram for construction of plasmid for mACE-2 shRNA expression driven by hSP-C promoter (hSP-C – mACE-2 shRNA).

RESULTS

ACE-2 mRNA is Downregulated in IPF Tissue

To obtain a quantitative assessment of ACE-2 expression in normal versus fibrotic human lung, real-time RT-PCR for ACE-2 and β-actin mRNAs was performed on total RNA isolated from lung biopsies from eight IPF patients and six normal subjects without fibrotic lung disease. In Figure 2.10, ACE-2 mRNA was found to be severely decreased by 92% in IPF lung tissue relative to the control specimens of human lung ($P<0.01$).

AngII Causes AT_1 – Dependent Decrease of ACE-2 mRNA that is Reversed by Ang1-7

Treatment of MLE12 mouse epithelial cells with 10^{-7} M Ang II in serum – free conditions for 20 hours resulted in a 45% decrease in relative ACE-2 mRNA levels compared to control untreated cells. Relative ACE-2 mRNA levels were measured by real-time RT-PCR (Figure 2.11). Pretreatment of MLE12 cells with the specific AT_1 receptor antagonist losartan (10^{-6} M) blocked Ang II – mediated decrease of relative ACE-2 mRNA levels (Figure 2.11). Figure 2.11 also shows that pretreatment of MLE12 cells with Ang 1-7 (10^{-7} M) blocked Ang II – mediated decrease of relative ACE-2 mRNA levels and increased ACE-2 mRNA levels by 200% of the control untreated cells ACE-2 mRNA level ($P<0.05$).

Alveolar Epithelial Cell – Specific Knock-Down of ACE-2

The constructed plasmid for mACE-2 shRNA expression driven by hSP-C promoter were used to transfect mouse alveolar epithelial and fibroblast cell-lines and tested for ACE-2 knockdown by Western blot detection of ACE-2 protein abundance. Western blotting of ACE-2 showed that transfection of plasmids si-1, si-2 or a mixture of si-1 and si-2 decreased ACE-2 protein abundance, indicated by band intensities, in MLE12 alveolar epithelial cell line but not in MLg lung fibroblast cell line (Figure 2.12) compared with transfection of pGP22 or mock transfection.

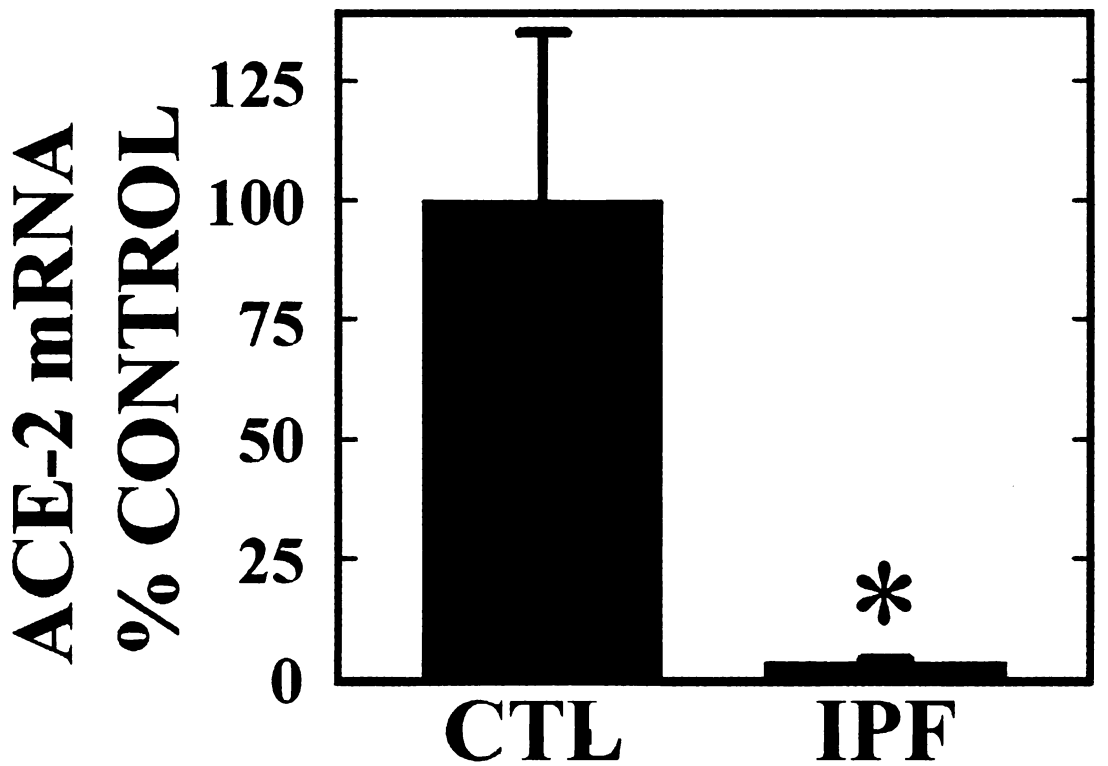


Figure 2.10.

Downregulation of ACE-2 mRNA in fibrotic human lung. Quantitative measurement of ACE-2 mRNA relative to β-actin by real-time RT-PCR in biopsies specimens from nonfibrotic (CTL) and Idiopathic Pulmonary Fibrosis (IPF) patients (see Methods). Bars are the mean + S.E.M. of n=8 for IPF and n=6 for CTL; *= $p<0.01$ vs. CTL by Student's t-test.

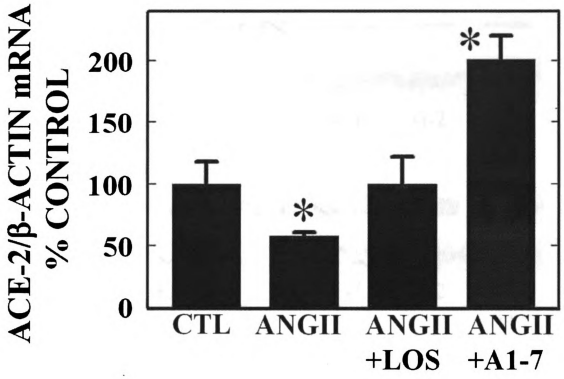


Figure 2.11.

Ang II causes AT_1 – dependent decrease of ACE-2 mRNA that is reversed by Ang 1-7. Quantitative measurement of ACE-2 mRNA relative to β-actin was performed by real-time RT-PCR in MLE-12 mouse epithelial cells. Cells were treated with or without losartan (LOS) or Ang 1-7 (A1-7) then challenged with Ang II (ANGII). Relative ACE-2 mRNA levels are compared with control cells (CTL) that received no treatment. Bars are means +S.E.M. of n=3; *= $p<0.05$ vs. CTL by ANOVA and Student-Newman-Keul's test.

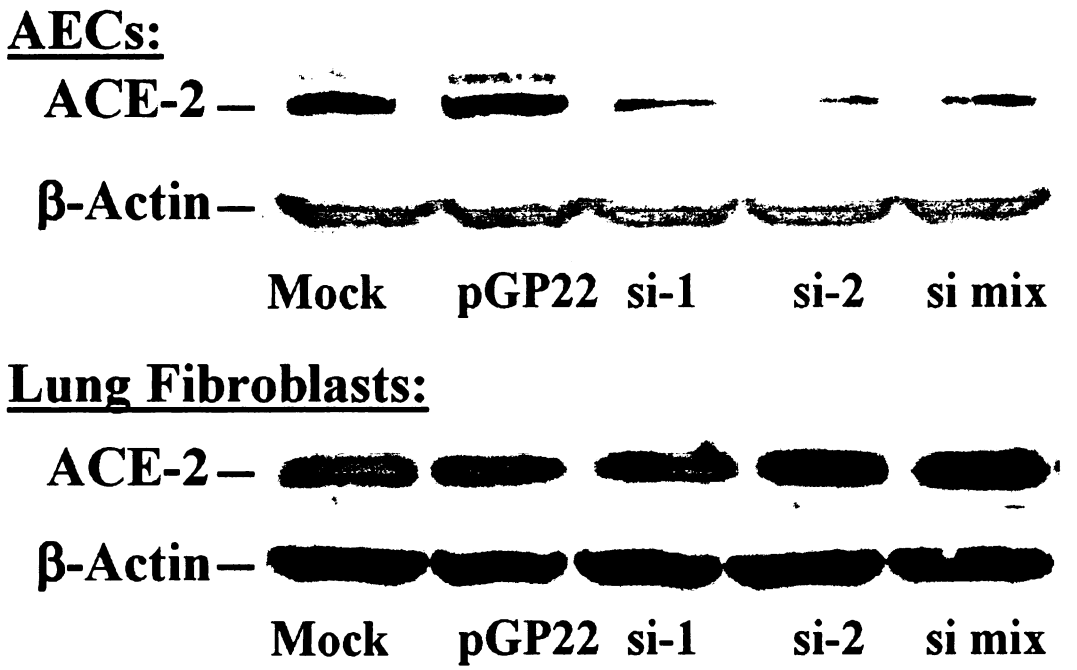


Figure 2.12.

Plasmids si-1 and si-2 cause knock-down of ACE-2 in AECs. Western blotting of ACE-2 and β-actin in whole cell lysates from MLE12 alveolar epithelial cells (AECs) and MLg fibroblast cells (Lung Fibroblasts). Whole cell lysates were prepared 96 hours after cell transfection with the hSP-C – mACE-2 shRNA plasmids si-1, si-2, or a mixture of si-1 + si-2 (si mix). For negative control, cells exposed to Lipofectamine2000 transfection reagent only (Mock) or cells transfected with pGP22 plasmid (pGP22) are used.

DISCUSSION

ACE-2 and its product Ang 1-7 were shown to play a protective role in experimental models of fibrosis. In cardiac fibrosis, ACE-2 expression was shown to be protective against angiotensin II-induced (Huentelman *et al.* 2005) and hypertension-induced (Díez-Freire *et al.* 2006) cardiac fibrosis. In liver fibrosis, both Ang 1-7 and ACE-2 provide protection against the development of liver injury and progression to cirrhosis (Warner *et al.* 2007). In experimental acute lung injury, ACE-2 also has protective effect associated with reduced levels of Ang II (Imai *et al.* 2005). These findings triggered questioning ACE-2 levels in IPF lung tissue. Consistent with the mentioned studies, the findings presented here show dramatic decrease (92%) of ACE-2 mRNA levels in IPF lungs compared to normal lung tissue (Figure 2.10). This mRNA down-regulation was also associated with a decrease in ACE-2 protein levels and activity in IPF lung (Li *et al.* 2008).

One of the histologic features of IPF is apoptosis of alveolar epithelial cells (Kuwano *et al.* 1996, Li *et al.* 2004b). Ang II was shown in many studies to induce apoptosis of alveolar epithelial cells through AT_1 receptor (Wang *et al.* 1999b, 1999c, 2000a, Li *et al.* 2003a). Ang II was also shown to down-regulate ACE-2 mRNA in rat brain astrocytes through AT_1 receptor (Gallagher *et al.* 2006). Taken together with the finding of decreased ACE-2 expression in IPF lung, it was interesting to see that Ang II down-regulates ACE-2 mRNA levels in alveolar epithelial cells (Figure 2.11). Reversal of this ACE-2 mRNA decrease by the angiotensin receptor AT_1 antagonist losartan indicates that Ang II – induced ACE-2 mRNA down-regulation is mediated via AT_1 receptor (Figure 2.11). Furthermore, the ability of the ACE-2 product Ang 1-7 to reverse the Ang II – induced ACE-2 mRNA down-regulation (Figure 2.11) supports antagonism

between the two angiotensin peptides in lung alveolar epithelial cells. Interestingly, the level of ACE-2 mRNA in cells treated with Ang 1-7 and Ang II is significantly higher than in control untreated or losartan and Ang II treated cells. This implies that the mechanism for Ang 1-7 for its action on ACE-2 mRNA levels is different from losartan's and suggests that Ang 1-7 acts on a receptor different from AT_1. The G-protein coupled receptor Mas proto-oncogene was identified to be a receptor for Ang 1-7 (Santos *et al.* 2003). The ACE-2 – Ang 1-7 – Mas axis is a putative pathway that is postulated to intrinsically regulate the RAS system and provide protection against fibrosis (Warner *et al.* 2007). Whether the actions of Ang 1-7 on ACE-2 mRNA are mediated via Mas receptor in alveolar epithelial cells is an interesting point for further investigation.

The roles of ACE-2 in lung fibrosis still need to be defined. Cell-type specific knockdown of ACE-2 would be a good tool to enable investigating *in vivo* roles of ACE-2 in experimental lung fibrosis models. One method to achieve this is to make a viral vector that expresses short hairpin RNA (shRNA) molecules for ACE-2 gene under the control of a cell-specific promoter. Alveolar epithelial cells represent good candidates for ACE-2 knockdown for the role epithelial apoptosis plays in fibrosis initiation (Li *et al.* 2004b) and the protective role ACE-2 plays in lung cells (Imai *et al.* 2005). Surfactant protein-C (SP-C) gene is only expressed in alveolar type II cells (Mulugeta & Beers 2006) and its promoter can be employed to confer alveolar type II cell-specific expression (Roper *et al.* 2003). To implement this approach, a plasmid expressing mouse ACE-2 (mACE-2) shRNA under the control of human SP-C promoter (hSP-C) designed and constructed (Figures 2.7 – 2.9). Knockdown was achieved in MLE12 cells, SP-C expressing mouse alveolar epithelial cells, transfected with the constructed plasmid for

mACE-2 shRNA expression driven by hSP-C promoter (Figure 2.12). Failure of the plasmid for mACE-2 shRNA expression driven by hSP-C promoter to knockdown ACE-2 in MLg mouse lung fibroblasts (Figure 2.12) indicate cell-specific knockdown of ACE-2. Future studies will be conducted to generate a viral vector from the plasmid for mACE-2 shRNA expression driven by hSP-C promoter to use the viral construct in vivo in experimental lung fibrosis models.

In summary, this study shows that in IPF ACE-2, that degrades Ang II, is down-regulated, and that Ang II downregulates ACE-2 mRNA. Further investigations on ACE-2 role(s) in pulmonary fibrosis need to be conducted. These investigations can be facilitated by the use of ACE-2 shRNA expression driven by hSP-C promoter.

CHAPTER 3:

JunD AND HIF-1α MEDIATE TRANSCRIPTIONAL ACTIVATION OF ANGIOTENSINOGEN BY TGF-β1 IN HUMAN LUNG FIBROBLASTS

ABSTRACT

Earlier work showed that TGF-β1 potently increases angiotensinogen (AGT) gene mRNA in primary isolates of human lung fibroblasts. Here the mechanism of TGF-β1 – induced AGT expression was studied in the IMR90 human lung fibroblast cell line. The increase in AGT mRNA (~11-fold) induced by TGF-β1 (2ng/ml) was completely blocked by actinomycin-D. TGF-β1 increased the activity of a full-length human AGT promoter-luciferase reporter (AGT-LUC, $p<0.001$) but did not alter AGT mRNA half-life. Serial deletion analyses revealed that 67% of TGF-β-inducible AGT-LUC activity resides in a small domain of the AGT core promoter at -46 to +22 bp from the transcription initiation site; this domain contains binding sites for HIF-1 and AP-1 transcription factors. TGF-β1 potently increased HIF-1α protein abundance and the activity of a hypoxia-responsive element reporter (HRE-LUC); transient transfection of HIF-1α increased basal AGT-LUC activity by 4-fold. Both oligonucleotide pulldown and chromatin immunoprecipitation assays revealed increased binding of JunD and HIF-1α to the -46 to +22 domain in response to TGF-β1. TGF-β1–inducible AGT-LUC was reduced by 88% ($p<0.001$) by a dominant-negative AP-1 construct (a-fos) or by 65% ($p<0.01$) by mutation of the AP-1 site. Gene silencing of either JunD or HIF-1α individually by siRNA partially reduced AGT-LUC by 54% or 33%, respectively. In contrast, simultaneous silencing of both JunD and HIF-1α completely eliminated TGF-β1–inducible AGT-LUC activity. These data suggest that TGF-β1 upregulates AGT transcription in human lung fibroblasts through a mechanism that requires both JunD and HIF-1α binding to the AGT core promoter. They also suggest a molecular mechanism linking hypoxia signaling and fibrogenic stimuli in the lungs.

INTRODUCTION

Activation of local renin angiotensin systems (RAS) plays a key role in the initiation and progression of fibrous tissue accumulation in a variety of organs including the heart (Weber & Sun 2000), liver (Yoshiji *et al.* 2001), kidney (Klahr & Morrissey 1997), pancreas (Ko *et al.* 2006) and lung (Wang *et al.* 2000a, Marshall *et al.* 2004, Uhal *et al.* 2007a). The activation of the local RAS in the lungs was shown to act by mechanisms independent of the blood-derived RAS (Li *et al.* 2003b, 2004a) but inhibitable by established blockers of angiotensin II (Ang II) production or receptor interaction. In animal and cell culture models of pulmonary fibrosis, myofibroblasts were shown to contribute to alveolar epithelial cell death by apoptosis *in vitro* (Uhal *et al.* 1995) and *in vivo* (Uhal *et al.* 1998) through production of the peptide Ang II *de novo* from its precursor angiotensinogen (AGT). Constitutive expression of AGT by myofibroblasts has been demonstrated *in vitro* (Wang *et al.* 1999a) after isolation of these cells from the lungs of patients with idiopathic pulmonary fibrosis (IPF). In biopsies of lung tissue from IPF patients, the production of AGT mRNA and protein was found to localize to myofibroblast foci (Li *et al.* 2006).

Transforming growth factor (TGF)-β1 is well known to induce the phenotypic transformation of fibroblasts to myofibroblasts in the lungs and other organs, and is thought to play a significant role in wound healing and/or subsequent fibrosis (Uhal *et al.* 2007a, Flanders 2004). Earlier work from this and other laboratories showed that Ang II activates TGF-β1 and collagen gene expression in the lungs and other organs (Uhal *et al.* 2007a, Kagami *et al.* 1994, Campbell & Katwa 1997, Yoshiji *et al.* 2001, Marshall *et al.* 2000). Moreover, induction of the transition to the myofibroblast phenotype in the

IMR90 normal human lung fibroblast cell line by TGF-β1 resulted in increased AGT mRNA (Uhal *et al.* 2007a); these results suggested the existence of an Ang II-TGF-β1 autocrine loop in human lung myofibroblasts. Given that TGF-β1 is a primary signal for the differentiation of normal lung fibroblasts to myofibroblasts and activation of the local RAS, it was of great interest to begin defining the molecular determinants of TGF-β1-induced AGT gene expression in human lung fibroblasts.

This chapter reports studies of the molecular mechanisms that mediate activation of AGT expression by TGF-β1 in the human lung fibroblast cell line IMR90. The data show that TGF-β1 stimulates AGT gene expression in IMR90 cells by increasing the binding of JunD and HIF-1α transcription factors to an AGT promoter domain close to the transcription start site. The implications of these findings are discussed in relation to disease pathogenesis theory and known genetic determinants of AGT expression that reside in the AGT core promoter.

METHODS

Reagents and Material

Porcine transforming growth factor-β1 (TGF-β1) was obtained from R&D Systems, Minneapolis, MN. A fos dominant negative construct (a-fos) was a kind gift from the laboratory of Dr. Charles Vinson (Olive *et al.* 1997). All other materials were of reagent grade and were obtained from Sigma Chemical Co., Saint Louis, MO.

Cell Culture

The human embryonic lung fibroblast cell line IMR90 was obtained from the American Type Culture Collection and cultured in Eagle's modified minimal essential media (EMEM) supplemented with 10% fetal bovine serum (FBS). IMR90 cells of passage number 15 or less were used. Treatment with TGF-β1 was performed in serum-free culture media after 24 hours of serum starvation.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted using Trizol Reagent® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First strand cDNA was synthesized from total RNA using Superscript II Reverse Transcriptase and oligo $(dT)_{12\text{-}18}$ (Invitrogen). Real-time RT-PCR was performed using cDNA synthesized from 50 ng total RNA, 2mM MgCl2, 1mM dNTP mix, 2.5U AmpliGold *Taq* Polymerase, 1x SYBR® Green PCR buffer (SYBR® Green PCR Core Reagents, Applied Biosystems, Foster City, CA) and 0.2 μM specific primers for human angiotensinogen (AGT), forward: 5`-GAG CAA TGA

CCG CAT CAG-3`, reverse: 5`-CAC AGC AAA CAG GAA TGG-3`, and for human β-actin, forward: 5`-AGG CCA ACC GCG AGA AGA TGA CC-3`, reverse: 5`-GAA GTC CAG GGC GAC GTA GC-3`, which produce PCR products of 151 and 332 bp respectively. The PCR thermal profile started with 10 minutes activation of the *Taq* polymerase at 95°C for 1 cycle followed by 30 seconds denaturation at 95°C, 37 seconds annealing at 55°C and 37 seconds extension at 72°C for 40 cycles ending with dissociation curve analysis to validate the specificity of the PCR products. Reactions were performed in a Mx3000P thermocycler (Stratagene, LaJolla, CA) and threshold cycle (CT) data was collected using MxPro-Mx3000P software version 3.0. The relative AGT expression was normalized to β-actin and calculated using the comparative CT method; folds change = $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{AGT} - CT_{\beta\text{-actin}}$ and $\Delta\Delta CT = \Delta CT_{Treatment} - \Delta CT_{Control}$.

Determination of mRNA Half-Life

IMR90 cells were serum starved for 24 hours and then were treated with or without TGF-β1 (2ng/ml) in serum-free media. Twenty hours later, actinomycin-D was added at 5 μg/ml. Cells were harvested for total RNA extraction at 0, 3, 6, 9 hours after actinomycin-D addition and used in real-time RT-PCR measures of AGT relative to β-actin.

Reporter Gene Assays and Transfections

Expression of human angiotensinogen was studied in IMR90 cells using a luciferase reporter driven by the full-length human angiotensinogen promoter,

specifically -991 to +22bp from the transcription initiation site, in pOLUC firefly luciferase reporter plasmid (AGT-LUC), a kind gift from Dr. Alan R. Brasier (The University of Texas Medical Branch, Galveston, Texas). Six deletion constructs of the AGT-LUC reporter were generated in our lab as described by Sherman and Brasier (Sherman & Brasier 2001).

For AP-1 binding site mutation, substitution of 2 bp was generated in the middle of AP-1 site at positions -13 and -12 from the transcription initiation site by site-directed mutagenesis using QuickChange kit (Stratagene, La Jolla, CA) on the full length AGT-LUC reporter. Each AGT-LUC firefly luciferase reporter construct was co-transfected with Renilla luciferase PRL-CMV plasmid in IMR90 cells using Fugene6 reagent (Roche) at 1:6 (μg:μl) ratio. Cells were treated with or without 2ng/ml TGF-β1 then harvested in lysis buffer and assayed using Dual Luciferase Reporter Assay kit (Promega, Madison, WI) according to manufacturer's instructions.

For JunD gene knockdown by siRNA for dual luciferase assay, 50nM human JunD siRNA or random sequence (control) siRNA (Santa Cruz Biotechnology), AGT-LUC and PRL-CMV were co-transfected into IMR90 cells using Lipofectamine 2000 transfection reagent (Invitrogen) at 1:25 lipofectamine:siRNA molar ratio. For HIF-1α gene knockdown and dual luciferase assay, sequential transfections of siRNA and reporter plasmids were utilized to avoid higher dose of lipofectamine. HIF-1α siRNA or random sequence duplex RNA were synthesized (Invitrogen) as in (Krick *et al.* 2005); HIF-1α, 5'-UGU GAG UUC GCA UCU UGA U dTdT-3', random, 3'-UAC ACC GUU AGC AGA CAC C dTdT-3' with two 5'-deoxythymidine overhangs. IMR90 cells were first transfected with 100nM HIF-1α or random siRNA using lipofectamine 2000. In

JunD – HIF-1α double knockdown the concentrations of siRNAs were scaled down to make the total transfected siRNA of 100nM concentration. Sequential co-transfection of AGT-LUC and PRL-CMV was performed 24 hours later using Fugene6 reagent as described above.

Transcription Factor Complex Pull-Down

Synthetic biotinylated oligonucleotide having AGT promoter sequence -46 to +22 (Integrated DNA Technologies, Coralville, IA) was immobilized to streptavidin magnetic beads (Promega) and washed in 0.1x SSC and twice in 1x PBS. Nuclear extracts were isolated from TGF-β1 treated IMR90 cells using Nuclear Extraction Kit (Panomics, Redwood City, CA). Nuclear extracts (500 μg) were allowed to bind to the immobilized AGT promoter in the presence of 20 μg poly d(I-C) (Roche) for competition of non-specific binding. Proteins bound to the biotinylated AGT promoter fragment were washed 3 times in 1x PBS. The "pull-down" proteins were eluted by heating in Laemmli sample buffer (for Western blot).

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed by a modification of Ray *et al.* (Ray *et al.* 2005). Briefly, TGF-β1 treated IMR90 cells were washed with PBS and proteins cross-linked to DNA by 1% formaldehyde at 37°C. Cells were collected by centrifugation and lysed in lysis buffer provided in Upstate Cell Signaling Solutions ChIP kit (Millipore, Billerica, MA). Cell lysates were sonicated to yield chromatin with DNA fragments ~ 200 – 400 bp in size. Immunoprecipitation was performed by primary antibodies for

JunD (Santa Cruz Biotechnology) or HIF-1α (BD Biosciences, San Jose, CA) and cleared by secondary immunoprecipitation reagent protein A-agarose. Reversal of the precipitate cross-links was done by heating at 65°C with 0.2M NaCl followed by proteinase-K digestion according to ChIP kit manufacturer instruction. DNA was extracted by phenol/chloroform extraction and ethanol precipitation. The extracted DNA was incorporated in SYBR Green real-time PCR reaction to amplify AGT promoter region (-47 to +32) using specific primers; forward = 5'- ATC CCC ACC CCT CAG CTA T- 3' and reverse = 5'- TGC TGT AGT ACC CAG AAC AAC G -3'. The relative amount of immunoprecipitated endogenous AGT promoter (-47 to +32) DNA (IP) was normalized to input chromatin DNA (input) and calculated using the comparative CT method; folds change = $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{IP} - CT_{input}$ and $\Delta\Delta CT = \Delta CT_{TGF\text{-}\beta1\ treatment} - \Delta CT_{Control}$, where CT (threshold cycle) is the fractional cycle number at which the fluorescence passes the fixed threshold.

Western Blotting

Where appropriate, whole cell extracts in NP40 buffer, nuclear extracts (Nuclear Extraction Kit, Panomics), or pull-down protein complex were separated by SDS-PAGE then transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) in Towbin buffer. Blotting membrane was blocked by 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. Western blot analysis was performed with antibodies against JunD, lamin A/C (Santa Cruz Biotechnology), HIF-1α (BD Biosciences), or β-actin (Cell Signaling Technology). Bands were visualized by HRP-conjugated secondary antibodies and the chemiluminescent substrate Super Signal West (Pierce, Rockford, IL).

RESULTS

TGF-β1 Induces AGT Gene Transcription

Figure 3.1 shows that TGF-β1 (2ng/ml) increased steady state AGT mRNA in IMR90 cells by 11-fold in 48 hours. To test whether TGF-β1 increases AGT mRNA via transcription, the RNA polymerase inhibitor actinomycin-D (5 μg/ml) was used to treat IMR90 cells. TGF-β1 – treated cells that had actinomycin-D treatment showed no increase of relative AGT mRNA levels due to inhibition of transcription initiation by actinomycin-D, while TGF-β1 increased AGT mRNA level without actinomycin-D treatment. AGT mRNA level was measured relative to the housekeeping gene β-actin mRNA level by real-time RT-PCR.

For AGT mRNA half-life comparison in cells treated with or without TGF-b1, sequential real-time RT-PCR measurements of AGT mRNA relative to β-actin mRNA after the addition of actinomycin-D were normalized to zero time-point level of each treatment group (with or without TGF-β1) being 100%. Normalized levels were plotted against time of incubation with actinomycin-D to reflect AGT mRNA stability relative to the stable housekeeping gene β-actin (Figure 3.2). The plots showed that TGF-β1 does not increase AGT mRNA stability (Figure 3.2).

Identification of AGT Promoter Region Responsive to TGF-β1

TGF-β1 also increased the expression of the AGT-promoter luciferase reporter construct AGT-LUC (Figures 2, 5 & 6) with similar fold increase to the increase in AGT mRNA. For identification of *cis*-regulatory region in AGT promoter that confers TGF-β1

effect, six deletion constructs of the AGT-LUC reporter were constructed. The full-length (-991 to +22bp) AGT-LUC reporter was used to generate the deletion mutants indicated in Figure 3.3. Each deletion firefly luciferase construct was cotransfected with PRL-CMV renilla luciferase plasmid into IMR90 cells followed by treatment (with or without) 2ng/ml TGF-β1 for 24hr to induce AGT-LUC as described in methods section. For each transfected deletion construct, firefly to renilla luciferase activity ratio for each treatment group (with or without TGF-β1). To test responsiveness to TGF-β1, the TGF-β1 – induced luciferase level for each deletion construct was expressed as folds increase of the firefly/renilla ratio for TGF-β1 treatment compared to no TGF-β1 control for the same construct. All deletion constructs had significant TGF-β1 – induced luciferase level (Figure 3.3). To compare the effect of the deletion mutants on the extent of TGF-β1 induction of AGT promoter, TGF-β1 – induced luciferase increase over control was calculated for each construct as a percent of the TGF-β1 – induced luciferase increase for the full length AGT-LUC (-991 to +22 construct), with the full length AGT-LUC having 100% induction. No statistically significant difference was found for percentage TGF-β1 – induction among the deletion constructs, with AGT promoter region from -46 to +22 able to produce ~67% of the TGF-β1 – induced AGT-LUC (Figure 3.3).

Screening for Candidate cis-Elements

To identify putative transcription factors binding sites within the AGT promoter region -46 to +22, Transcription Element Search Software (TESS, URL: http://www.cbil.upenn.edu/tess) was utilized to search this DNA sequence for consensus sequences in the TRANSFAC database of transcription factors binding sites. Figure 3.4 is

a diagram representing results of the site search showing AGT promoter region -46 to +3 with binding sites for several transcription factors including SP1, AP-1, AP-2, USF-1, RAR, AGCE1 and c-Myc/HIF.

To test the role of AP-1 element at position -10 to -15 bp (Figure 3.4) in AGT regulation by TGF-β1, full-length AGT-LUC construct with mutation at -13 bp was transfected into IMR90 cells and challenged with TGF-β1. The promoter with the *cis*-element mutation within the AP-1 site showed significant reduction in the TGF-β1 – inducible AGT-LUC by 60% (Figure 3.5).

JunD, AP-1 Family Member, Plays Role in TGF-β1 Induced AGT Transcription

To show role of AP-1 transcription factors in *trans*, the dominant negative a-fos construct, which eliminates AP-1 transcription factors – DNA binding activity, was cotransfected with AGT-LUC reporter in IMR90 cells and challenged with TGF-β1. Figure 3.6 shows that a-fos reduced the TGF-β1 – induced AGT-LUC expression by 72%.

Eickelberg *et al.* previously showed that JunD mediates TGF-β-inducible collagen gene expression in primary cultures of human lung fibroblasts (Eickelberg *et al.* 2001). To test the possibility that JunD might also regulate TGF-β-inducible AGT expression at the core promoter, JunD binding was assessed with synthetic oligonucleotide pulldown and chromatin immunoprecipitation assays (ChIP). In Figure 3.7, TGF-β1 increased the binding of immunoreactive JunD to a biotinylated oligonucleotide with the sequence -46 to +22 (see Figure 3.4) in transcription factor complex pulldown assay applied to IMR90 nuclear extracts, followed by western blotting for JunD (see methods section). ChIP assay

with antibodies specific for JunD also detected increased binding to the -47 to +32 domain of AGT after TGF-β1 stimulation, reflecting 2.6 folds more binding to AGT core promoter as ChIP DNA was quantified by realtime PCR (Figure 3.8).

For testing functional role of JunD, gene silencing of JunD with specific siRNAs was applied to IMR90cells. Western blot results in Figure 3.9 shows successful knockdown for both the 34kDa and 39kDa isoforms of human JunD using siRNA. Application of the siRNAs prior to stimulation with TGF-β1 (Figure 3.10) reduced TGF-β-inducible AGT-LUC by 54% ($p<0.001$), but random sequence RNAs of the same base composition did not reduce TGF-β-inducible AGT-LUC.

TGF-β1 Stimulates HIF-1 – Regulated Gene Transcription in Human Lung Fibroblasts

TGF-β1 treatment potently increased HIF-1α protein abundance in IMR90 in both nuclear extracts (Figure 3.11) and in whole cell lysates (Figure 3.16) as detected by western blot band intensities, showing stronger HIF-1α band intensities in TGF-β1 treatment relative to control samples. Hypoxia responsive element activity, measured by HRE-LUC expression, showed higher induction of luciferase activity in TGF-β1 treated cells by 5 folds ($P<0.001$) compared to control cells (Figure 3.12).

JunD and HIF-1 Together Mediate TGF-β1 – Inducible AGT Expression in Human Lung Fibroblasts

The ability of HIF-1α to bind AGT promoter region –46 to +22 was tested by transcription factor complex pull-down and ChIP assay. Figure 3.13 shows identification

of HIF-1α among transcription factor complex proteins bound to AGT core promoter in TGF-β1 treated IMR90 cells by the transcription factor complex pull-down and western blotting. ChIP assay indicated increased binding of HIF-1α to AGT core promoter region (-47 to +32) by 7.2 folds ($p<0.001$) in response to TGF-β1 stimulation of IMR90 cells (Figure 3.14). Furthermore, transient transfection of HIF-1α together with HIF-1β into IMR90 cells revealed that overexpression of HIF-1 in IMR90 cells is sufficient to activate AGT-LUC expression by 3.5-fold ($p<0.01$) (Figure 3.15).

Knockdown of HIF-1α in IMR90 cells using siRNA specific for human HIF-1α (Krick *et al.* 2005) was tested by detecting decrease in HIF-1α protein abundance and function. Immunoreactive HIF-1α protein abundance, detected by western blot band intensity, was decreased after IMR90 cells transfection with HIF-1α siRNA compared to TGF-β1 treated or control non-treated IMR90 cells (Figure 3.16). siRNA – mediated knockdown of HIF-1α completely eliminated stimulation of HRE-LUC by TGF-β1 (detected by dual luciferase assay) compared to mock transfection or control siRNA (Figure 3.17).

HIF-1α knockdown using HIF-1α siRNAs alone reduced TGF-β-inducible AGT-LUC by only 33% ($p<0.05$) (Figure 3.18). However, a double knockdown of HIF-1α and JunD together using the siRNAs to both HIF-1α and JunD applied simultaneously showed that silencing of both HIF-1α and JunD together could reduce TGF-β-inducible AGT-LUC by over 76% ($p<0.05$) to a value not significantly different from the control (Figure 3.18, rightmost columns).

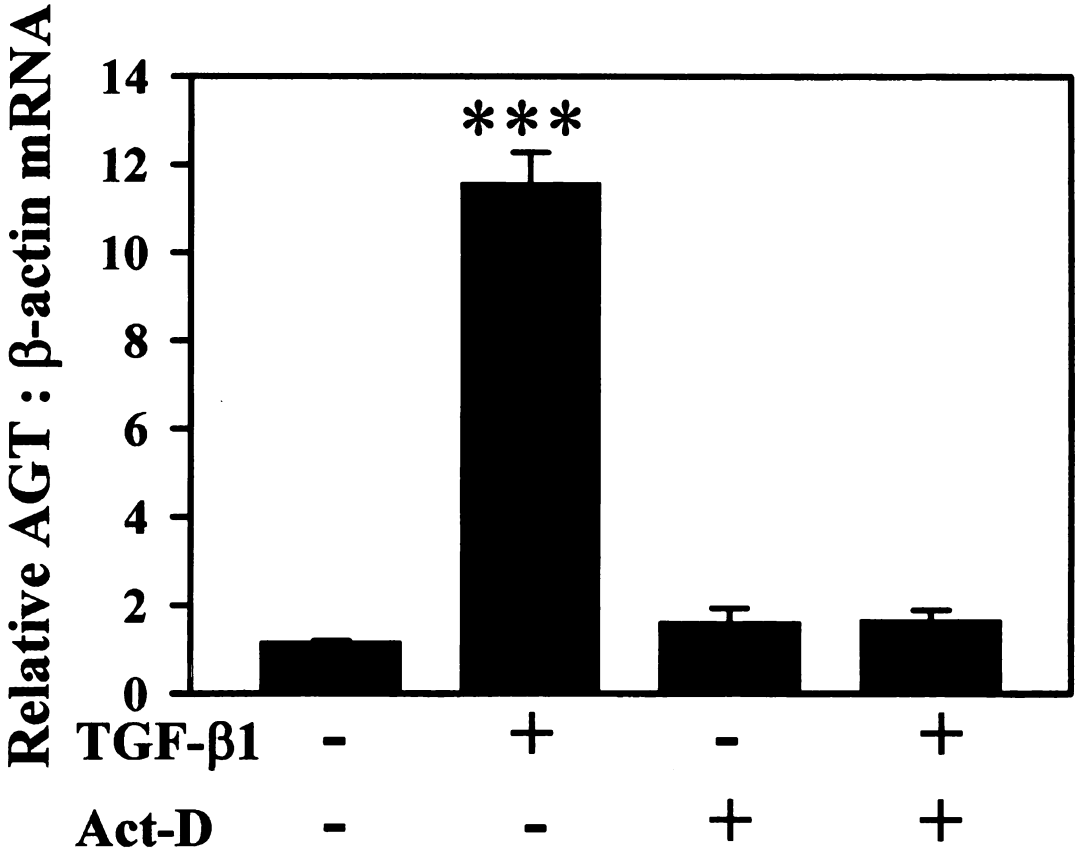


Figure 3.1.

TGF-β1 upregulates angiotensinogen gene transcription in IMR90 fibroblasts. TGF-β1 (2ng/ml) was added to IMR90 cells in presence or absence of actinomycin-D (Act-D, 5μg/ml) for 48 hours in serum-free medium. Data represent realtime RT-PCR of AGT mRNA relative to β-actin. Bars are the means + S.E.M. of N=3; * = $p<0.001$ by ANOVA and Student-Newman-Keul's test.

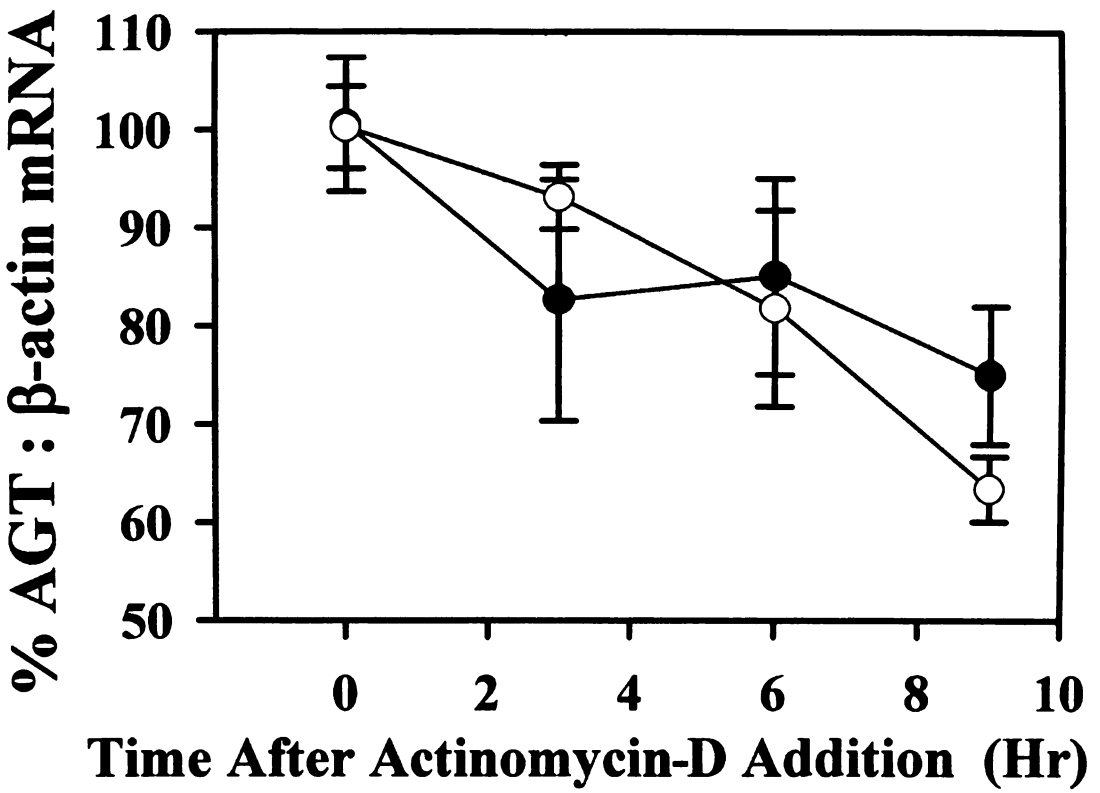


Figure 3.2.

TGF-β1 does not increase AGT mRNA stability. IMR90 cells were treated with (white circles) or without (black circles) TGF-β1 (2ng/ml) for 48 hours then actinomycin-D (5μg/ml) was added for 0, 3, 6, 9 hours after which total RNA was extracted. Real-time RT-PCR was performed to measure relative levels of AGT and β-actin mRNA and normalized to zero time-point level of each treatment group being 100%. Normalized levels are plotted against time of incubation with actinomycin-D to reflect angiotensinogen mRNA stability relative to the stable housekeeping gene β-actin. Bars are the mean ± S.E.M of n=3.

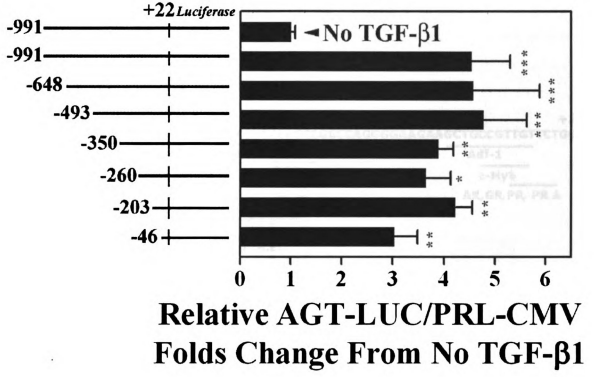


Figure 3.3.

Deletion analysis of AGT promoter reveals TGF-β1 response domain close to transcription start site. Data are expressed as TGF-β-induced fold increase in AGT-LUC over the no TGF-β1 control for each construct (only -991 to +22 control shown). Note that most (~67%) TGF-β-inducible AGT-LUC resides in the domain -46 to +22bp. Bars are the means + S.D. of n=6; *=p< 0.05, **=p<0.01, ***=p<0.001 vs. no TGF-β1 for the corresponding construct by ANOVA and Student-Newman-Keul's test.

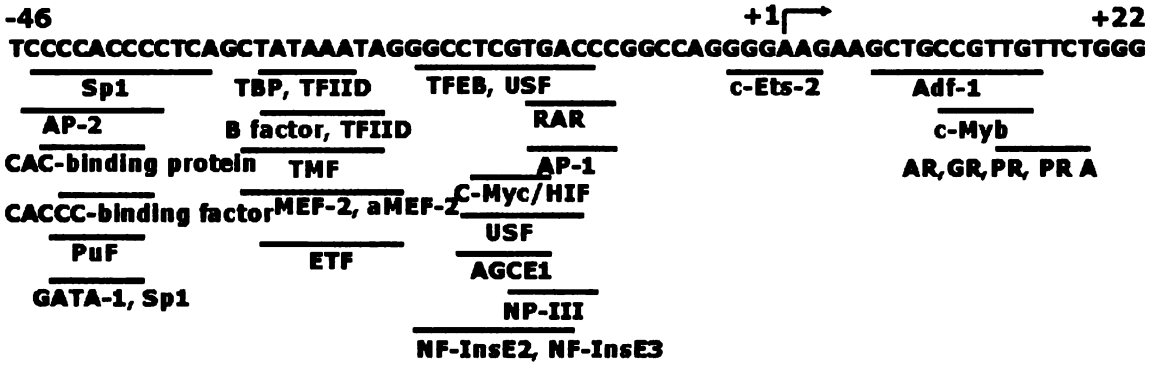


Figure 3.4.

Human AGT promoter proximal sequence (-46 to +22) with known and postulated transcription factor binding domains. Results of TESS search in TRANSFAC database shown, lines indicate DNA sequence for corresponding transcription factors binding. +1 indicate transcription start site. AR: androgen receptor, GR: glucocorticoid receptor, PR: progestron receptor, HIF: hypoxia inducible factor, USF: upstream stimulating factor, RAR: retinoic acid receptor.

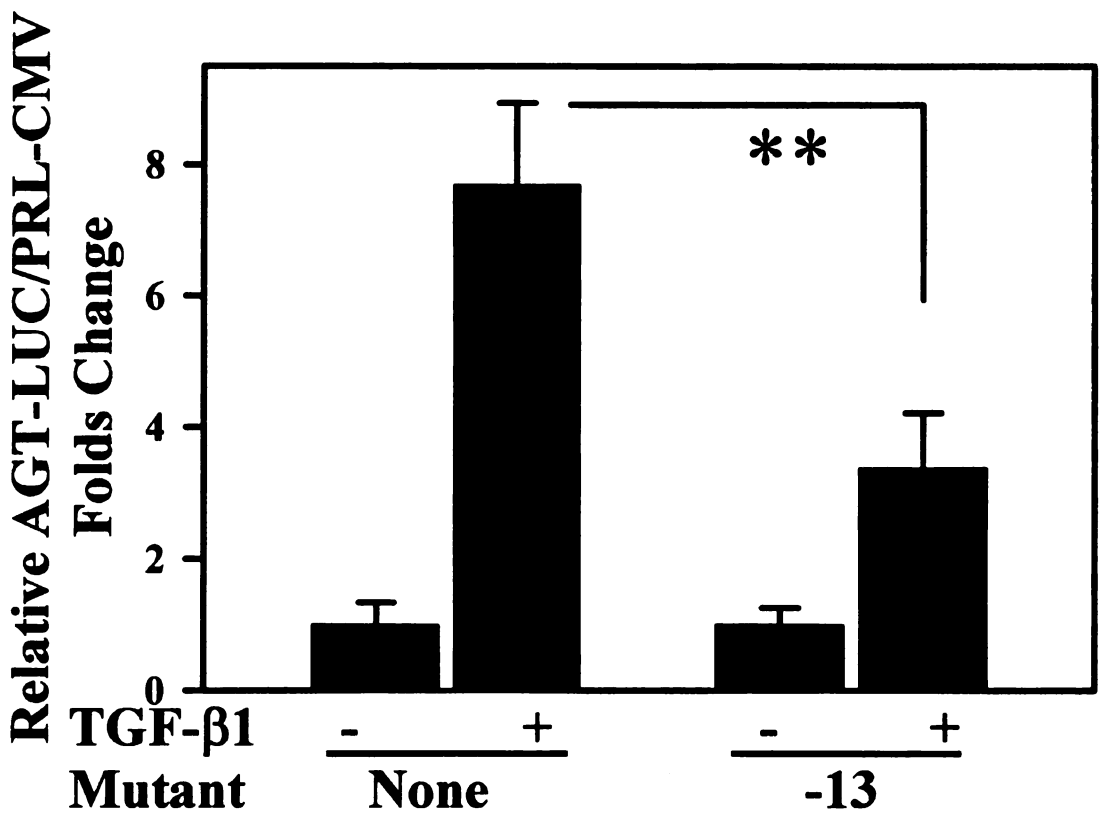


Figure 3.5.

Mutation of AP-1 site at -13bp reduces TGF-β1 – induced AGT-LUC. Site-directed mutagenesis was used to mutate the full-length AGT-LUC reporter at -13/-12bp, in the AP-1 site (see methods section, Figure 3.4). The WT and mutant constructs were transfected into IMR90 cells followed by 24hr TGF-β1 (2ng/ml) treatment. Bars are means + S.E.M; *** = $p < 0.001$, n=3 by ANOVA and Student-Newman-Keul's test.

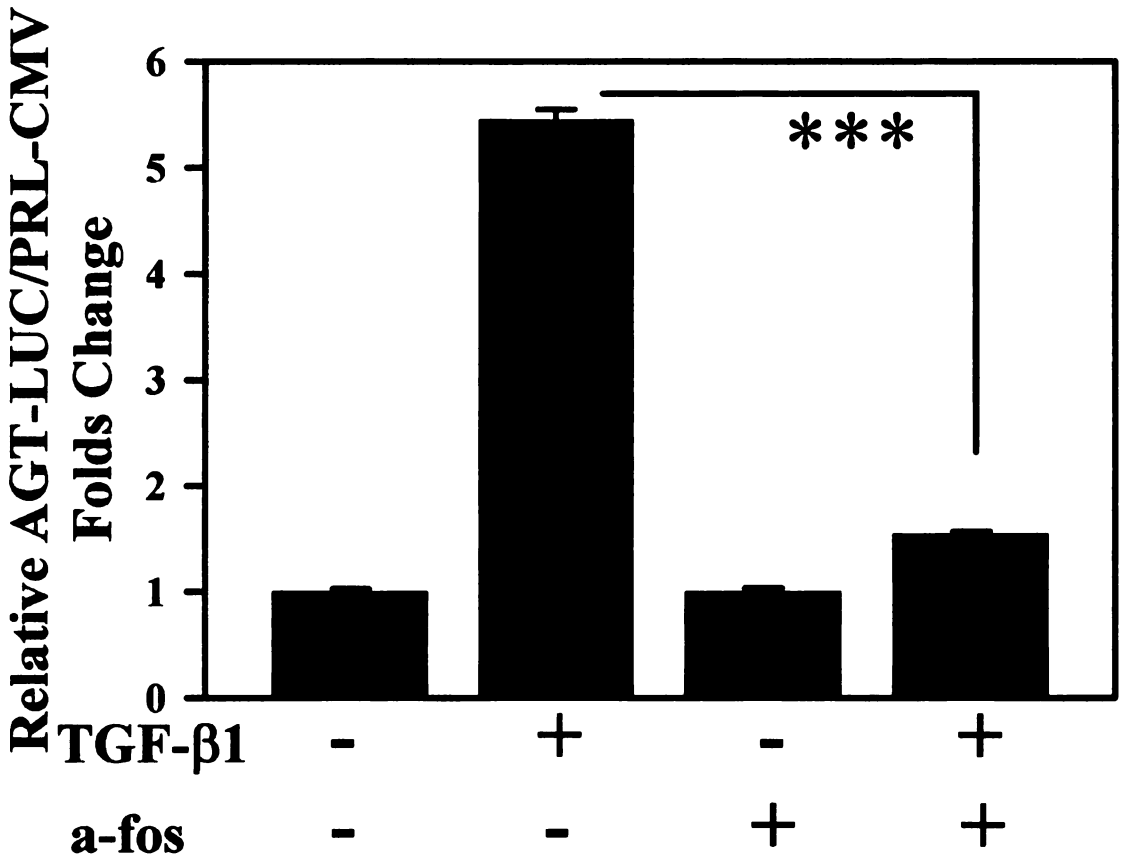


Figure 3.6.

A-fos, an AP-1 dominant negative, reduces TGF-β1 – induced AGT-LUC. AP-1 dominant-negative fos construct (a-fos), that inhibits AP-1 – DNA binding activity, was cotransfected into IMR90 cells together with the full length AGT-LUC construct. Transfection was followed by 48hr TGF-β1 (2ng/ml) treatment. Bars are means + S.E.M, n=3; * = $p < 0.001$, by ANOVA and Student-Newman-Keul's test.

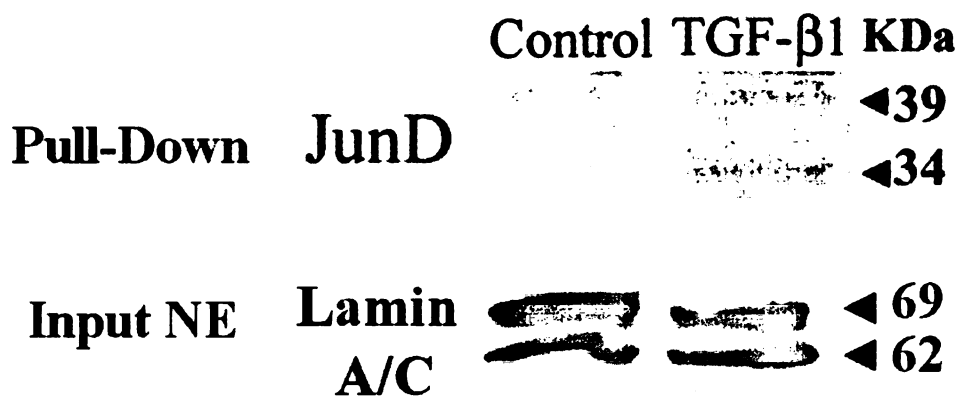


Figure 3.7.

Detection of JunD binding to AGT core promoter by western blotting. Pull down proteins bound to AGT promoter -46 to +22 (see methods) from untreated (control) cells or TGF-β1 treated cells for 24 hr were used in JunD Western blot. Western blot of the nuclear proteins lamin A/C is used as loading control for input nuclear extract (NE).

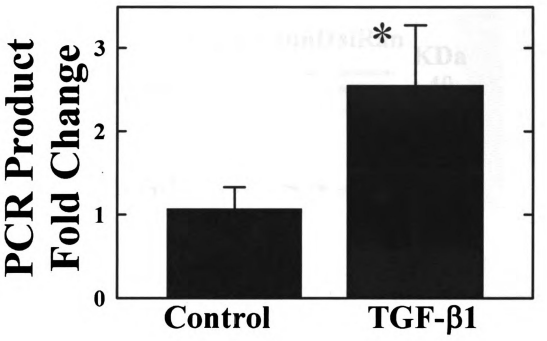


Figure 3.8.

Increased JunD binding to AGT core promoter in IMR90 genome by TGF-β1. Real-time PCR quantitation of Chromatin immunoprecipitation (ChIP) DNA using antibodies against the AP-1 transcription factor (JunD) and specific primers for AGT core promoter. Quantitation is expressed in folds change from control. Bars are means + S.E.M; *= $p<0.05$ by Paired t test; n=3.

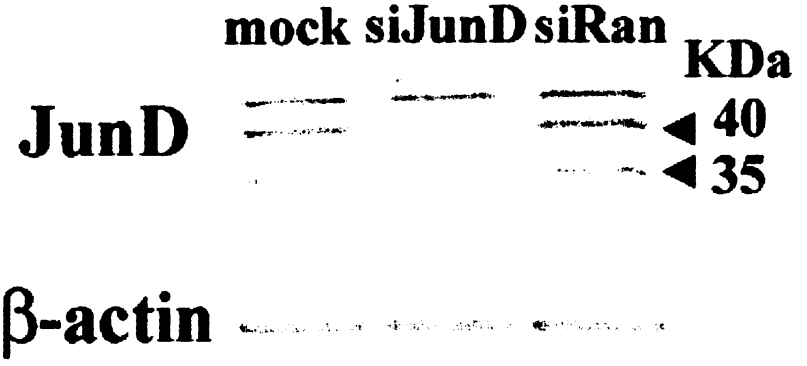


Figure 3.9.

Testing JunD knockdown in IMR90 cells. IMR90 cells were transfected with human JunD siRNA (siJunD), random sequence siRNA (siRan) or mock transfection (mock). Cell were lysed 24hr post transfection and used for JunD detection by western blotting. Western blot of the housekeeping protein β-actin is used as loading control.

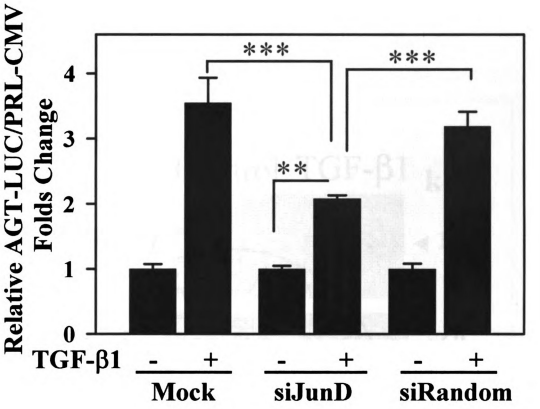


Figure 3.10.

JunD knockdown reduces the TGF-β1 induced AGT-LUC expression. IMR90 Cells were cotransfected with human JunD siRNA (siJunD), random sequence siRNA (siRandom) or mock transfection (mock) together with the full length AGT-LUC construct and PRL-CMV and incubated in serum free conditions for 24hr before TGF-β1 treatment for another 24hr then used in dual luciferase assay. Bars are means + S.E.M; *** = $p < 0.001$, ** = $p < 0.01$, n=6 by ANOVA and Student-Newman-Keul's test.

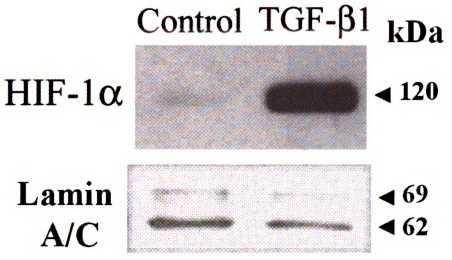


Figure 3.11.

TGF-β1 treatment increases HIF-1α protein nuclear abundance. Western blot results for HIF-1α in nuclear extracts of IMR90 cells treated without (Control) or with TGF-β1 2ng/ml for 24 hr. Western blot of the nuclear proteins lamin A/C is used as loading control.

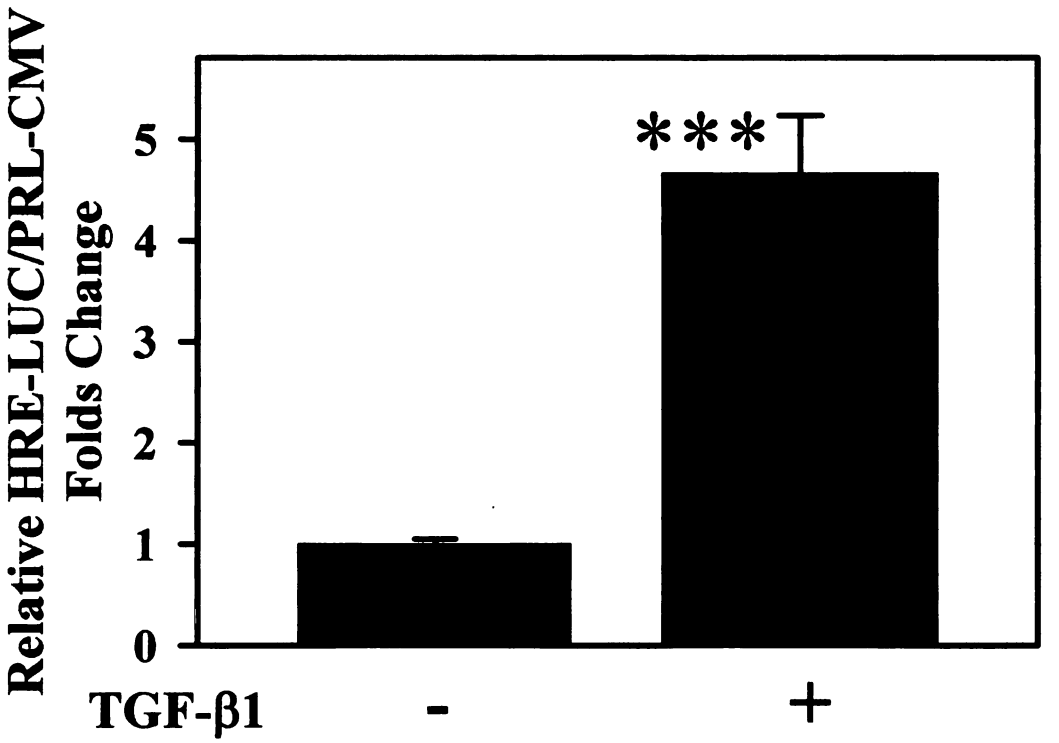


Figure 3.12.

Stimulation of hypoxia responsive element by TGF-β1 treatment. IMR90 cells were cotransfected with HRE-LUC and PRL-CMV then treated with TGF-β1 for 24hr and used in dual luciferase assay. Bars are means + S.E.M; *** = $p < 0.001$, n=6 by unpaired t-test.

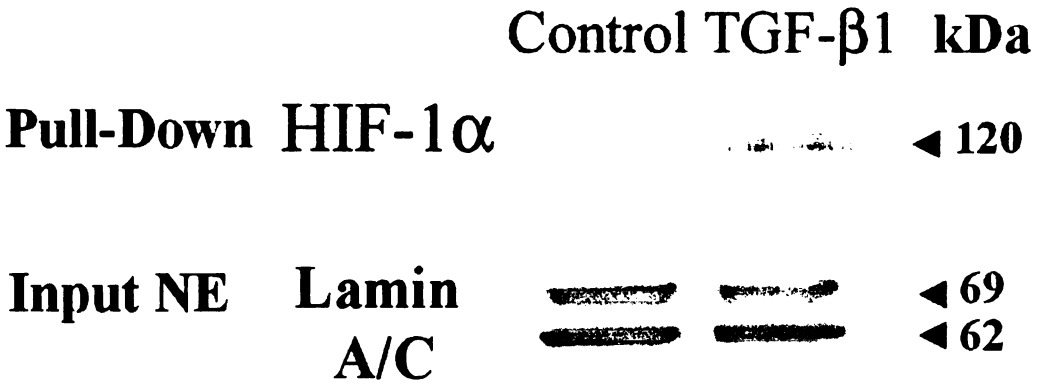


Figure 3.13.

HIF-1α to binds to AGT core promoter in response to TGF-β1. Pull down proteins bound to AGT promoter -46 to +22 (see methods) from untreated (control) cells or TGF-β1 treated cells were used in HIF-1α Western blot. Western blot of the nuclear proteins lamin A/C is used as loading control for input nuclear extract (NE).

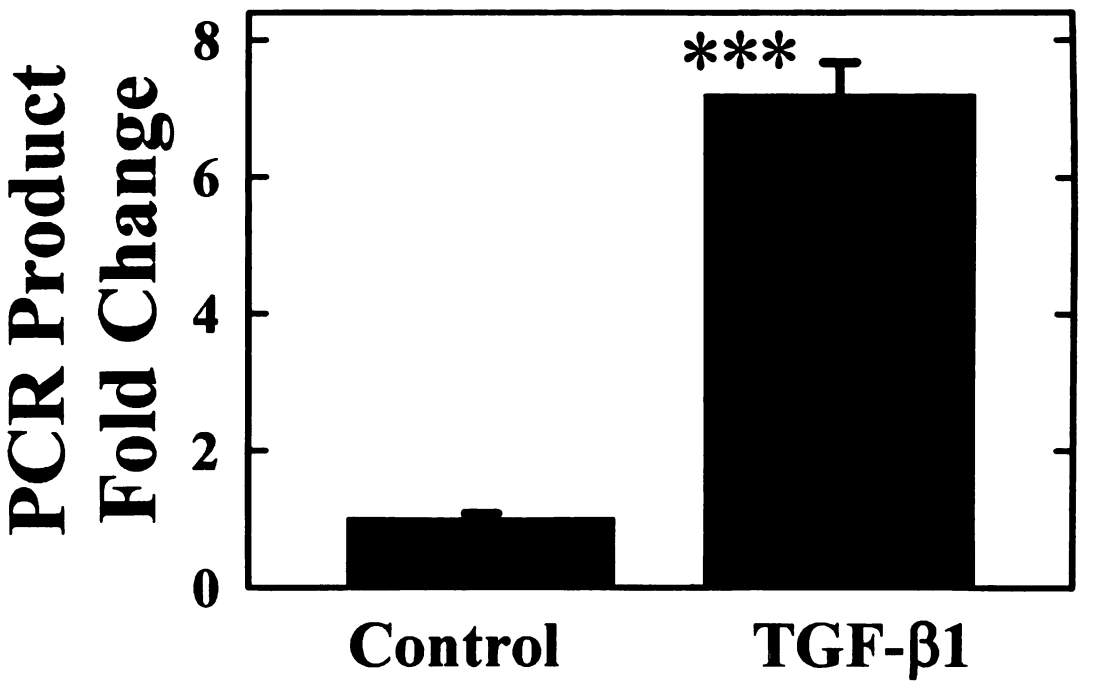


Figure 3.14.

Increased HIF-1α binding to AGT core promoter in IMR90 genome by TGF-β1. Real-time PCR quantitation of Chromatin immunoprecipitation (ChIP) DNA using antibodies against the HIF-1α and specific primers for AGT core promoter. Quantitation is expressed in folds change from control. Bars are means + S.E.M; ***= $p<0.001$ by Unpaired t-test; n=3.

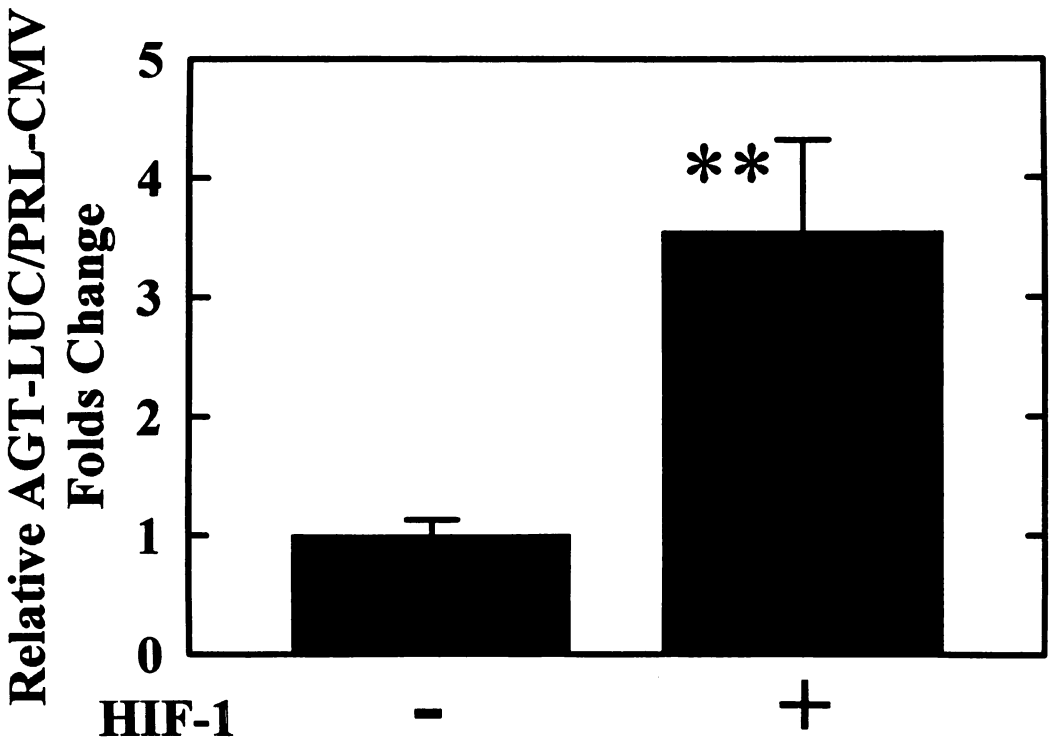


Figure 3.15.

Overexpression of HIF-1 increases basal activity levels of AGT-LUC. IMR90 cells were cotransfected with AGT-LUC, PRL-CMV plus/minus two mammalian expression plasmids encoding HIF-1α and HIF-β. Cell lysates 24hr post transfection were used for dual luciferase assay. Bars are means +S.E.M. by unpaired t-test; **=$p<0.01$.

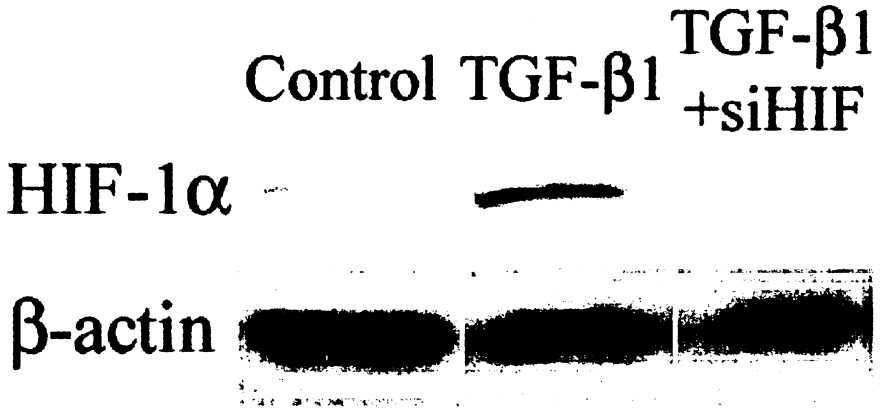


Figure 3.16.

Testing HIF-1α knockdown in IMR90 cells. HIF-1α and β-actin Western blot results obtained from IMR90 whole cell lysates for cells with no treatment (Control), treated with TGF-β1 or transfected with human HIF-1α siRNA (siHIF) and treated with TGF-β1.

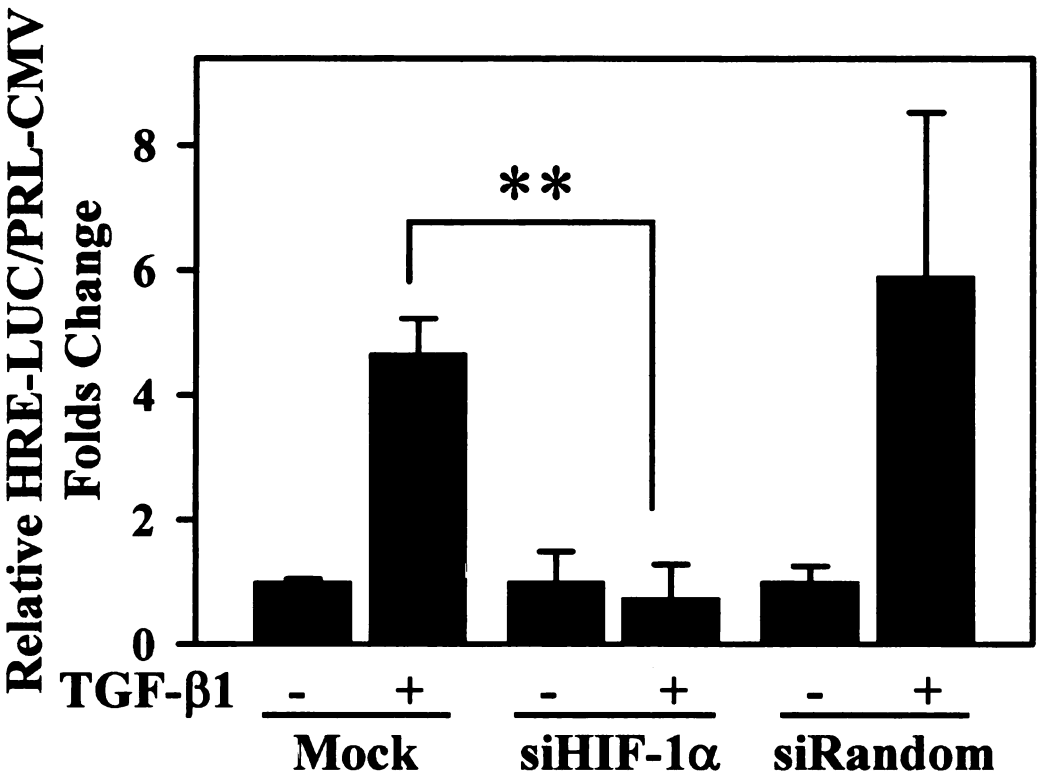


Figure 3.17.

HIF-1α knockdown reduces the TGF-β1 induced HRE-LUC expression. IMR90 Cells were sequentially transfected with human HIF1α siRNA (siHIF-1α), random sequence siRNA (siRandom) or mock transfection (mock) then 24hr later with hypoxia responsive element (HRE)-LUC and PRL-CMV. Cells were treated with TGF-β1 for 24hr and used in dual luciferase assay. Bars are means + S.E.M; ** = $p < 0.01$, n=6 by ANOVA and Student-Newman-Keul's test.

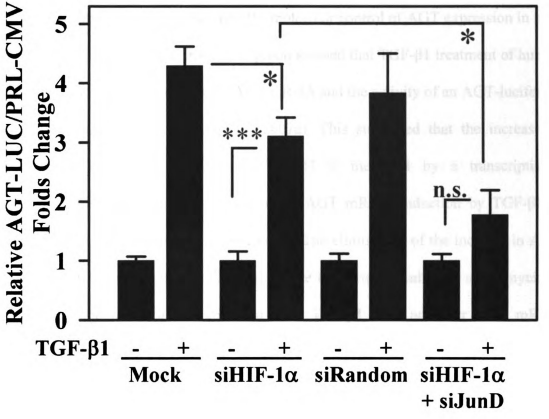


Figure 3.18.

HIF-1α knockdown reduces the TGF-β1 induced AGT-LUC expression while double knockdown of HIF-1α and JunD together completely eliminated the TGF-β1 – induced AGT-LUC. IMR90 Cells were sequentially transfected with human HIF-1α siRNA (siHIF-1α), random sequence siRNA (siRandom), mixture of human HIF-1α and JunD siRNA (siHIF-1α +siJunD), or mock transfection (mock) then 24hr later with AGT-LUC and PRL-CMV.IMR90 Cells were sequentially transfected with then 24hr later with AGT-LUC and PRL-CMV. Cells were treated with TGF-β1 for 24hr and used in dual luciferase assay. Bars are means + S.E.M; ***= $p<0.001$, * = $p<0.05$, n.s.= not significant, $n\geq6$ by ANOVA and Student-Newman-Keul's test.

DISCUSSION

This study is the first to examine the molecular control of AGT expression in lung cells by TGF-β1. Earlier work from Uhal group showed that TGF-β1 treatment of human lung fibroblasts increased endogenous AGT mRNA and the activity of an AGT-luciferase reporter to similar degrees (Uhal *et al.* 2007a). This suggested that the increase in endogenous AGT mRNA induced by TGF-β1 is mediated by a transcriptional mechanism. This transcriptional mechanism of AGT mRNA induction by TGF-β1 is confirmed in the work presented in this chapter. The elimination of the increase in AGT endogenous mRNA in response to TGF-β1 by the transcription inhibitor actinomycin-D (Figure 3.1), together with data indicating that TGF-β1 does not alter AGT mRNA stability (Figure 3.2), support that TGF-β1 increases AGT mRNA transcription in human lung fibroblasts.

Serial deletion analyses of the AGT-LUC reporter construct strongly suggest that functional *cis*-regulatory sequences in the AGT core promoter region -46 to +22 confer the majority of AGT transcription in response to TGF-β1. A search of the TRANSFAC database revealed the presence of many overlapping transcription factor binding sites in the AGT core promoter region -46 to +22; this suggested that many possible *cis*-regulatory elements might be involved in the AGT promoter response to TGF-β1. Although Smad-mediated signaling by TGF-β has been implicated in the pathophysiology of pulmonary fibrosis (Flanders 2004), a search of the full-length AGT promoter (-991 to +22) and AGT promoter region -46 to +22 did not identify any Smad binding sites and thereby ruled out Smad as a transcription factor directly interacting with the AGT promoter in IMR90 cells. The data reported here strongly suggest that almost all

of TGF-β1 – induced AGT-LUC activity is mediated via pathways resulting in increased JunD and HIF-1α binding to AGT core promoter.

In studies of normal lung fibroblasts, Eickelberg *et al.* showed that the AP-1 transcription factor family member JunD mediates TGF-β1 induced collagen gene expression (Eickelberg *et al.* 2001). In addition, AGT gene expression control in the lung has been studied in pulmonary epithelial cells, where the cardiovascular drug "amiodarone" induced AGT transcription through the AP-1 site between the TATA box and transcription start site (Uhal *et al.* 2007b, Chapter 2 and appendix B). These findings led to the hypothesis that TGF-β1 might regulate AGT expression through JunD binding to the AP-1 site between the TATA box and transcription start site. This hypothesis is supported by the data presented here that the TGF-β1 – induced increase in AGT-LUC in IMR90 cells was reduced by a-fos, an AP-1 transcription factor dominant negative (Figure 3.6), by the AP-1 *cis* element mutant -13 (Figure 3.5) or by siRNAs against JunD (Figure 3.10) and that with IMR90 cells exposure to TGF-β1, JunD binding increases to both synthetic AGT core promoter *in vitro* by pull-down assay (Figure 3.7) and endogenous AGT promoter in the genome of the cells by ChIP assay (Figure 3.8). Although at least three AP-1 sites are identified in the full-length AGT promoter by TRANSFAC search, the ability of the -13 AP-1 site mutant to eliminate 67% of the TGF-β-inducible AGT-LUC activity supports the argument that the single AP-1 site in the core promoter (Figure 3.5) confers the majority of the AP-1 – dependent signaling of AGT transcription induced by TGF-β1.

Interactions between TGF-β1 and hypoxia-inducible factors have been described in a number of experimental systems. For example, hypoxia itself induces the expression

of TGF-β1 and Smad-dependent TGF-β signaling through HIF-1 in rat pulmonary artery walls (Jiang *et al.* 2007) and in human hepatic stellate cells (Shi *et al.* 2007). In HepG2 cells, TGF-β1 was shown to induce stabilization of HIF-1α protein through a mechanism involving Smads and the inhibition of prolyl hydroxylase-2 (McMahon *et al.* 2006). Whether or not a similar mechanism explains the increase in HIF-1α protein abundance (Figures 11, 16), and HIF – mediated transcription on HRE-LUC (Figure12) induced by TGF-β1 in the present study will be an interesting topic for future inquiry.

Hypoxia inducible factors have been suggested to be involved in the pathogenesis of lung fibrosis, on the basis that hypoxia was found to induce apoptosis of primary alveolar epithelial type II cells (Krick *et al.* 2005) and HIF-1α overexpression was detected in the hyperplastic epithelium of fibrotic lungs (Tzouvelekis *et al.* 2007). However, this study is the first to suggest a molecular mechanism that links hypoxia signaling (HIF-1) with fibrogenic molecules (angiotensin and TGF-β1) in the lungs. The increased HIF-1α abundance and function by TGF-β1 in our system raised the question whether HIF-1α is involved in TGF-β1 – induced AGT transcription. Evidence for HIF-1α involvement is presented here, as HIF overexpression alone was capable to induce AGT-LUC expression (Figure 3.15) and that IMR90 cells exposure to TGF-β1 HIF-1α binding to AGT core promoter increases both *in vitro* by the use of synthetic oligos for AGT core promoter (-46 to +22) (Figure 3.13) and in the genome of the cells as indicated by ChIP assay reflecting binding to the endogenous promoter (Figure 3.14). Furthermore, the TGF-β1 – induced increase in AGT-LUC in IMR90 cells was reduced by siRNAs against HIF-1α (Figure 3.18). Complete elimination of TGF-β1 – induced AGT-LUC by the use of both JunD and HIF-1α specific siRNAs in the double knockdown experiment

(Figure 3.18), indicated cooperation between the two transcription factors in mediating TGF-β1 – induced AGT transcription.

Similarly, interactions between JunD and HIF-1 have been shown to regulate the activation of other genes. In A549 cells, JunD competes with HIF-1 for binding to the TGF-β1 promoter at the site of the -1347 SNP (Shah *et al.* 2006). In rat mesangial cells, JunD and HIF-1 interact to induce the expression of vascular endothelial growth factor (VEGF) in response to overexpression of glucose transporter 1 (GLUT-1, Pfäfflin *et al.* 2006), but the exact site(s) of action of these transcription factors on the VEGF promoter is not currently known. To our knowledge, the present study is the first to document the regulation of AGT by cooperative interactions between JunD and HIF-1 at the AGT core promoter.

The AGT core promoter includes a region between the TATA box and transcription start site that contains three single nucleotide polymorphisms (SNPs), the G-6A, C-18T and A-20C SNPs (Jeunemaitre 2004), that have been studied extensively in the context of liver fibrosis and hypertension. These SNPs have been associated with alterations in basal AGT promoter – driven transcription in liver cells and transcription factor binding to the variant DNA sequences (Inoue *et al.* 1997, Xiao *et al.* 2006). Together, those findings suggest a critical role for this domain in the AGT core promoter in the control of AGT expression by the liver.

The AGT core promoter polymorphisms A-20C and G-6A were associated with liver cirrhosis in patients with chronic hepatitis B (Xiao *et al.* 2006). Further, a relationship was found between the inheritance of a high TGF-β1 producing genotype, together with the A allele of the AGT G-6A SNP, and the development of progressive

hepatic fibrosis after chronic hepatitis C infection (Powell *et al.* 2000). A similar relationship was found between these two SNPs and non-alcoholic fatty liver disease in obese patients (Dixon *et al.* 2003). In pulmonary fibrosis, TGF-β1 gene polymorphisms are suggested to affect disease progression in patients with idiopathic pulmonary fibrosis (Xaubet *et al.* 2003). The AGT promoter G-6A polymorphism was found to associate with idiopathic pulmonary fibrosis progression (Molina-Molina *et al.* 2008); this study is discussed in chapter 4.

In summary, this chapter shows that TGF-β1 upregulates AGT transcription in the human lung fibroblast cell line IMR90 through a mechanism that requires both JunD and HIF-1α as key components of a transcription factor complex. This complex acts primarily at the core promoter of AGT through a domain that contains three single-nucleotide polymorphisms; one of these (G-6A) already known to associate with idiopathic pulmonary fibrosis progression. The roles of the AGT sequence variants conferred by the three SNPs in lung fibroblast AGT expression and idiopathic pulmonary fibrosis are currently being investigated.

CHAPTER 4:

EFFECTS OF ANGIOTENSINOGEN PROMOTER SINGLE NUCLEOTIDE POLYMORPHISMS ON TGF-β1 – INDUCED ANGIOTENSINOGEN IN LUNG FIBROBLASTS

INTRODUCTION

The angiotensinogen gene (AGT) core promoter has 3 single nucleotide polymorphisms (SNPs) in the sequences between the TATA box and transcription start site. These SNPs are G-6A, C-18T and A-20C. These SNPs have shown variations in the AGT promoter – driven transcription and in transcription factor binding to DNA sequences (Inoue *et al.* 1997, Yanai *et al.* 1997a). The G-6A SNP was shown to associate with hypertension and affect reporter gene expression in HepG2 liver cells and 293 kidney cells. The expression levels of reporter genes were shown to be higher with nucleotide A in position -6. The difference in basal transcriptional activity between the two nucleotide promoter variants ranged from 20 to 70% according to the cell-type and vector used in transfection (Inoue *et al.* 1997). SNPs C-18T and A-20C were also shown to affect AGT gene transcription in reporter gene expression studies. The presence of variants -20A and -18T nucleotides in AGT promoter displayed reduced basal transcription (40%) compared to the transcriptional activity when variants -20C and -18C were present. In the same study, the effect of -20 and -18 SNPs was shown on AGCF1 (angiotensinogen core promoter element binding factor 1) transcription factor binding to AGT promoter by gel shift assays. These showed that binding of AGCF1 to -20C, -18C > -20A, -18C > -20A, -18T, corresponding to the transcription activity order (Yanai *et al.* 1997a). Other AGT promoter SNP – transcription factor interactions include A-20C with estrogen receptor and the orphan receptor Arp-1(Zhao *et al.* 1999, Narayanan *et al.* 1999) and the G-6A interaction with the nuclear factor YB1 (Nakajima *et al.* 2002).

In a recent study by our lab group, the AGT gene G-6A polymorphism was shown to influence disease progression in IPF in a case – control study in a Spanish population.

In this study, 438 DNA specimens extracted from blood of 214 IPF patients and 224 normal subject controls were analyzed for their G-6A SNP genotype. Although distribution of G–6A genotypes and alleles did not significantly differ between cases and controls, AA genotype at -6 SNP was associated with disease progression. Higher alveolar arterial oxygen tension changes over time were observed in patients with AA genotype indicating increased deterioration in gas exchange and pulmonary function in patients with IPF (Molina-Molina *et al.* 2008). In addition, other studies on fibrosis of the liver and other organs have demonstrated association between AGT core promoter SNPs and development and/or progression of fibrosis (Xiao *et al.* 2006). In some studies, this progression of fibrosis was also associated with profibrotic genotype of the TGF-β1 gene (Powell *et al.* 2000, Dixon *et al.* 2003, Hume *et al.* 2006).

The AGT core promoter SNPs are overlapping or nearby two transcription factors binding domains, AP-1 and HIF1 (Figure 4.1). The profibrotic drug, Amiodarone, was shown to up-regulate AGT gene expression in human alveolar epithelial cells through a mechanism mediated by AP-1 family transcription factors through the AP-1 binding site between the TATA box and transcription start site (Uhal *et al* 2007b, Chapter 2). In addition to the AP-1 site between the TATA box and transcription start site, TGF-β1 up-regulates AGT gene expression in human lung fibroblasts through HIF-1 (Chapter 3).

In this chapter, having different variant sequences representing AGT core promoter SNPs is shown to affect HIF-1α binding to AGT core promoter in response to TGFβ1 in IMR90 human lung fibroblasts.

METHODS

Cell Culture and Treatment

The human embryonic lung fibroblast cell line IMR90 was obtained from the American Type Culture Collection and cultured in Eagle's modified minimal essential media (EMEM) supplemented with 10% fetal bovine serum (FBS). IMR90 cells of passage number 14 were used. Cells were serum starved at ~70% confluence level for 24 hours followed by treatment with TGF-β1 (2ng/ml) for another 24 hours before harvesting and nuclear extraction.

Nuclear Extraction

Nuclear extracts were isolated from TGF-β1 treated IMR90 cells using Nuclear Extraction Kit (Panomics, Redwood City, CA). Cytosolic extracts were first separated from nuclei by scraping IMR90 cells in buffer containing 10mM HEPES, pH 7.9, 10mM KCl, 10 mM EDTA, 1 mM DTT and 0.4% IGEPAL supplemented with protease inhibitor cocktail followed by centrifugation. Nuclear proteins were extracted from nuclei by shaking in a buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and protease inhibitor cocktail at 4°C. Protein concentration in the nuclear extracts was assayed using Bradford protein assay (BioRad).

DNA/Protein Pull-Down

Two double stranded 5'-biotinylated oligonucleotides having AGT core promoter sequence were synthesized (IDT DNA Technologies); one oligo has the nucleotides -20C, -18C, -6A, and the other -20A, -18T, -6G (Figure 4.2 A). Each of the two variant

sequence AGT promoter biotin-oligos was immobilized to MagneSphere® streptavidin magnetic beads (Promega). Equal amounts of nuclear extracts (20 μg) were allowed to bind to 1 μg of each immobilized AGT promoter variant in the presence of 10 μg poly d(I-C) (Roche) for competition of non-specific binding for 2 hours at 4°C. Proteins bound to the biotinylated AGT promoter fragment were washed 3 times in 1x PBS. The bound "pull-down" proteins were then eluted by heating in equal volume of Laemmli sample buffer (for Western blot).

Western Blotting

Equal volume of eluted pull-down protein complexes were separated by SDS-PAGE then transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) in Towbin buffer and used for western blotting as mentioned previously (page 37 chapter 2) using antibodies HIF-1α (BD Biosciences), HRP-conjugated antimouse secondary antibodies (Santa Cruz Biotechnology) an chemiluminescent substrate detection.

RESULTS

AGT Core Promoter SNPs Affect HIF-1α Binding

Immunoreactivity for HIF-1α was detected by western blotting of pull-down protein complexes from TGF-β1 – treated IMR90 cells in both AGT promoter sequence variants. As shown in Figure 4.2 B, the band for HIF-1α in variant type 1 (-20C, -18C, -6A) was of more intensity than for type 2 (-20A, -18T, -6G). This shows that AGT sequence variants at bases -20, -18 and -6 in AGT promoter alter HIF-1α binding to AGT promoter fragment -46 to +22 in IMR90 cells treated with TGF-β1.

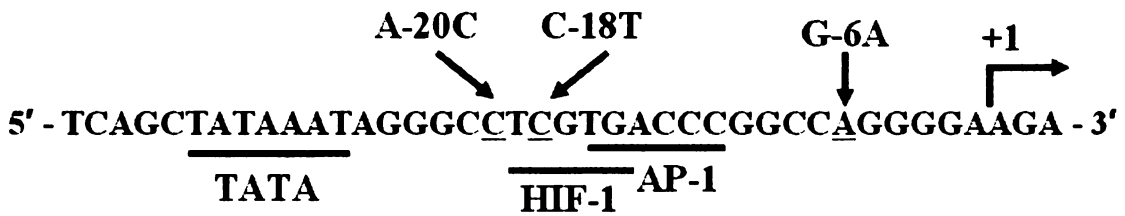


Figure 4.1.

Human AGT promoter proximal sequence between the TATA box and transcription start site. Indicated sequences are transcription factor binding domains of AP-1 and HIF-1 and known SNPs at positions -6, -18 and -20 from the transcription start site (+1).

A.

B.

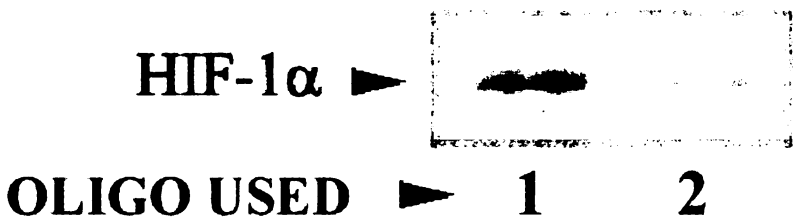


Figure 4.2.

Sequence variants reflecting SNPs of AGT alter HIF-1α binding to AGT promoter. (A) Sequences for oligonucleotides used in the DNA-protein pull-down assay. Each double stranded oligo represented AGT core promoter sequence spanning region -46 to +22 (68-mer oligo). Only the region of sequence variations shown here as indicated at positions -20, -18, and -6 from the transcription start site of AGT gene. (B) AGT core promoter sequence variants affect HIF-1α binding. HIF-1α western blotting of pull down proteins from TGF-β1 treated cells bound to the 2 types of AGT promoter oligo variants in (A) (see methods).

DISCUSSION AND FUTURE DIRECTIONS

Genetic polymorphisms association with IPF disease progression was found for both TGF-β1 and AGT genes. The presence of proline allele in codon 10 of the TGF-β1 gene or the presence of AA genotype in -6 position of AGT gene promoter were associated with increased deterioration in gas exchange in patients with IPF (Xaubet *et al.* 2003, Molina-Molina *et al.* 2008). These genetic associations of AGT and TGF-β1 genes with IPF progression indicate roles for these genes in IPF pathogenesis. Cross-talk between AGT and TGF-β1 was discovered to form an "autocrine loop" in myofibroblasts that is speculated to form an autonomous persistent profibrotic microenvironment as in human IPF (Uhal *et al.* 2007a). Furthermore, stimulation of AGT gene expression by TGF-β1 challenge in human lung fibroblasts was shown to be mediated via induction of AGT gene transcription and HIF-1α and JunD transcription factors binding to AGT core promoter (Chapter 3).

The AGT gene core promoter single nucleotide polymorphisms (SNPs) contribute to variations of AGT expression level via differential binding to transcription factors (Inoue *et al.* 1997, Yanai *et al.* 1997a, Zhao *et al.* 1999, Narayanan *et al.* 1999, Nakajima *et al.* 2002). AGT core promoter SNPs at positions -6 and -20 showed association with progression of fibrosis in liver when co-inherited with profibrotic genotype of TGF-β1 (Xiao *et al.* 2006, Powell *et al.* 2000, Dixon *et al.* 2003, Hume *et al.* 2006).

Considering the above mentioned studies together, AGT core promoter SNPs (i) play role in controlling AGT gene transcription, (ii) affect binding to transcription factors, (iii) associate with fibrotic diseases, (iv) promote fibrosis progression in the presence of profibrotic TGF-β1 genotype, (v) and that these SNPs are present in

overlapping/near by sequence to AP-1 and HIF-1 binding domains controlling AGT transcription, we hypothesized that AGT core promoter SNPs can affect binding of transcription factors to AGT promoter in response to TGF-β1 stimulation. Consistent with this hypothesis, DNA/protein pull-down of bound transcription factors identified variation in the transcription factor HIF-1α binding to AGT core promoter with the alternative bases present at the SNP positions (Figure 4.2).

According to studies on liver cells (Inoue *et al.* 1997, Yanai *et al.* 1997a), basal AGT expression is expected to be highest with the haplotype having nucleotides C, C, A are present at SNPs -20, -18, -6 respectively versus the haplotype with A, T, G nucleotides at these positions. To test whether this is the case for TGF-β1 – induced AGT expression in IMR90 human lung fibroblasts, synthetic oligonucleotides representing these two haplotypes were tested for their ability to differentially bind to TGF-β1 – inducible transcription factors. Type 1 sequence variant had nucleotides -20C, -18C, -6A to represent the highest expected AGT expression haplotype while Type 2 sequence variant had nucleotides -20A, -18T, -6G representing the lowest expected AGT expression haplotype (Figure 4.2A, oligos 1 and 2). Consistent with the expected AGT expression levels, type 1 sequence variant oligo showed more HIF-1α binding in response to TGF-β1 than type 2 indicated by DNA/protein pull-down and western blotting (Figure 4.2B).

This variation in transcription factor binding to AGT core promoter as a result of DNA sequence variation at SNP positions suggests that AGT expression in response to TGF-β1 may be dependent on individual's haplotype. *De novo* pulmonary synthesis of AGT was shown to be required for lung fibrogenesis in animal models (Li *et al.* 2007).

Local pulmonary synthesis of AGT in pulmonary fibrosis was shown to be increased in animal models (Li *et al.* 2003a), human primary cell cultures (Wang *et al.* 1999a) and IPF patients' lung tissues (Li *et al.* 2006). In addition, IPF patients' progression of fibrosis associated with the AGT core promoter SNP at -6 position (Molina-Molina *et al.* 2008). Together with the results presented here, these suggest that severity of IPF disease and progression may correlate with individual's haplotype due to its impact on the expression of the profibrotic precursor AGT.

Increased plasma levels of AGT protein and elevated blood pressure were shown to associate with -6A AGT promoter SNP (Sethi *et al.* 2003, Markovic *et al.* 2005). However, meta-analysis of the AGT polymorphisms association with hypertension showed dual role for the A-20C SNP according to population tested and excluded association of the A-6G variant with hypertension (Pereira *et al.* 2008). One possible explanation of the controversy on the A-6G AGT promoter SNP association with hypertension is the presence of linkage disequilibrium between A-6G and A-20C. In studies demonstrating role of the A-6G, the population tested could have stronger linkage disequilibrium with A-20C so that the results could actually be reflecting association with A-20C. In IPF, the A-6G SNP showed association with IPF disease progression and deterioration of gas exchange in pulmonary function (Molina-Molina *et al.* 2008). However, in that study A-6G SNP did not associate with the incidence of IPF. Future assessment of the IPF patients AGT SNP A-20C genotype could reflect association with IPF incidence similar to the meta-analysis study results on hypertension. Taking into consideration the role of the AGT core promoter region on AGT expression under

fibrotic conditions, genotyping IPF patients and testing association of the other two SNPs in this region, at positions -20 and -18, should be warranted.

Although the AGT promoter SNPs A-20C and C-18T are in linkage disequilibrium with G-6A (Jeunemaitre 2004), this linkage was not shown to be absolute to the best of knowledge. Presence of the alternative bases at these three SNPs can give rise to eight possible combinations or haplotypes. If genotyping results for IPF patients reveal association of either or both -20 and -18 SNPs with IPF progression, this would enable the prediction of a predisposing haplotype to fibrotic lung progression. The prediction of such haplotype would stimulate investigation of this haplotype's effect of AGT transcription in the lung fibroblasts for both basal and TGF-β1 – induced transcription to further elucidate the presence of functional association of these SNPs. This can be done by promoter reporter studies similar to studies on the effect of the -18 and -20 variants on basal AGT expression in liver cells (Yanai *et al.* 1997a).

In summary, data in this chapter show that sequence variants reflecting AGT core promoter SNPs alter transcription factor binding to AGT promoter in lung fibroblasts stimulated with TGF-β1. AGT core promoter SNPs association with IPF disease and their effect on TGF-β1 – induced AGT transcription is an interesting topic for future studies.

CHAPTER 5:

SUMMARY AND CONCLUSIONS

UPREGULATION OF ANGIOTENSINOGEN IN IPF

In IPF Lung tissues

Investigations on cell cultures and animal models of pulmonary fibrosis prior to this study showed that inducers of epithelial apoptosis (Wang *et al.* 1999b, 2000a, Li *et al.* 2003b) and pulmonary fibrosis induce local production of angiotensinogen (AGT) (Li *et al.* 2007), the precursor of the profibrotic peptide angiotensin II (Ang II). In this study, increased local production of AGT mRNA and protein was found in the lung tissues of human IPF patients compared to normal human lung tissues (Chapter 2). Two cell types showed this increased AGT production; alveolar epithelial cells and myofibroblasts.

In Alveolar Epithelial Cells

Induction of alveolar epithelial cell apoptosis is an essential event in the initiation of pulmonary fibrosis (Uhal 2003). The profibrotic antiarrhythmic drug amiodarone was shown to induce alveolar epithelial cell apoptosis (Bargout *et al.* 2000) and cause pulmonary fibrosis (Uhal *et al.* 2003) through the involvement of renin angiotensin system. Other inducers of apoptosis, such as Fas ligand (Wang *et al.* 1999b, 1999c), tumour necrosis factor-α (Wang *et al.* 2000a) and the antineoplastic agent bleomycin (Li *et al.* 2003b) were shown to upregulate AGT expression in alveolar epithelial cells. In this study (chapter 2), amiodarone is also found to upregulate AGT gene expression by transcriptional mechanism to increase AGT mRNA levels. This upregulation is mediated by activation protein-1 (AP-1) family transcription factors and requires an AP-1 binding site on AGT promoter between the TATA box and transcription start site.

In Myofibroblasts

Myofibroblasts are considered key mediators of pulmonary fibrosis as increased numbers of fibroblastic foci are associated with disease progression and worse prognosis in IPF (King *et al.* 2001). Transforming growth factor-beta1 (TGF-β1) plays an essential role in the differentiation of fibroblasts to myofibroblasts and the maintenance of myofibroblast survival (Phan 2002). Induction of the phenotypic transition to the myofibroblast in the IMR90 normal human lung fibroblast cell line by TGF-β1 resulted in increased AGT mRNA (Uhal *et al.* 2007a). Research presented here focused on the molecular determinants of TGF-β1-induced AGT gene expression in human lung fibroblasts.

Data presented in chapter 3 indicated that in human lung fibroblasts TGF-β1 induces AGT gene expression through transcriptional mechanism. Analyses of AGT promoter specified sequence from -46 to +22 region to be responsive to TGF-β1. Screening for candidate cis-elements by TESS search in TRANSFAC database gave rise to many possible candidate transcription factors that can be mediating TGF-β1 – induced AGT including AP-1 and hypoxia inducible factor (HIF) binding domains in the AGT promoter -46 to +22 region.

In normal lung fibroblasts, Eickelberg *et al.* showed that JunD, an AP-1 transcription factors family member, mediate TGF-β1 – induced collagen expression (Eickelberg *et al.* 2001). Mutation of the AP-1 binding site or AP-1 knockdown with a dominant negative to AP-1 transcription factors reduced full-length angiotensinogen promoter stimulation by TGF-β1, indicating a role of AP-1 transcription factors and AP-1

binding site proximal to AGT transcription start site in TGF-β1 – induced AGT transcription. Stimulation of JunD binding to AGT promoter in response to TGF-β1 and partial reduction of AGT promoter induction by TGF-β1 after JunD gene silencing indicated that the AP-1 family member, JunD, plays a role in TGF-β1 induced AGT transcription.

Recent data in lung fibrosis suggests that hypoxia is involved in the pathogenesis of lung fibrosis (Krick *et al.* 2005, Tzouvelekis *et al.* 2007). Having HIF binding site in AGT promoter region responsive to TGF-β1, it was interesting to find that TGF-β1 increases HIF-1α protein abundance and stimulates HIF-1 – regulated gene transcription in IMR90 human lung fibroblasts. Stimulation of HIF-1α binding to AGT promoter in response to TGF-β1 and partial reduction of AGT promoter induction by TGF-β1 after HIF-1α gene silencing indicated that HIF-1 plays role in TGF-β1 induced AGT transcription. Complete elimination of TGF-β1 – induced AGT promoter stimulation by gene silencing of both JunD and HIF-1α indicated cooperation between the two transcription factors at the AGT core promoter in mediating TGF-β1 – induced AGT transcription.

In Presence of AGT Sequence Variants

The AGT core promoter has 3 single nucleotide polymorphisms (SNPs) in the sequences between the TATA box and transcription start site at positions -6, -18 and -20 from transcription start site. These SNPs have shown variations in the AGT promoter – driven transcription and in transcription factor binding to variant DNA sequences (Inoue *et al.* 1997, Yanai *et al.* 1997a). The AGT promoter G-6A SNP was shown to influence

disease progression in IPF in a case – control study (Molina-Molina *et al.* 2008). The AGT core promoter SNPs are in the same promoter region containing AP-1 and HIF binding sites with the HIF binding site overlapping with the -18 and -20 SNPs. Here we show that having sequence variants representing AGT core promoter SNPs alter HIF-1α transcription factor binding to AGT core promoter in TGF-β1 – treated human lung fibroblasts (Chapter 4). This variation in transcription factor binding to AGT core promoter as a result of DNA sequence variation at SNP positions suggests that AGT expression in response to TGF-β1 may be dependent on individual's haplotype.

DOWNREGLATION OF ANGIOTENSIN CONVERTING ENZYME-2 IN IPF

In IPF Lung Tissues

Angiotensin converting enzyme-2 (ACE-2) cleaves one amino acid from the carboxyl terminal of the profibrotic peptide Ang II to form the peptide angiotensin 1-7 (Ang 1-7). ACE-2 and its product Ang 1-7 were shown to play a protective role in experimental models of cardiac fibrosis (Huentelman *et al.* 2005, Díez-Freire *et al.* 2006), liver fibrosis (Warner *et al.* 2007) and in experimental acute lung injury (Imai *et al.* 2005). In the study presented here (Chapter 2), ACE-2 levels in IPF lung tissue showed dramatic decrease of ACE-2 mRNA levels compared to normal lung tissue. This mRNA down-regulation was also associated with a decrease in ACE-2 protein levels and activity in IPF lung (Li *et al.* 2008). These findings are consistent with the protective roles of ACE-2 in fibrosis.

In Alveolar Epithelial Cells

Ang II was shown in many studies to induce apoptosis of alveolar epithelial cells, one of the histologic features of IPF, through AT_1 receptor (Wang *et al.* 1999b, 1999c, 2000a, Li *et al.* 2003a). Ang II was also shown to down-regulate ACE-2 mRNA in rat brain astrocytes through AT_1 receptor (Gallagher *et al.* 2006). In this study, Ang II was shown to decrease ACE-2 mRNA levels in alveolar epithelial cells (Chapter 2) that could be reversed by the angiotensin receptor AT_1 antagonist "losartan" and by the ACE-2 product "Ang 1-7". These findings indicate that Ang II – induced ACE-2 mRNA down-regulation is mediated via AT_1 receptor and support the presence of antagonism between the two angiotensin peptides in lung alveolar epithelial cells. Interestingly, Ang 1-7 increased levels of ACE-2 mRNA in alveolar epithelial cells to a level higher than basal ACE-2 mRNA levels suggesting that the mechanism for Ang 1-7 for its action on ACE-2 mRNA levels is mediated through a receptor different from AT_1 and raises the possibility of involvement of the Ang 1-7 G-protein coupled receptor "Mas" protooncogene in the protection from fibrosis.

Cell Type – Specific ACE-2 Knockdown

To enable future investigations of the *in vivo* roles of ACE-2 in experimental lung fibrosis models, alveolar epithelial cell – specific ACE-2 short hairpin RNA (shRNA) plasmid was constructed. The choice of alveolar epithelial cell specificity is based the role epithelial apoptosis plays in fibrosis initiation (Li *et al.* 2004b) and the protective role ACE-2 plays in lung cells (Imai *et al.* 2005). The designed and constructed plasmids

expressing mouse ACE-2 (mACE-2) shRNA under the control of the alveolar epithelial cell – specific human SP-C promoter (hSP-C) successfully knocked down ACE-2 in cultured transfected alveolar epithelial cells but not in lung fibroblasts (Chapter 2). This indicated the feasibility for the use of these clones to construct a viral vector for future *in vivo* testing of ACE-2 knockdown in alveolar epithelial cells.

CONCLUSIONS

This study shows that in IPF the Ang II peptide precursor AGT gene local expression is up-regulated, while the Ang II degrading enzyme ACE-2 gene local expression is down-regulated in the fibrotic lung. These indicate that in IPF, pulmonary gene expression patterns favor Ang II production and hinder Ang II degradation causing impairment of balance between Ang II production and degradation in fibrosing conditions promoting Ang II accumulation. In light of the profibrotic roles of Ang II peptide, targeting the reversal of this imbalance towards the decrease of Ang II accumulation represents a possible therapeutic approach for IPF. For this targeting, pathways leading to this imbalance should be defined. In this study, highlights on ACE-2 down-regulation by Ang II and molecular determinants for up-regulation of AGT expression were investigated. Data presented in this study show additional roles for Ang II peptide acting on AT_1 receptor in progression of fibrogenesis by causing a decrease in the protective ACE-2 enzyme gene expression. The protective role of the Ang II degradation product "Ang 1-7" is further illustrated through its effects in up-regulating the protective ACE-2 enzyme gene expression.

The AGT gene expression up-regulation in fibrosing conditions for both alveolar epithelial cells and lung fibroblasts is mediated via transcriptional mechanism. The activation protein-1 (AP-1) family of transcription factors is involved in up-regulation of AGT gene transcription in both alveolar epithelial cells and lung fibroblasts in response to the profibrotic drug amiodarone and cytokine TGF-β1 respectively. In addition to the AP-1 family member "JunD", AGT transcription by TGF-β1 in lung fibroblasts was found to be mediated by the hypoxia inducible factor-1 (HIF-1). These findings suggest a

molecular mechanism that links hypoxia signaling (HIF-1) with fibrogenic molecules (angiotensin and TGF-β1) in the lungs.

The TGF-β1 – induced HIF-1 binding to AGT promoter sequence variants reflecting single nucleotide polymorphism (SNPs) of AGT showed altered transcription factor binding. The AGT promoter G-6A SNP has been shown to associate with IPF progression. This association could be explained by the effects of SNP variants at position -6 and/or at positions -18 and -20, that are in linkage disequilibrium with the -6 SNP, on AGT promoter stimulation by fibrotic factors and induction of *de novo* AGT gene expression. These results suggest that severity of IPF disease and progression may correlate with individual's haplotype due to its impact on the expression of the profibrotic precursor AGT and that choice of a successful therapeutic approach may be dependent on the patient's genotype.

APPENDICES

Appendix A:

EXTRAVASCULAR SOURCES OF LUNG ANGIOTENSIN PEPTIDE SYNTHESIS IN IDIOPATHIC PULMONARY FIBROSIS

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ABSTRACT

Previous work from this laboratory demonstrated *de novo* synthesis of angiotensin (ANG) peptides by apoptotic pulmonary alveolar epithelial cells (AEC) and by lung myofibroblasts *in vitro* and within bleomycin-treated rat. To determine if these same cell types also synthesize ANG peptides *de novo* within the fibrotic human lung *in situ,* paraffin sections of normal and fibrotic human lung (Idiopathic Pulmonary Fibrosis, IPF) were subjected to immunohistochemistry (IHC) and in situ hybridization (ISH) to detect ANG peptides and angiotensinogen (AGT) mRNA. These were analyzed both alone and in combination with cell-specific markers of AEC (mAB MNF-116) and myofibroblasts (α-smooth muscle actin mAB, α-SMA) and an *in situ* DNA end labeling method (ISEL) to detect apoptosis. In normal human lung, IHC detected AGT protein in smooth muscle underlying normal bronchi and vessels, but not elsewhere. Realtime RT-PCR and western blotting revealed that AGT mRNA and protein were 21-fold and 3.6-fold more abundant, respectively, in IPF lung biopsies relative to biopsies of normal human lung (both $p<0.05$). In IPF lung, both AGT protein and mRNA were detected in AEC that double-labeled with mAB MNF-116 and with ISEL, suggesting AGT expression by apoptotic epithelia *in situ.* AGT protein and mRNA also colocalized to myofibroblast foci detected by α-SMA mAB, but AGT mRNA was not detected in smooth muscle. These data are consistent with earlier data from isolated human lung cells *in vitro* and with bleomycin-induced rat lung fibrosis models, and suggest that apoptotic AEC and myofibroblasts constitute key sources of locally derived ANG peptides in the IPF lung.

Keywords: Lung fibrosis; Myofibroblast; Alveolar Epithelial Cells; Apoptosis

INTRODUCTION

Angiotensin II (ANGII) is known to play a key role in tissue fibrogenesis in a variety of organs including the heart, kidney and liver (Klahr & Morrissey 1997, Weber & Sun 2000, Yoshiji *et al.* 2001). In experimental animal models of lung fibrosis, a key role for ANGII has been implicated by demonstrations that inhibitors of ANG converting enzyme (ACEis) or blockers of ANG receptor AT_1 could reduce or abrogate fibrogenesis in response to bleomycin (Marshall *et al.* 2004, Wang *et al.* 2000b), monocrotaline (Molteni *et al.* 1985), gamma irradiation (Molteni *et al.* 2000) or the antiarrhythmic agent amiodarone (Uhal *et al.* 2003). A key role for ANGII in the pathogenesis of lung fibrosis is further supported by the finding that knockout mice deficient in ANG receptor AT_{1a} are resistant to bleomycin-induced lung collagen deposition (Li *et al.* 2003a). The profibrotic potential of ANGII is believed to be mediated by upregulation of collagen gene expression in lung fibroblasts (Marshall *et al.* 2000, 2004), by induction of apoptosis in AECs (Li *et al.* 2003b) and other profibrotic actions (Marshall 2003).

Several lines of evidence suggest that the source(s) of precursor for the ANGII synthesis that drives fibrogenesis in the lung is generated locally, i.e., within the lung tissue itself. Cultured alveolar epithelial cells (AEC) of either human or rat origin synthesize angiotensinogen (AGT) mRNA and secrete ANG peptides upon exposure to proapoptotic stimuli such as Fas ligand (Wang *et al.* 1999b), TNF-alpha (Wang *et al.* 2000a) or bleomycin (Li *et al.* 2003b). ANGII itself also induces apoptosis of AECs through ANG receptor AT_1 (Wang *et al.* 1999c). In addition, myofibroblasts isolated from the lungs of patients with Idiopathic Pulmonary Fibrosis (IPF) also synthesize AGT mRNA constitutively and secrete ANG peptides (Wang *et al.* 1999a). The notion that

local pulmonary synthesis of ANGII *de novo*, i.e. from the precursor AGT within the lung, is required for lung fibrogenesis is supported by the recent demonstration that intratracheal administration of antisense oligonucleotides against AGT mRNA could prevent bleomycin-induced lung fibrosis in rats without affecting circulating levels of AGT protein (Li *et al.* 2007).

To date, the evidence in support of local pulmonary synthesis of AGT protein *de novo* is derived from either animal models (Li *et al.* 2003a, 2007) or indirect studies of primary cells isolated from fibrotic human lung (Wang *et al.* 1999a). In the present study it was therefore of interest to directly examine fibrotic human lung tissue in an attempt to identify local tissue sources of ANG peptide generation. We report here the findings of ANG peptide expression in at least two cell types, apoptotic AEC and myofibroblasts, in lung tissue from patients with IPF.

MATERIALS AND METHODS

Materials

Monoclonal antibodies to alpha-smooth muscle actin (Clone 1A4), propidium iodide, purifed renin, purified agiotensin converting enzyme, purified angiotensinogen from human serum and biotinylated deoxyuridine trisphosphate (Bio-dUTP) were obtained from Sigma Chemical, Saint Louis, MO. Anticytokeratin monoclonal antibody MNF-116 was purchased from Dako Cytomation, Carpinteria, CA. Antibodies recognizing angiotensinogen and ANG peptides were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. DNA Polymerase I was obtained from Promega Corp., Madison, WI. Biotinylated oligonucleotides for in situ hybridization were obtained from Invitrogen Corp., Carlsbad, CA. Solutions A and B of the Vectastain ABC Elite formulation were purchased from Vector Laboratories, Burlingame, CA. All other materials were of reagent grade.

Tissue Samples and Handling

Human lung tissue was obtained by open lung biopsy or video-assisted thoracoscopic surgery performed at the Thorax Institute, Hospital Clínic of Barcelona, Spain. Fibrotic lung tissue was obtained from twelve patients with Idiopathic Pulmonary Fibrosis (IPF); biopsies were obtained from more than one lung lobe. All patients had clinical, functional and radiologic features which fulfill the diagnostic criteria for an ILD (Katzenstein & Myers 1998). Briefly, all had progressive dyspnea, bilateral reticulonodular images on chest roentgenogram, restrictive lung functional impairment with decreased lung volumes and reduced single-breath carbon monoxide diffusing

capacity, and hypoxemia at rest that worsened with exercise. Patients with IPF had neither antecedents of any occupational or environmental exposure nor other known cause of ILD. None of the IPF patients had received steroids or other immunosupressant therapy at the time of clinical sample collection. Normal human lung tissue was obtained from individuals undergoing surgical treatment for spontaneous pneumothorax with no history of pulmonary disease. No histopathologic evidence of disease was found in those tissue samples. Written, informed consent was obtained from the patients according to institutional guidelines, and the study was approved by the Ethics Committee of Hospital Clinic de Barcelona.

All tissue was fixed in 10% neutral buffered formalin for 16 hours and embedded in paraffin. Sections were cut at 5.0um thickness and mounted on glass cover slips. Human lung tissues designated for RNA isolation were immediately immersed in ice-cold TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) after excision and were processed immediately. Lung tissues designated for analysis of proteins were flash-frozen in liquid nitrogen and stored at –80C until protein isolation as described below.

In Situ End Labeling of Fragmented DNA

Tissue sections were deparaffinized by passing through xylene, xylene:alcohol 1:1, 100% alcohol and 70% alcohol for 10 minutes each. In situ end labeling (ISEL) of fragmented DNA was conducted by a modification of the method of Mundle *et al.* (Mundle *et al.* 1994) performed as described earlier on IPF lung tissue (Uhal *et al.* 1998). Briefly, ethanol was removed by rinsing in distilled water for at least 10 minutes. The cover slips were then placed in 0.23% periodic acid (Sigma Chemical, St. Louis, MO) for

30 minutes at 20C. Samples were rinsed once in water, three times in 0.15M phosphate buffered saline (PBS) for 4 minutes each, and were then incubated in saline sodium citrate solution (SSC, 0.3 M NaCl and 30mM sodium citrate in water, pH 7.0) at 80C for 20 minutes. After four rinses in PBS and four rinses in Buffer A (50mM Tris/HCl, 5mM MgCl, 10mM β-mercaptoethanol and 0.005% BSA in water, pH 7.5), the cover slips were incubated at 18C for two hours with ISEL solution (1.0μM Bio-dUTP, 20U/ml DNA Polymerase I and 0.01mM each of dATP, dCTP and dGTP in Buffer A). Afterward the sections were rinsed thoroughly five times with Buffer A and three additional times in 0.5M PBS. For detections based on diaminobenzidine, the tissue was then incubated at 20C with a solution consisting of 80ul each of reagents A (avidin solution) and B (biotin-peroxidase solution) of the Vectastain Elite Kit (Burlingame, CA) in 3.84ml Buffer B (1% BSA and 0.5% TWEEN 20 in 0.5M PBS). After 30 minutes the sections were washed four times in PBS and were then immersed for 10 minutes into a solution of 0.25mg/ml diaminobenzidine (DAB) in 0.05M Tris/HCl, pH7.5 containing 0.01% hydrogen peroxide. Alternatively, detection of incorporated dUTP was achieved with a Fast Blue chromogen system. The tissues were rinsed in distilled water three times and mounted under Fluoromount Solution (Southern Biotechnology Associates, Birmingham, AL).

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry (IHC) for ANG peptides, type II cell-specific cytokeratins and α–smooth muscle actin (α-SMA) was performed with anti-ANG peptide antibody (Santa Cruz, 1:50 dilution), anticytokeratin antibody MNF-116 (Dako, 1:50 dilution) and

an α-SMA-specific monoclonal antibody (Sigma, 1:100 dilution). Deparaffinized lung sections were blocked with a solution of 3% bovine serum albumin in PBS for 1 hour; the primary antibody was then applied overnight at 4°C in 3% bovine serum albumin/PBS. After washing in PBS, the antibody was detected with a biotin-conjugated secondary antibody and avidin-linked chromogen system. Chromogens were either diaminobenzidine (DAB, brown) or nitroblue tetrazolium (dark grey or black). For double labeling for ANG peptides and ISEL, ISEL was performed first as described above and the following day, ANG peptide primary antibody was applied for 2 hours follwed by detection with DAB chromogen. For double labeling with ANG peptide antibody and MNF-116 antibody, both primary antibodies were applied together overnight, and differential detection was achieved with anti-goat-HRP secondary antibody (ANG peptide) or anti-mouse-AP (MNF-116) secondary antibody. Negative controls were obtained by completing the same procedure described above, but with omission of the primary antibody from the 3% BSA/PBS solution.

In situ hybridization was performed essentially as described previously (Wang *et al.* 1999b). Deparaffinized slides were hybridized with digoxigenin-labeled antisense oligonucleotide DNA probes specific for AGT, which were detected with an amplified biotin/avidin system linked to nitro blue tetrazolium chromogen (purple). The digoxigenin-labeled probes used were: 5'-AGGGTGGGGGAGGTGCTGAACAGC-3', as described by Lai *et al.* (Lai *et al.* 1998). A digoxigenin-labeled probe of the same base composition, but with scrambled sequence, was used as the control.

Microscopy and Image Acquisition

The prepared lung sections were photographed under transmitted or epifluorescent light on an Olympus BH2 epifluorescence microscope fitted with a SPOT Slider digital camera. Images of green fluorescence (alpha-smooth muscle actin-FITC) were acquired through a 520nm bandpass filter, and images of red fluorescence (propidium iodide) were acquired through a >570 long pass filter. .

RNA Isolation and RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described earlier (Wang *et al.* 1999b, 2000b), and realtime RT-PCR was performed in the Physiology Department, Michigan State University. The annealing temperatures for PCR reactions were optimized for each primer by preliminary trials. The identity of the PCR products was determined by expected size in 1.6% agarose gels and by DNA sequencing of the PCR product excised from agarose gels (not shown). Total RNA was extracted from biopsies using TRI Reagent® (Molecular Research center, Inc., Cincinnati, OH) according to the manufacturer's protocol. First strand cDNA was synthesized from 1 μg total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo $(dT)_{12\text{-}18}$. Real-Time RT-PCR was performed using cDNA synthesized from 50 ng total RNA, SYBR® Green PCR Core Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and 0.2 μM specific primers for: human angiotensinogen (AGT), forward = 5`-GAG CAA TGA CCG CAT CAG-3` and reverse = 5`-CAC AGC AAA CAG GAA TGG-3`, and β-actin, forward = 5`-AGG CCA ACC GCG AGA AGA TGA CC-3` and reverse = 5`-GAA GTC CAG GGC GAC GTA GC-3`,

which produce PCR products of 151 and 332 bp respectively. The PCR thermal profile started with 10 minutes activation of the *Taq* polymerase at 95°C followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 37 seconds and extension at 72°C for 37 seconds ending with dissociation curve analysis to validate the specificity of the PCR products. Reactions were performed in Mx3000P machine (Stratagene, LaJolla, CA) and CT data was collected using MxPro-Mx3000P software version 3.0. The relative AGT expression was normalized to β-actin and calculated using the comparative CT method of $2^{-\Delta\Delta CT}$.

Western Blotting

Protein was extracted from biopsy samples by tissue homogenization in ice-cold Tris Buffered Saline pH 8.0, supplemented with protease inhibitor cocktail (Roche, Mannhiem Germany) and tributylphosphine. Soluble protein extracts (10 μg) were diluted 1:2 in Laemmli Sample Buffer (BioRad, Hercules, CA) and loaded on 10% Tris-HCl polyacrylamide gel and separated by SDS-PAGE then transferred to Immun-Blot® PVDF membrane (BioRad, Hercules, CA) in Towbin buffer. Blotting membrane was blocked by 5% nonfat dry milk in TBST (0.1% Tween-20 in Tris Buffered Saline). Western blot analysis of angiotensinogen was performed using anti-ANG peptide antibody, 1:400 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bands were visualized by Horseradish peroxidase-conjugated donkey antigoat secondary antibody, 1:2000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the chemiluminescent substrate Super Signal® West Femto Maximum Sensitivity (Pierce, Rockford, IL). Images of the chemiluminescence-exposed film were analyzed for band

intensity using Scion Image software, release Beta 4.0.2, and normalized to total protein band intensities obtained by silver staining of SDS-polyacrylamide gels of replicate biopsy extracts. Silver staining was performed with a commercially available kit (Silver Stain Plus, BioRad, Hercules, CA) according to the manufacturer's instructions.

Cell Culture

The human lung cell line A549 was cultured as described previously (Wang *et al.* 1999a, 1999b, 1999c). The primary human lung fibroblast strain N13, isolated from normal human lung, was recovered from cryostorage and cultured as described earlier (Wang *et al.* 1999a). Prior to analysis, cells were switched from growth medium containing fetal bovine serum to serum-free medium (Ham's F12) for at least two days before harvesting. Immunoreactive AGT was detected by western blotting of cells lysed in buffer containing NP40 detergent and commercially available protease inhibitor cocktail.

RESULTS

Earlier cell culture studies from this laboratory showed that apoptotic alveolar epithelial cells or myofibroblasts isolated from IPF lung tissue synthesize ANG peptides *de novo*, i.e. from the precursor AGT (Wang *et al.* 1999c, 2000b). To begin determining if these same cell types synthesize ANG peptides in the fibrotic human lung *in situ*, paraffin sections of histologically normal human lung tissue and lung tissue from a patient with IPF were subjected to immunohistochemistry (IHC) with antibodies that recognize ANG peptides; the specificity of the antibodies is discussed further in Figure A.3. As shown in Figure A.1, normal human lung (Panels A-C) showed immunoreactivity (dark brown color) in smooth muscle underlying airways (arrowheads) and occasional alveolar wall cells (arrow), but did not label in the absence of the primary antibody (Panel C). Smooth muscle underlying large vessels of normal lung also was immunoreactive for ANG peptides (Panel B, brown). In the human IPF lung (Panel D), intense immunoreactivity was observed in numerous areas resembling fibroblastic foci throughout the parenchyma (arrows) and in cells resembling cuboidal epithelia within the surfaces of airspaces (arrowheads).

To obtain a quantitative assessment of ANG peptide expression in normal versus fibrotic human lung, realtime RT-PCR for AGT and β-actin mRNAs was performed on total RNA isolated from lung biopsies from five IPF patients and four patients without fibrotic lung disease. In Figure A.2, AGT mRNA was found to be 21-fold more abundant in IPF lung tissue relative to the control specimens (CTL) of human lung ($p<0.05$). Figure A.3 shows quantitation of immunoreactive AGT in total protein extracts from a panel of lung biopsy specimens and purified human lung cells. By western blotting with

the same antibodies used for IHC, two bands of differing molecular mass, which represent the two isoforms of human AGT, were detected in a commercially available purified AGT standard isolated from human serum (HS, Lane 1) and in IPF lung (Lanes 8-12). In human serum, the higher (~61kDa) and lower MW isoforms (~58kDa) of AGT were detected in apparently similar abundance. Cultured human lung epithelial cells (A549, Lane 2) and primary human lung fibroblasts (N13, Lane 3) expressed exclusively the 61kDa isoform of AGT. Lung biopsies from both normal lung (Lanes 4-7) and IPF lung (Lanes 8-12) contained primarily the higher MW isoform of AGT expressed by the isolated lung cells, although IPF biopsies did contain some of the low MW isoform. Densitometric quantitation of the higher MW isoform of AGT (~61kDa) only, relative to total protein (bar graph, Figure A.3) revealed 3.6-fold higher levels of the 61kDa isoform of AGT in IPF lung biopsies compared to nonfibrotic lung ($p<0.05$).

Due to the severely altered structure of IPF lung, the determination of cell type in tissue sections is difficult if based on morphology alone. To begin identifying the cell types labeled by the ANG peptide antibodies in Figure A.1, lung tissue from a patient with IPF was subjected to IHC with an antibody that recognizes type II pneumocytes (mAB MNF-116, Fehrenbach *et al.* 2000), applied alone or together with the ANG peptide antibody. In Figure A.4, immunoreactivity for mAB MNF-116 (Panel A, black) was observed in cuboidal epithelial cells that were usually located in airspace corners, or occasionally in attenuated cells in the airspace surfaces (arrowhead). In Panel B, double labeling with both mAB MNF-116 and ANG peptide mAB revealed ANG peptide immunoreactivity (brown) within MNF-116-positive cells (black). Panel C shows ANG peptide reactivity detected with an NBT-based chromogen system (dark grey) in cuboidal

epithelial cells that appeared to contain lamellar bodies (arrows). In some regions, cells with the morphology of alveolar macrophages showed positive labeling with anti-ANG peptide antibody but negative reactivity with MNF-116 (not shown).

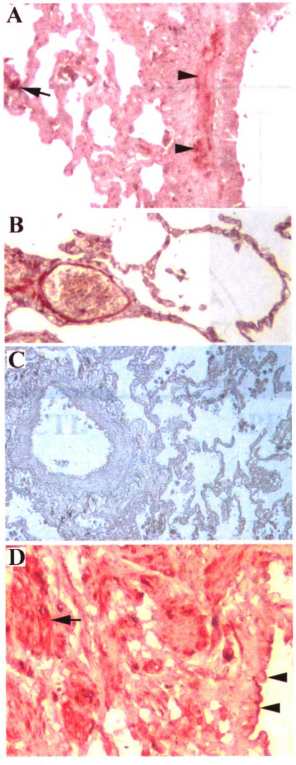
To determine if ANG peptide expression by epithelial cells in IPF lung was associated with apoptosis *in situ* as it is in cell culture (Wang *et al.* 1999b, 2000b), sections of normal human lung and sections from a patient with IPF were subjected to IHC with ANG peptide mAB, together with simultaneous double-labeling by In Situ End Labeling of fragmented DNA (ISEL) or propidum iodide plus DNAse-free ribonuclease (PI/RNAse). In Figure A.5A, double-labeled normal human lung revealed occasional ANG peptide-positive cells within alveolar walls (brown, arrowheads) or alveolar spaces (arrow), but very few ISEL-positive cells (blue). In contrast, double-labeling of IPF lung (Panel B) revealed numerous foci of cuboidal epithelia that were both ISEL positive (blue) and ANG peptide-positive (brown). Note the ISEL-positive epithelial nuclei that also displayed chromatin condensation (arrowhead) or margination against the nuclear envelop (arrow), morphological hallmarks of apoptosis. Panels C and D show two views of the same microenvironment of IPF lung that was double-labeled with ANG peptide antibodies (C) and PI/RNAse (D); paired black and white arrows highlight the condensed and marginated chromatin morphology of ANG peptide-positive cuboidal epithelial cells. For contrast, the normal nuclei of underlying stromal cells that are ANG peptide-negative are denoted by arrowheads (Panel D). Within the alveolar spaces, some of the cells labeled by ANG peptide antibody also had the morphology of alveolar macrophages (Panel C), and did not colabel with the epithelial marker MNF-116 (not shown). Panel E shows no labeling of IPF lung upon omission of the primary antibody.

Expression of ANG peptides *de novo* has been demonstrated in cultured myofibroblasts isolated from the IPF lung (Wang *et al.* 1999a). To begin determining if myofibroblasts within the IPF lung *in situ* also express ANG peptides, lung tissue from a patient with IPF was subjected to IHC with antibodies that recognize the myofibroblast marker α-smooth muscle actin (α-SMA) and ANG peptide. In Figure A.6, moderate immunoreactivity with α-SMA and ANG peptide antibodies (green and brown, respectively) was found in smooth muscle underlying vessels (paired white and black arrowheads, Panels A and B), but intense ANG peptide immunoreactivity was found in myofibroblast foci (paired arrows, Panels A and B). In Panels C and D, higher magnification revealed precise colocalization of ANG peptide immunoreactivity (D) with small microfoci of myofibroblasts (C) denoted by paired arrows. In Panel D, the arrowhead denotes ANG peptide labeling within nearby epithelium.

In Situ Hybridization (ISH) of IPF lung specimens (Figure A.7) for AGT mRNA revealed positive labeling in cuboidal epithelial cells and in occasional unidentified stromal cells (Panel A). AGT mRNA also was detected in foci (Panel B) that colabeled with antibodies against the myofibroblast marker alpha-smooth muscle actin (Panel C) applied to adjacent serial sections. In contrast, no evidence for AGT mRNA was detected in the smooth muscle underlying airways (Panel D) or large vessels (Panel E), nor was label deposited by scrambled-sequence control oligonucleotides (Panel F). Little positive ISH signal was detected in normal lung (not shown).

Figure A.1.

ANG peptide immunoreactivity in normal and fibrotic human lung. Paraffin sections of histologically normal human lung tissue (Panels A and B) and lung tissue from a patient with IPF (Panels C and D) were subjected to immunohistochemistry (IHC) with an antibody that recognizes ANGI and AGT. A: Normal human lung, airway wall and nearby parenchyma. Immunoreactivity = dark brown color; arrowheads = smooth muscle underlying airways, arrows = occasional alveolar wall cells. B: Normal human lung, vessel wall and nearby parenchyma. C: Normal human lung, same IHC procedure but with the primary antibody replaced by BSA. D: Human IPF lung, small airspace and nearby parenchyma; arrow = possible myofibroblast focus, arrowheads = cuboidal epithelia. See subsequent figures for phenotype markers.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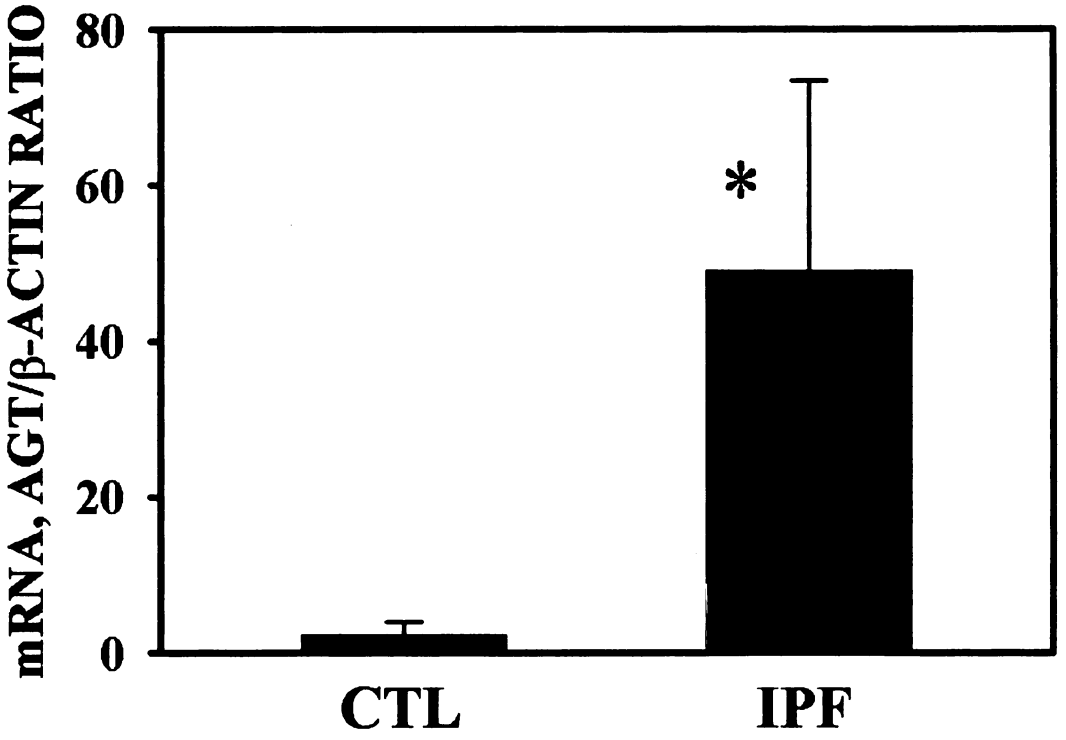
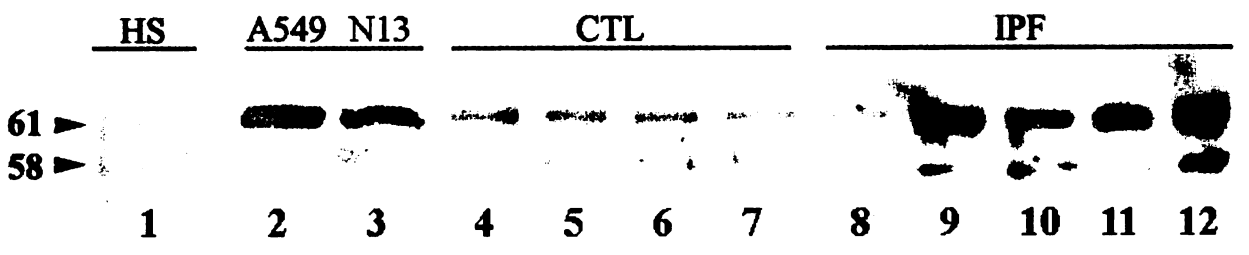


Figure A.2.

Quantitative RT-PCR of AGT mRNA in normal and fibrotic human lung. Fresh IPF or normal control (CTL) lung tissue was obtained by biopsy and immediately prepared for isolation of total RNA (see Methods). Realtime RT-PCR was performed for both AGT and β-ACTIN mRNAs as described in Methods. Bars are the mean + S.E.M. of data collected from the biopsies of four (CTL) and five (IPF) separate patients; * = $p<0.05$ by Mann-Whitney test.

Figure A.3.

Western blotting of AGT protein in normal and fibrotic human lung and isolated lung cells. Fresh IPF or normal control (CTL) lung tissue was obtained by biopsy and immediately flash-frozen for isolation of total protein (see Methods). Western blotting was performed as described in Methods with the same antibodies used for IHC in Figure A.1; equal amounts of total protein per lane were loaded for Lanes 4-12. Lane 1: Positive control of AGT protein purified from human serum (HS); note two isoforms of AGT at ~58 and 61kDa in similar abundance. Lanes 2-3: Positive AGT protein controls of A549 cells (Lane 2) or N13 primary human lung fibroblasts isolated from normal lung (Lane 3, see Wang *et al.* 1999a). Note expression of high MW isoform (upper band, ~61kDa) of AGT by isolated lung cells. Lanes 4-12: Immunoreactive AGT in lung biopsies from normal (CTL, Lanes 4-7) or IPF (Lanes 8-12) patients. Bar Graph: Densitometry of 61kDa AGT/total protein ratio, determined as described in Methods. Bars are the mean + S.E.M. of data collected from biopsies of four (CTL) and five (IPF) separate patients; * = $p<0.05$ by Student's t-test.


HS
A549
N13
CTL
IPF
61
58
1
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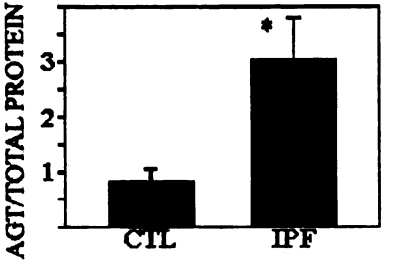

AGT/TOTAL PROTEIN
3
2
1
*
CTL
IPF

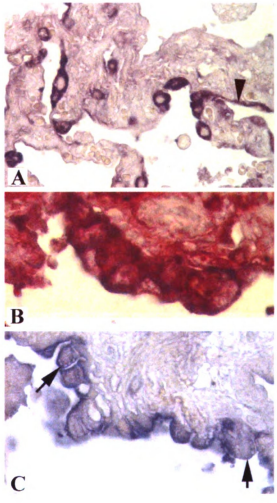
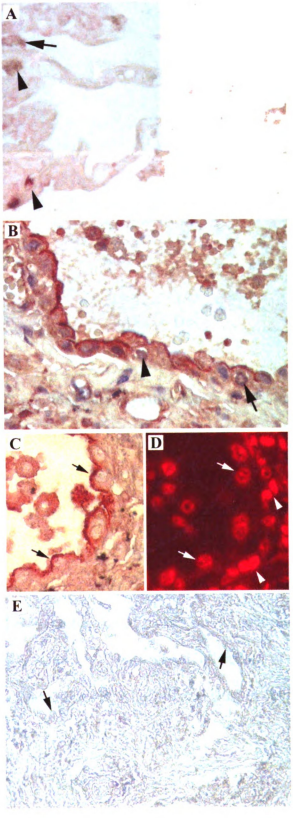


Figure A.4.

Colocalization of ANG peptides with alveolar epithelial cell markers in IPF lung. Paraffin sections of lung tissue from a patient with IPF were subjected to IHC with an antibody that recognizes type II pneumocytes (mAB MNF-116, Panels A and B) or with the ANG peptide antibody (Panels B and C). A: Immunoreactivity for mAB MNF-116 (black) in cuboidal epithelial cells and occasionally in attenuated cells in airspace surfaces (arrowhead). B: Double labeling with mAB MNF-116 and ANG peptide antibody showed ANG peptide immunoreactivity (brown) in MNF-116-positive cells (black). C: ANG peptide immunoreactivity in cuboidal epithelial cells containing perinuclear inclusion bodies (arrows).

Figure A.5.

Colocalization of ANG peptides with markers of apoptosis in epithelial cells within IPF lung. Lung sections from normal human lung (A) or from a patient with IPF (B-E) were subjected to IHC with ANG peptide antibodies with simultaneous double labeling by In Situ End Labeling of fragmented DNA (ISEL, A and B) or propidum iodide plus DNAse-free ribonuclease (PI/RNAse) to highlight chromatin morphology (C and D). A: Double-labeled normal human lung with occasional ANG/AGT-positive cells but very few ISEL-positive cells (blue). B: IPF lung showing foci of cuboidal epithelia that were both ISEL positive (blue) and ANG peptide-positive; note chromatin condensation (arrowhead) or margination (arrow) in ISEL-positive nuclei. C and D: Double labeling of IPF lung with ANG peptide antibody (C) and PI/DNAse (D); note chromatin morphology of ANG peptide-positive epithelia (arrows, C and D) compared to normal nuclei in ANG peptide-negative stromal cells (arrowheads, D). E: Negative labeling of IPF lung after IHC with the primary antibodies for ANG peptide replaced by BSA. Note lack of label in cuboidal epithelia (arrows).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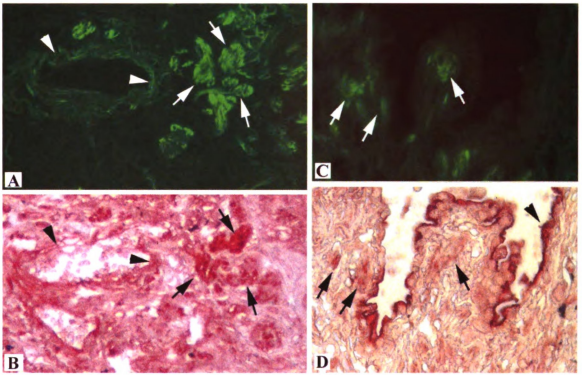
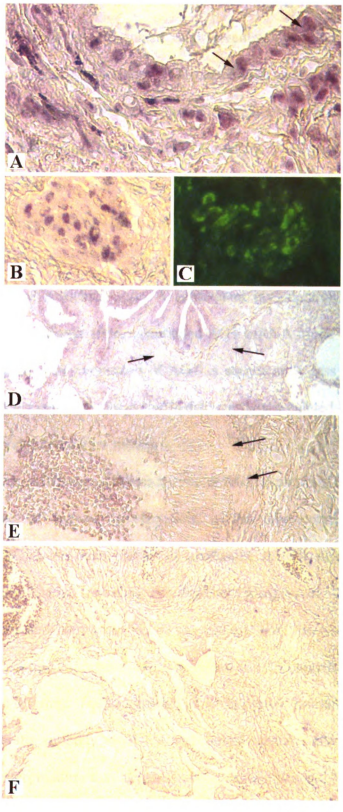


Figure A.6.

Colocalization of ANG peptides with myofibroblasts in IPF lung. Lung tissue from a patient with IPF was subjected to IHC with an antibody that recognizes the myofibroblast marker α-smooth muscle actin (α-SMA mAB = green, Panels A and C) or with ANG peptide antibody (brown, B and D). A and B: Adjacent serial sections of IPF lung reveal mild ANG peptide mAB immunoreactivity in vessel smooth muscle (arrowheads) but intense ANG peptide immunoreactivity in myofibroblast foci (paired white and black arrows). C and D: Higher magnification of reveals precise colocalization of ANG peptide immunoreactivity (black arrows, D) with small microfoci of myofibroblasts (white arrows, C). In D, arrowhead denotes ANG peptide labeling in epithelial cells.

Figure A.7.

In Situ Hybridization for AGT mRNA in IPF lung. Lung tissue from a patient with IPF was subjected to ISH with antisense oligonucleotides that bind AGT mRNA (Panels A-E), or with scrambled-sequence control oligonucleotides (Panel F, see Methods). A: In IPF lung, positive signal for AGT mRNA (purple) was observed in cuboidal epithelial cells lining many of the same airspaces that also labeled with ISEL (see Figure A.5). Labeling by ISH appeared to localize to both epithelial nuclei and cytosol (arrows, Panel A), as well as in some unidentified stromal cells. B: Positive signal for AGT mRNA (purple) in a putative focus of myofibroblasts; C: Alpha-smooth muscle actin (α-SMA) immunolabeling of the same focus shown in Panel B, but performed on an adjacent serial section. Note colocalization of ISH signal with α-SMA-positive cells. D: Negative signal for AGT mRNA in smooth muscle (arrows) underlying an airway. E: Negative signal for AGT mRNA in smooth muscle (arrows) underlying a large vessel. F: Negative signal in IPF lung prepared for ISH with scrambled-sequence control oligonucleotides. Magnification = 400X (Panel A), 200X (Panels B and C), 100X (Panels D and E) and 50X (Panel F); see Results and Methods sections for details.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DISCUSSION

Earlier *in vitro* studies from this laboratory had shown that two cell types found in the fibrotic human lung, myofibroblasts and alveolar epithelial cells undergoing apoptosis, where capable of synthesizing ANG peptides *de novo*, at least in cell culture (Wang *et al.* 1999a, 1999b). The studies herein provide evidence from intact fibrotic human lung that these same cell types also synthesize ANG peptides *in situ*. The observations that airway and vascular smooth muscle are immunoreactive for ANG peptide antibodies (Figure A.1 Panels A and B) agree with earlier findings by Ohkubo *et al.* (Ohkubo *et al.* 1986) that ISH of rat lung detected AGT mRNA in fibroblast-like cells adjacent to vessels and bronchial walls. A subsequent investigation by Campbell and Habener (Campbell & Habener 1986) quantitated AGT mRNA in the lungs, and showed that even though pulmonary expression of AGT is significantly less than that of liver, heart or kidney, it is present in the lungs and is differentially regulated by nephrectomy or hormone treatment relative to other tissues.

Determinations of the mechanisms by which AGT expression is regulated in lung cells are the subject of ongoing investigations in this laboratory. Cultured alveolar epithelial cells (AEC) have been shown to synthesize ANGII in response to many proapoptotic stimuli *in vitro*, including Fas ligation (Wang *et al.* 1999b), TNF-alpha (Wang *et al.* 2000b) and bleomycin (Li *et al.* 2003b). For this reason we sought to determine if ANG peptide expression might be found in AEC together with markers of apoptosis within the IPF lung. The localization of ANG peptide immunoreactivity in cells that bind anticytokeratin antibody MNF-116 and contain putative lamellar bodies (Figure A.2) supports the interpretation that at least some of the cells expressing ANG peptides

are type II pneumocytes. Moreover, the finding of positive labeling by ISH for AGT mRNA in both cuboidal epithelia and alpha-actin-positive foci (Figures 7A, B and C) supports the contention that the ANG peptides are being synthesized in AEC *de novo* in AECs and myofibroblasts rather than being sequestered, for example, from other sources such as the blood. On the other hand, negative labeling for AGT mRNA in the smooth muscle underlying airway and vessel walls (Figures 7D and E) suggests that the positive IHC labeling of these cells (Figure A.1) may reflect uptake of ANG peptides rather than *de novo* synthesis.

Some cells with the morphology of alveolar macrophages and negative immunoreactivity to MNF-116 also were found to label with the ANG peptide antibody and by ISH for AGT mRNA (not shown), but positive markers of macrophage phenotype were not available for positive phenotype analyses. Although far from definitive, these preliminary observations are consistent with the studies of normal human alveolar macrophages by Dezso *et al.* (Dezso *et al.* 1989) and suggest that alveolar macrophages may also be a source of ANG peptides in fibrotic human lung. The possibility of ANG peptide expression by alveolar macrophages will be an interesting topic for further study.

The ANG peptide antibody used for these studies, which was derived against the peptide ANGI, recognizes ANGI, ANGII and the two isoforms of AGT found in serum (Figure A.3). Given that the ~58 and ~61kDa isoforms of AGT are both found in human serum, whereas isolated lung cells express only the 61kDa isoform (Figure A.3), the finding of both isoforms in IPF lung biopsies suggests that some of the increase in lung tissue AGT in the IPF specimens may be due to serum-derived AGT. On the other hand, the observation that the 61kDa isoform expressed by isolated lung cells was more highly

abundant that the 58kDa form in both normal and IPF lung supports the theory that most of the increase in lung tissue AGT in IPF occurs through *de novo* synthesis of AGT within the lung.

Due to the reactivity of the ANG peptide antibodies to AGT, ANGI and ANGII, it is not possible to determine from the IHC studies alone if the labeled regions contain primarily precursor AGT or the processed ANG peptides ANGI and ANGII. On the other hand, *in vitro* experiments with human lung myofibroblasts isolated from IPF biopsies (Wang *et al.* 1999a) or cultured human or rat AECs (Wang *et al.* 1999b, 2000b) show that both myofibroblasts and apoptotic lung epithelial cells can constitutively convert newly synthesized AGT to ANGII, apparently by autocrine mechanisms. Thus, it seems likely that at least some of the immunoreactivity observed within foci of apoptotic AECs and myofibroblasts consisted of the processed peptides ANGI and ANGII. Determinations of the abundance of these processed peptides in human lung and the kinetics of their appearance will be interesting topics for future investigations

Regardless, the detection of ANG peptide immunoreactivity in epithelial cells that simultaneously labeled positively for fragmented DNA by ISEL (Figure A.3B) and also exhibited chromatin condensation and margination against the nuclear envelop (Figures 3 C and D) strongly suggests ANG peptide expression by apoptotic AECs in IPF lung *in situ*. Thus, these findings are consistent with earlier studies of cultured AEC exposed to proapoptotic stimuli *in vitro* (Wang *et al.* 1999b, 2000b). Nonetheless, the stimuli which caused the apoptosis of AECs detected in the IPF lung biopsies studied here are unknown. The observation that occasional epithelial cells in the normal human lung were found to be ANG peptide-positive but ISEL negative (Figure A.3A) might be explained

by the kinetics of apoptosis; the DNA fragmentation detected by ISEL is a relatively late downstream event in apoptosis (Bursch *et al.* 1990), whereas AGT expression occurs within 2-3 hours (Li *et al.* 2007). Thus, depending on the timing of tissue fixation, not all ANG-positive cells would be expected to colabel by ISEL, and the images of fibrotic lung are consistent with that interpretation (Figure A.3B). Regardless, the findings herein also are consistent with an earlier investigation if IPF lung biopsies that revealed ISEL within epithelial cells adjacent to foci of myofibroblasts and heavy collagen deposition (Lai *et al.* 1998).

Regardless, the findings that foci of α-SMA-positive cells also label heavily with the ANG peptide antibodies (Figure A.4) and AGT mRNA (Figures 7B and C) are consistent with other investigations showing ANG peptide expression by myofibroblasts of the fibrosing heart (Weber & Sun 2000), kidney (Klahr & Morrissey 1997) and liver (Yoshiji *et al.* 2001). They are also consistent with our earlier study of myofibroblasts isolated from IPF lung biopsies, which synthesize and secrete ANG peptides in culture (Wang *et al.* 1999a). Given that primary cultures of AECs undergo apoptosis upon exposure to purified ANGII (Wang *et al.* 1999c), TNF-α (Wang *et al.* 2000b) or Fas ligand (Wang *et al.* 1999b), it is difficult to know which of these proapoptotic factors were responsible for the induction of DNA fragmentation in AECs within the IPF biopsies studied here (Figure A.3).

In animal models, both ACE inhibitors (Molteni *et al.* 1985, Wang *et al.* 2000a) and ANG receptor AT_1 antagonists (Li *et al.* 2003b, Molteni *et al.* 2000, Uhal *et al.* 2003) have been shown to prevent radiation and chemically-induced experimental lung fibrosis; further, knockout mice deficient in ANG receptor AT_{1a} are resistant to bleomycin-

induced lung fibrosis (Ohkubo *et al.* 1986). On that basis, it is speculated that the production of ANG peptides by apoptotic AEC and myofibroblasts is an important component of the molecular mechanisms that maintain a profibrotic environment within the IPF lung. Experiments are in progress to determine if blockade of ANG receptors in short term explant cultures of IPF lung tissue can reduce the expression of profibrotic genes.

In summary, immunolabeling and in situ hybridization studies of normal and fibrotic human lung have identified at least two extravascular sources, myofibroblasts and apoptotic AECs, that synthesize angiotensin peptides *de novo* in the IPF lung. These results confirm earlier investigations of cultured human AEC and myofibroblasts isolated from IPF tissue, and are consistent with reports of local angiotensin generating systems in other fibrosing organs. Given the known roles of angiotensin II in stimulating collagen deposition in the lungs (Li *et al.* 2003b, Marshall *et al.* 2004) and other organs (Klahr & Morrissey 1997, Weber & Sun 2000, Yoshiji *et al.* 2001), it is theorized that the ANG peptides produced by apoptotic AEC and myofibroblasts contribute to the fibrogenic response in IPF lung. To evaluate this theory in human lung tissue, experiments designed to test for reduction of α-I-collagen mRNA in IPF lung explants by angiotensin receptor antagonists are currently underway.

Appendix B:

AMIODARONE INDUCES ANGIOTENSINOGEN GENE EXPRESSION IN LUNG ALVEOLAR EPITHELIAL CELLS THROUGH AP-1

Bruce D. Uhal, Huiying Zhang, Amal Abdul-Hafez, Ruijie Shu and Xiaopeng Li

Data presented in this appendix is published in: Basic & Clinical Pharmacology & Toxicology. (2007) 100(1):59-66.

ABSTRACT

Previous work from this laboratory showed that amiodarone induces alveolar epithelial cell apoptosis that was abrogated by antagonists of angiotensin II (Bargout *et al.* 2000). In this study, amiodarone (AMIO) upregulated angiotensinogen (AGT) mRNA and protein in primary cultures of rat type II pneumocytes and in the human A549 cell line. The mechanism of AMIO-induced AGT expression was studied in A549 cells with a human AGT promoter-luciferase reporter (AGT-LUC). AMIO (3ug/ml) induced both AGT-LUC and endogenous AGT mRNA; the latter was completely blocked by actinomycin-D. AMIO did not affect the halflife of endogenous AGT mRNA. Deletion analyses of AGT-LUC identified at least two AMIO-responsive domains in the AGT promoter between –350 to –260bp and –203 to –46bp. DNA/Protein array and electrophoretic mobility shift assays showed that AMIO increases DNA binding of both AP-1 and STAT-5 transcription factors. Site-directed mutagenesis of three IL-6-responsive STAT binding sites within the AMIO-response domains had no effect on AMIO-induced AGT-LUC expression. In contrast, AMIO-induced AGT-LUC expression was abrogated by a dominant-negative fos construct and was stimulated over 5-fold by c-fos and c-jun expressed together but not separately. Mutagenesis of the AP-1 binding site at -15 to –12bp completely eliminated the response to AMIO. These data show that AP-1 family transcription factors mediate AMIO-induced AGT expression in human alveolar epithelial cells and identify an AP-1 site, located between the TATA box and the transcription initiation site, that is required for the response.

INTRODUCTION

The benzofuran derivative amiodarone is widely used and very effective for the treatment of intratractable cardiac arrhythmias (Wilson & Lippmann 1990, Piepoli *et al.* 1998). Although it is generally considered safe, amiodarone has several side effects that limit the maximal dose (Kennedy *et al.* 1987); the most severe of these is pulmonary toxicity that affects approximately 6% of patients receiving the drug, with a mortality rate of 5-10% of affected patients. In longterm therapy, the pulmonary toxicity in humans includes a fibrotic response that is reproducible in animal models subjected to longterm (6-12 months) oral administration of amiodarone (Carvalho *et al.* 1996; Uhal *et al.* 2003).

Although the mechanisms by which amiodarone induces lung toxicity are not entirely clear, several lines of evidence suggest that direct cytotoxicity of amiodarone or its primary toxic metabolite, desethylamiodarone, to various lung cell types could contribute to the lung toxicity. In cell culture studies, amiodarone has been shown directly cytotoxic, at physiologically relevant concentrations, to alveolar macrophages (Ogle & Reasor 1990), lung fibroblasts (Martin & Howard 1985), human pulmonary artery endothelial cells (Powis *et al.* 1990) and lung alveolar epithelial cells (Bargout *et al.* 2000). In the latter case, amiodarone was shown to induce apoptosis of alveolar type II pneumocytes in a manner that was inhibitable by antagonists of angiotensin II (ANGII). The inhibition occurred at the level of either ANGII production, as demonstrated by angiotensin converting enzyme inhibitors (ACEi's), or at the level of binding of ANGII to the receptor AT_1 as demonstrated by the AT_1-selective antagonist losartan (ibid). Those data are consistent with the more recent demonstration that systemic administration of either an ACEi or losartan could prevent amiodarone-induced lung

fibrosis in rats (Uhal *et al.* 2003).

The in vitro data just discussed also are consistent with previous work from this laboratory that showed upregulation of angiotensinogen (AGT) expression in alveolar epithelial cells by other apoptosis inducers, including Fas ligand (Wang *et al.* 1999b), tumor necrosis factor alpha (Wang *et al.* 2000a) and the antineoplastic agent bleomycin (Li *et al.* 2003b). Accordingly, this study addressed the possibility that amiodarone might also induce AGT expression in alveolar epithelial cells, and begins to examine the mechanisms controlling AGT gene expression in these cells. We report here that AMIO upregulates AGT gene expression in human alveolar epithelial cells through a mechanism mediated by AP-1 family transcription factors and which requires an AP-1 binding site close to the transcription start site.

MATERIALS AND METHODS

Reagents and Materials

Amiodarone (AMIO) and desethylamiodarone were kindly provided by Wyeth/Ayerst Research, Princeton, NJ. A fos dominant negative construct (a-fos) was a kind gift from the laboratory of Dr. Charles Vinson (Olive *et al.* 1997). Plasmids for the overexpression of c-jun and c-fos were obtained from Dr. Richard Pope, Northwestern University School of Medicine. All other materials were of reagent grade and were obtained from Sigma Chemical Co., Saint Louis, MO.

Cell Culture

The human lung adenocarcinoma cell line A549 was obtained from the American Type Cell Culture Collection and cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). Primary alveolar epithelial cells isolated from adult male Wistar rats as described earlier (Wang *et al.* 1999c). The primary cells were studied at day two of culture, a time at which they are type II cell-like by accepted morphologic and biochemical criteria (Paine & Simon 1996). Primary cell preparations were of better than 90% purity assessed by acridine orange staining as described previously (Wang *et al.* 1996). All cells were grown in 24- or 6-well chambers and were analyzed at subconfluent densities of 80-90%. All subsequent incubations with AMIO and/or other test agents were performed in serum-free medium.

RT-PCR

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

For RTPCR of rat-specific gene products, the following primers were used: for angiotensinogen, coding = 5'-CCT CGC TCT CTG GAC TTA TC-3', and uncoding = 5'-CAG ACA CTG AGG TGC TGT TG-3', which yields a PCR product of 205bp by single-step RTPCR. For β-microglobulin, the primers used were: coding = 5'-CTCCCCAAA-TTCAAGTGTACTCTCG-3', and uncoding = 5-GAGTGACGTGTTTAACTCTGCA-AGC-3', which yields a product of 249bp. For RT-PCR from human A549 cells, the following primers were used: for angiotensinogen, coding = 5'-GAG CAA TGA CCG CAT CAG-3', and uncoding = 5'-CAC AGC AAA CAG GAA TGG-3'. These primers yield a final PCR product of 151bp. For β-actin, single-step RTPCR was used with the primers: coding = 5'-AGGCCAACCGCGAGAAGATGACC-3', and uncoding = 5'-GAAGTCCAGGGCGACGT-AGC-3', which produces a PCR product of 332bp.

Halflife Determinations of AGT mRNA

A549 cells were serum-starved for 24h and were then treated with or without amiodarone (3ug/ml) in serum-free media. Twenty hours later, actinomycin-D was added at 2ug/ml. Cells were then harvested for total RNA extraction at 0, 3, 6, 9 hours after actinomycin-D addition. Triplicate treatments for each time point were used in real-time RT-PCR measures of AGT relative to β-actin.

Reporter Gene Assays and Transfections

Expression of human angiotensinogen was studied in A549 cells using a luciferase reporter (firefly) driven by the human angiotensinogen promoter, specifically –991 to +22bp from the transcription initiation site. The plasmid was obtained from Dr. Allan R. Brasier of the University of Texas at Galveston (Sherman & Brasier 2001). The reporter construct (AGT-LUC) was cotransfected with a control luciferase construct (Renilla) driven by the CMV promoter (PRL-CMV) with the transfection reagent FuGene6 (Roche Diagnostics, Indianapolis, IN) for 20 hours, as described by the manufacturer. AMIO was applied to the cells thereafter for an additional 20 hours before assay of luciferase. Firefly and renilla luciferase activities were measured with the Dual Luciferase Reporter Assay System (Promega Corp., Madison WI) as described by the manufacturer. Transfections of other plasmids were conducted with FuGene6 reagent as described for AGT-LUC.

Site directed mutagenesis

Six deletion constructs of the AGT-LUC reporter were generated as described by

Sherman and Brasier (Sherman & Brasier 2001); all six constructs and the full-length plasmid were sequenced in the MSU Genomics Technology Support Facility to confirm proper orientation and fragment length. For mutation of STAT binding sites, small block deletions (8bp) were generated to eliminate the entire consensus sequence (see Figure B.6A) without creating new protein binding sites. For mutation of AP-1 binding sites, 2bp substitutions were generated in the middle of each site (GTCA to GTAC). In all cases, the generated mutations did not create new binding sites detectable by TFSearch or TRANSFAC software (not shown). Site directed mutagenesis was performed on the full length AGT-LUC reporter with the QuikChange Kit (Stratagene, La Jolla, CA) as directed by the manufacturer, and products were sequenced in the MSU Sequencing Facility to verify mutations.

RESULTS

When applied to primary cultures of type II pneumocytes isolated from rats, amiodarone upregulated angiotensinogen (AGT) mRNA and protein (Figure B.1) at a drug concentration close to that reached in the serum of patients receiving amiodarone in longterm therapy (Canada *et al.* 1983). Amiodarone (AMIO) also upregulated AGT mRNA in A549 cells; the increase was completely blocked by actinomycin D (Figure B.2). Moreover, sequential real-time RT-PCR measurements of AGT mRNA after the addition of actinomycin D (Figure B.3) revealed no affect of AMIO on the halflife of the mRNA.

To begin determining the mechanisms by which amiodarone induces AGT gene expression, the effect of amiodarone (AMIO) on A549 cells was studied with a reporter construct containing the firefly luciferase gene immediately downstream of a portion of the human AGT gene, specifically 1013 bp (-991 to +22) of the AGT promoter (Sherman & Brasier 2001). In Figure B.4, AMIO induced expression of the AGT-LUC reporter in a concentration-dependent manner, with maximal induction at 3 ug/ml. The primary metabolite of AMIO, desethylamiodarone, also induced AGT-LUC expression with the same potency as the parent compound (Figure B.5).

To facilitate the identification of AMIO-responsive domains within the human AGT promoter, deletion mutants of the AGT-LUC promoter-reporter were constructed and were tested for the ability to eliminate responsiveness to AMIO. In Figure B.6, truncations that removed bases down to –350 had no effect on AMIO-induced AGT-LUC activity relative to the full-length reporter. On the other hand, further deletion of the promoter region between –350 and –260 reduced AMIO-induced AGT expression by

51%, and additional deletion of the region between –203 and –46 further reduced expression to a value not significantly different from the control (no AMIO).

These two domains contain STAT binding sites known to be important in the liver; Figure B.7A shows the specific locations of STAT sites, within or near these two promoter regions, which are known to mediate the response of human liver AGT to Interleukin-6 (Sherman & Brasier 2001). To test the theory that these same STAT binding sites might be involved in the response of A549 cells to AMIO, block deletion mutants (8bp) that eliminated each of these STAT sites were generated by site-directed mutagenesis of the full length AGT-LUC (-991 to +22). Figure B.7B shows that deletion of each STAT binding site, alone or in combination, had no effect on the response to AMIO.

DNA/protein array screening of transcription factor binding activity in response to AMIO (Figure B.8A) revealed increased binding of AP-1 and STAT-5 transcription factors, but no changes in STAT-1 or STAT-3 binding. The AMIO-induced increase in AP-1 binding was confirmed by EMSA (Figure B.8B). In light of the failure of STAT site deletion (see Figure B.7) to diminish the response to AMIO, the well-described dominant negative construct a-fos (Olive *et al.* 1997) was evaluated for its effect on AMIO-induced AGT expression. The a-fos construct, which eliminates binding of AP-1 by interfering with the DNA binding domain of jun proteins, reduced AMIO-induced AGT expression to a value not significantly different from the control (Figure B.9). In Figure B.10, overexpression of AP-1 through cotransfection of both c-fos and c-jun induced AGT-LUC expression by over 5-fold, whereas expression of c-fos or c-jun individually made no significant stimulation of AGT-LUC.

The human AGT promoter contains eight AP-1 binding sites; Figure B.11 shows the locations of the three AP-1 sites within the AMIO-responsive domains identified by the deletion constructs described in Figure B.6. In Figure B.12, mutants of the full length AGT-LUC were generated with 2bp substitutions within each of the three AP-1 sites (see Methods) and were tested for the ability to inhibit AMIO-induced AGT-LUC expression. Mutation of only one of the AP-1 sites, located at –15, eliminated AMIO-induced AGT-LUC expression.

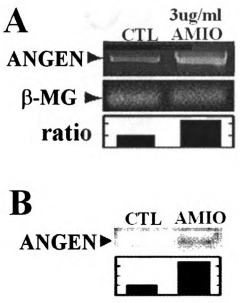

Figure B.1.

Amiodarone upregulates angiotensinogen gene expression in isolated type II pneumocytes. Primary cultures of rat type II pneumocytes were exposed to amiodarone (AMIO) in serum-free medium for 20 hours, beginning on day 2 after isolation . A: Conventional RTPCR for angiotensinogen (AGT) mRNA, relative to β-microglobulin (β-MG) as loading control. The bargraph plots the AGT/β-MG ratio obtained by densitometry. B: Western blot of lyophilized cell culture medium collected from the cells harvested for RTPCR in panel A. Bargraph = densitometry of the AGT band; loading was normalized to cell lysate protein as described in Methods.

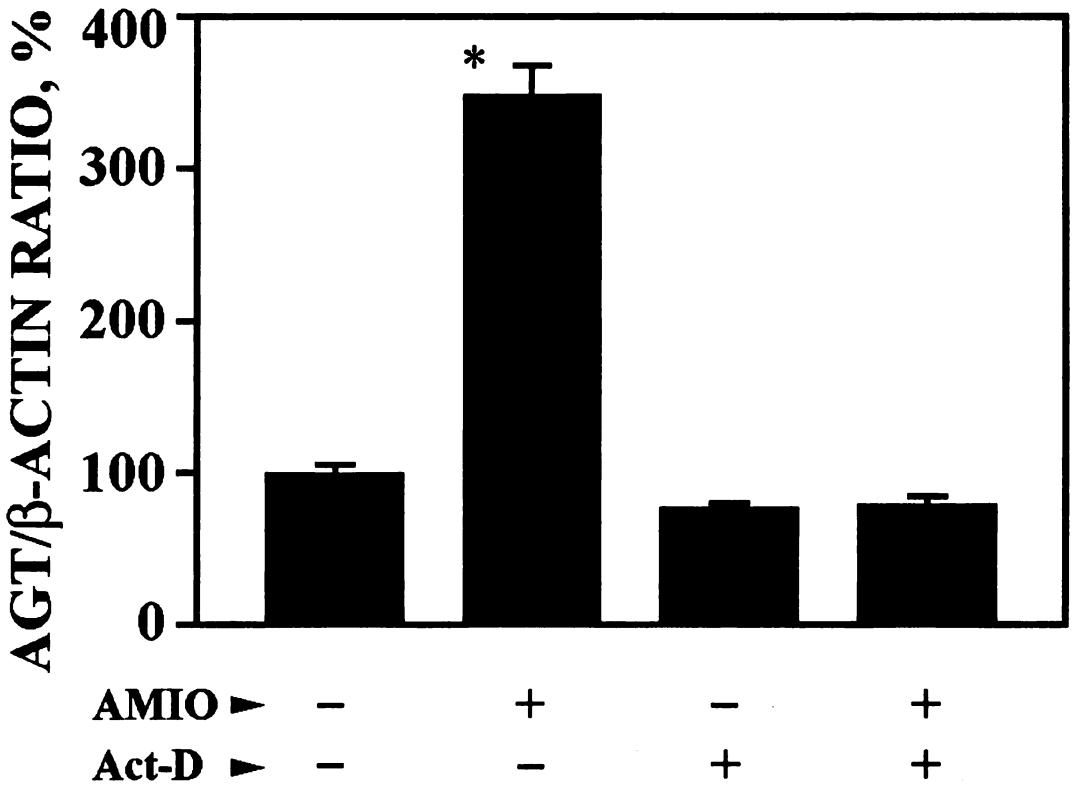

Figure B.2.

Amiodarone upregulates angiotensinogen gene expression in the A549 adenocarcinoma cell line. A549 cells were exposed to amiodarone (AMIO, 3ug/ml) in serum-free medium for 20 hours, in the presence and absence of actinomycin–D (ACT-D, 2ug/ml). Data represent realtime RTPCR measurements of AGT mRNA, relative to β-actin. Bars are the means + S.E.M. of N=6; * = $p<0.01$ by ANOVA and Student-Newman-Keul's test.

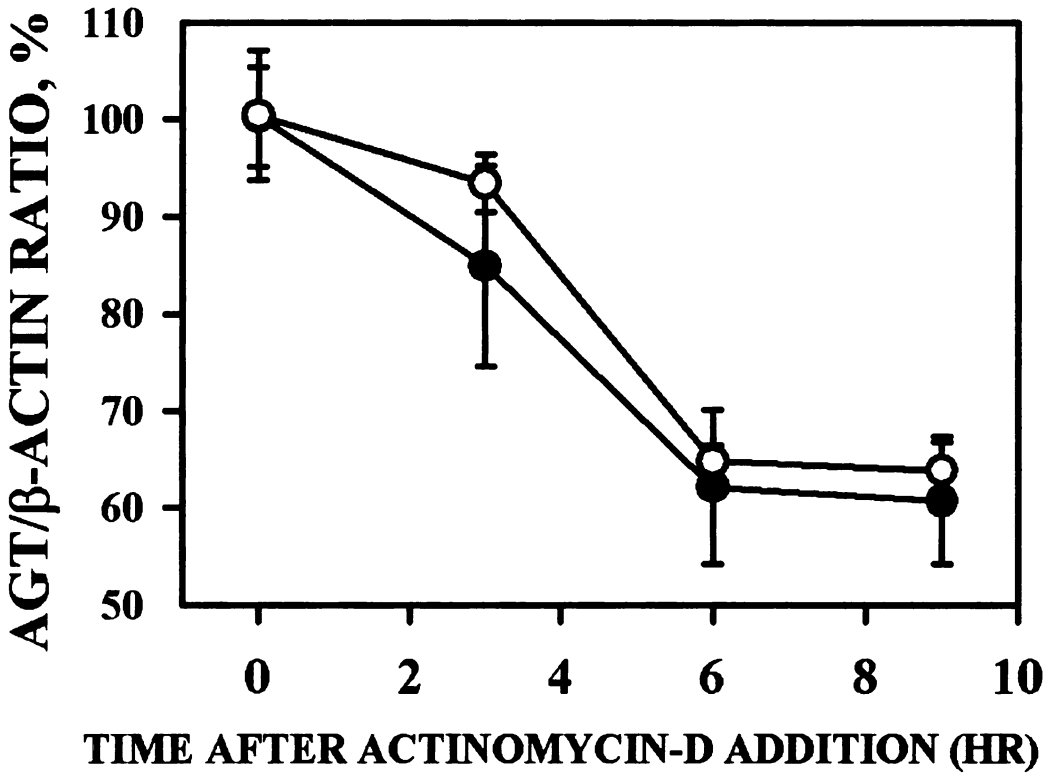

Figure B.3.

Amiodarone does not affect the decay rate of AGT mRNA. A549 cells were treated with (white circles) or without (black circles) AMIO (3ug/ml) in serum-free media. Twenty hours later, actinomycin-D was added at 2ug/ml. Cells were harvested for total RNA extraction at 0, 3, 6, 9 hours after actinomycin-D addition. Data represent real-time RT-PCR measurements of AGT mRNA, relative to β-actin. Bars are the means + S.E.M. of N=6.

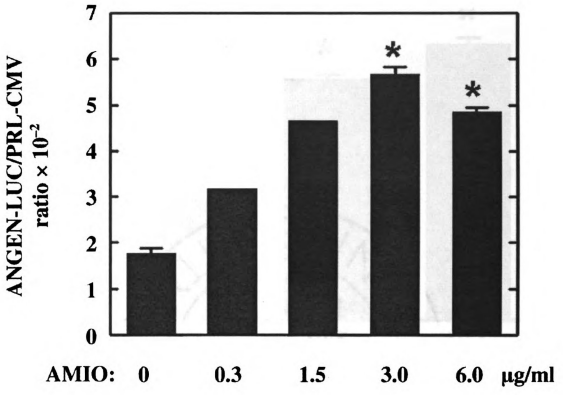

Figure B.4.

Amiodarone upregulates AGT-LUC reporter expression in the A549 adenocarcinoma cell line. A549 cells were transfected with AGT-LUC and control PRL-CMV vectors, and were then exposed to amiodarone at the indicated concentrations for 20 hours in serum-free medium. The transfections and calculation of LUC/PRL-CMV ratio are described in the Methods section. Bars are the mean + S.E.M. of 3 observations; * = $p<0.05$ versus control (0.0 dose) by ANOVA and Student-Newman-Keul's test.

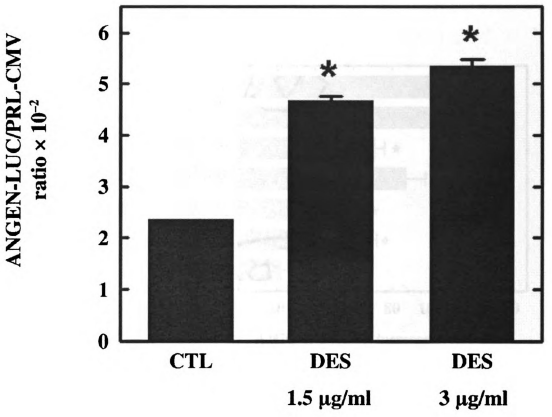

Figure B.5.

Desethylamiodarone upregulates AGT-LUC reporter expression in A549 cells. A549 cells were transfected with AGT-LUC and control PRL-CMV vectors, and were then exposed to desethylamiodarone (DES) at the indicated concentrations for 20 hours in serum-free medium. Bars are the mean + S.E.M. of 3; * = $p<0.05$ versus control (CTL) by ANOVA and Student-Newman-Keul's test.

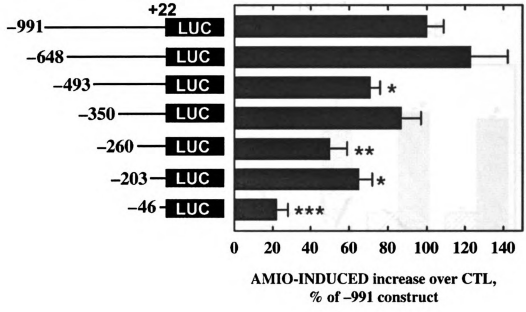

Figure B.6.

Identification of amiodarone-responsive domains in the human angiotensinogen promoter. The indicated deletion constructs of the AGT-LUC promoter-reporter were assembled as described in the Methods section. A549 cells were transfected with the deletion mutants and PRL/CMV, and were exposed to AMIO (3ug/ml) thereafter as in Figure B.2. Data are expressed as the LUC/PRL-CMV ration for each construct, as a percentage of the full length (-991 to +22) reporter. Bars are the mean + S.E.M. of 3 observations; * = $p<0.05$ vs. –648; ** = $p<0.05$ vs. –350 and *** = $p<0.05$ vs. –203 by ANOVA and Student-Newman-Keul's test.

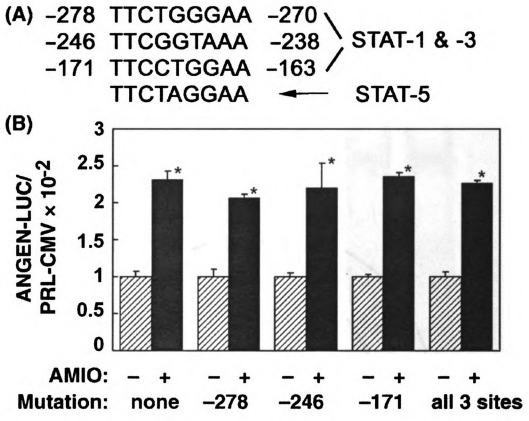

Figure B.7.

Mutation of STAT binding sites does not affect amiodarone-induced angiotensinogen expression in A549 cells. A549 cells were transfected with mutants of the full-length (-991 to +22) AGT-LUC in which the indicated STAT sites were deleted (see Methods) and were thereafter challenged with AMIO as in Figure B.6. A: Sequences and locations of STAT binding sites known to mediate interleukin-6-induced AGT expression in hepatocytes (Sherman & Brasier 2001). Sequence labeled STAT5 is that of the probe used to assess STAT5 binding by DNA/Protein array (see Figure B.8).

B: Effect of STAT site mutations on amiodarone-induced AGT-LUC reporter expression in A549 cells. Bars are the mean + S.E.M. of at least three observations;

* = $p<0.05$ vs. corresponding control (-AMIO) by Student's t-test.

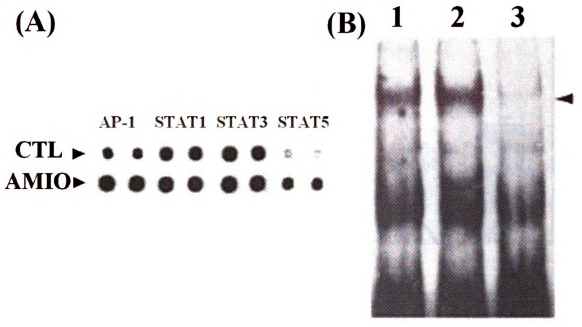

Figure B.8.

Amiodarone increases AP-1 binding to DNA. A549 cells were exposed to AMIO for 20hr, and nuclear extracts were prepared as described in the Methods section. A: Nuclear extracts were analyed by DNA/Protein array; each pair of dots corresponds to probes for the indicated transcription factors. B: Extracts were assessed by electrophoretic mobility shift assay (EMSA) with consensus probes for AP-1 binding sites. Lane 1 = control; Lane 2 = AMIO; Lane 3 = AMIO + excess of unlabeled AP-1-selective oligonucleotide. Arrowhead indicates mobility of specific AP-1/DNA complexes.

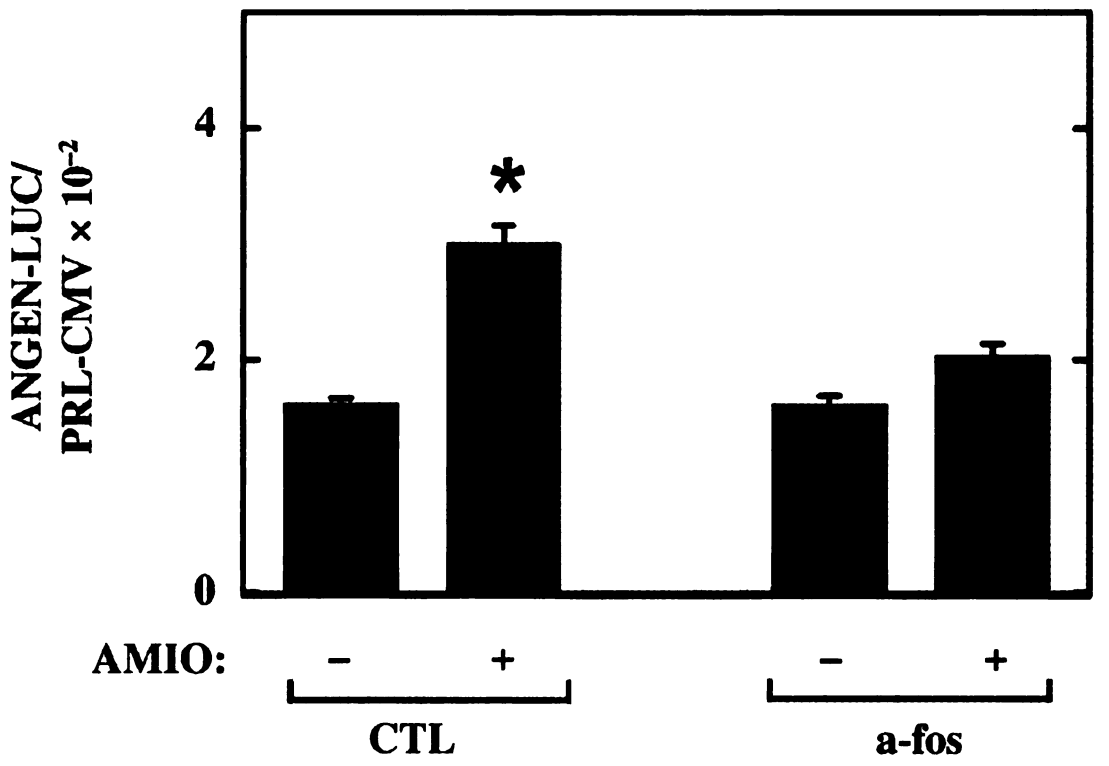

Figure B.9.

Abrogation of amiodarone-induced AGT expression by an AP-1 dominant negative construct. A549 cells were cotransfected with AGT-LUC and a dominant negative fos construct (a-fos) that prevents DNA binding by AP-1 (Olive *et al.* 1997). Cells were thereafter challenged with AMIO as in Figure B.5. Bars are the mean + S.E.M. of at least three observations; * = $p<0.05$ vs. corresponding control (-AMIO) by Student's t-test.

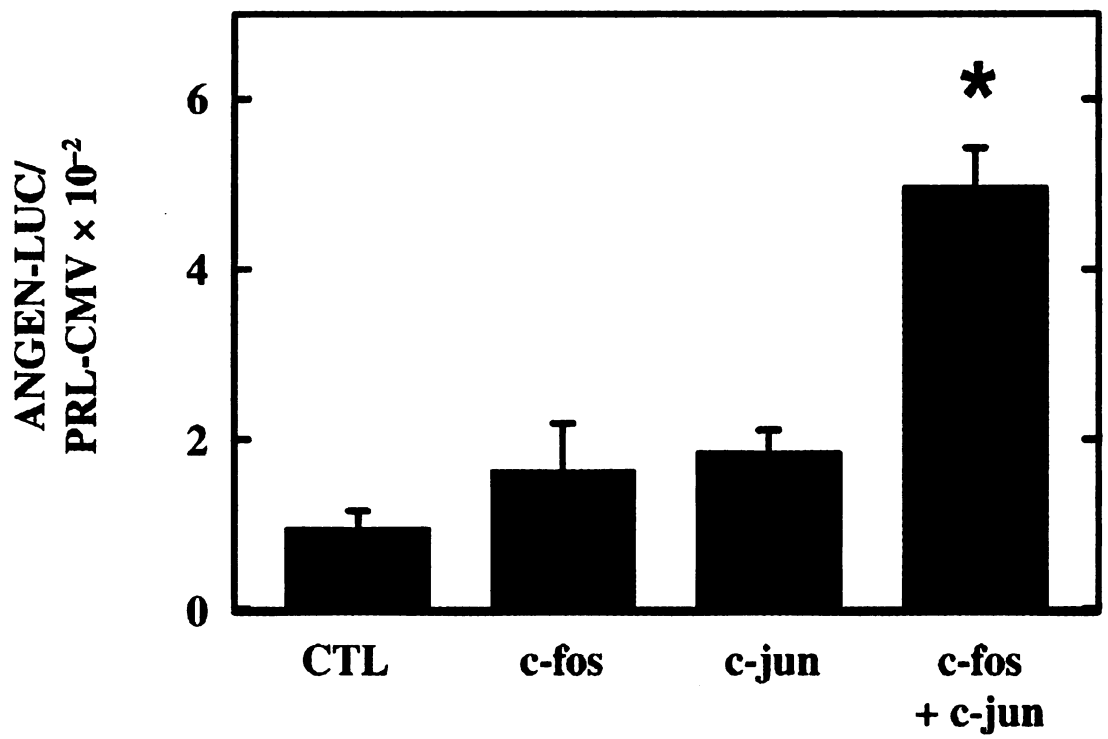

Figure B.10.

Expression of AP-1 is sufficient to induce angiotensinogen expression by A549 cells. A549 cells were cotransfected with expression vectors for c-fos and c-jun. AGT-LUC expression was detected as described in Figure B.3. Bars are the mean + S.E.M. of 3; * = $p<0.05$ versus control (CTL) by ANOVA and Student-Newman-Keul's test.

```
-360 tgggggtaca tctcccgggg ctgggtcaga aggcctgggt ggttggcctc aggctgtcac
                              AP-1_-336                      AP-1_-305
-300 acacctaggg agatgctccc gtttctggga accttggccc cgactcctgc aaacttcggt
-240 aaatgtgtaa ctcgaccctg caccggctca ctctgttcag cagtgaaact ctgcatcgat
-180 cactaagact tcctggaaga ggtcccagcg tgagtgtcgc ttctggcatc tgtccttctg
-120 gccagcctgt ggtctggcca agtgatgtaa ccctcctctc cagcctgtgc acaggcagcc
-60  tgggaacagc tccatcccca cccctcagct ataaataggg cctcgtgacc cggccagggg
                                    TATA box          AP-1_-15
+1   aagaagctgc cgttgttctg ggtactacag
```

Figure B.11.

Locations of AP-1 binding sites in the angiotensinogen promoter. The human AGT promoter sequence was analyzed for the presence of AP-1 binding sites as described in the Methods section. Map displays only those sites found within AMIO-responsive domains identified in Figure B.5. The effect of individual site mutations is reported in Figure B.12.

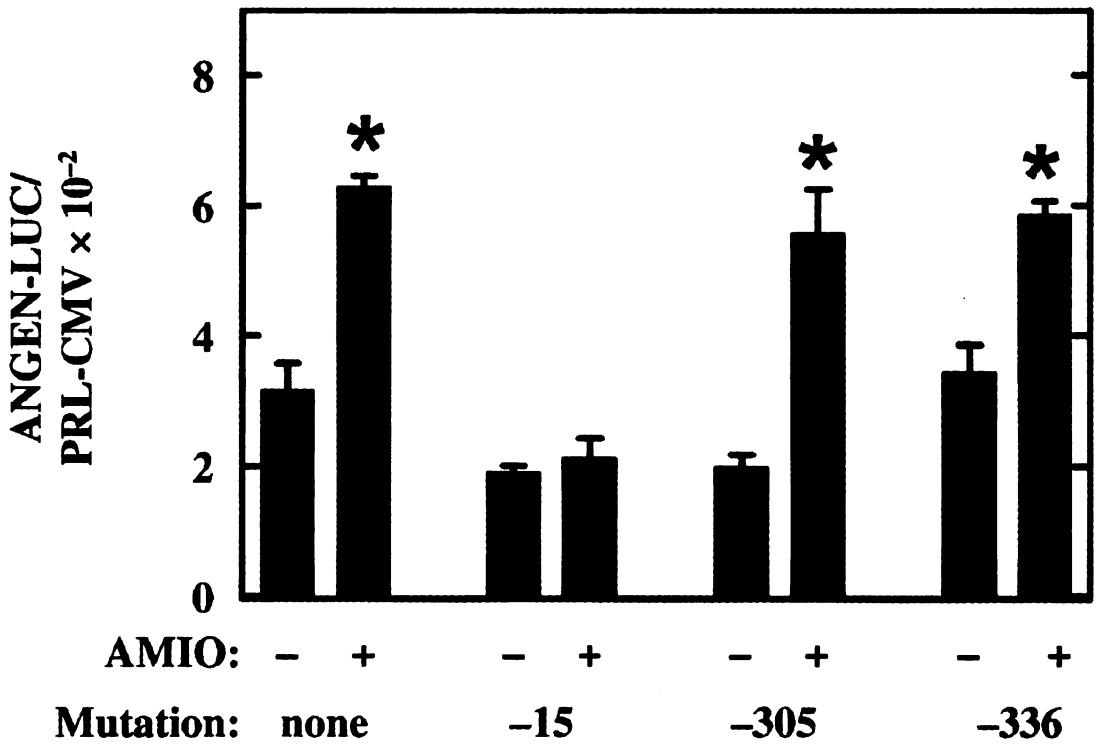

Figure B.12.

Effect of AP-1 binding site mutation on AGT-LUC expression in A549 cells. A549 cells were transfected with mutants of the full-length (-991 to +22) AGT-LUC reporter generated by 2bp substitutions within each of the indicated AP-1 sites (see Methods). The cells were thereafter challenged with AMIO as in Figure B.6. Bars are the mean + S.E.M. of at least three observations; * = $p<0.05$ vs. corresponding control (-AMIO) by Student's t-test.

DISCUSSION

Previous work from this laboratory demonstrated that exposure of alveolar epithelial cells (AEC) to the apoptosis inducers Fas, TNF-alpha or bleomycin resulted in upregulation of angiotensinogen (AGT) gene expression (Wang *et al.* 1999b; Wang *et al.* 2000a; Li *et al.* 2003b). In each of those prior studies, the proteolytic processing of AGT to the peptide ANGII was found to be required for the apoptotic response. For this reason, an earlier study of AEC exposed to AMIO, which demonstrated blockade of AMIO-induced apoptosis by the angiotensin converting enzyme inhibitor Captopril or the angiotensin receptor antagonist Saralasin (Bargout *et al.* 2000), suggested that AMIO might also upregulate AGT gene expression in AEC.

The experiments reported here provide direct evidence in support of this theory by demonstrating that AMIO upregulates AGT mRNA in either primary rat AEC or in the human AEC-derived A549 cell line. The notion that the upregulation occurs primarily at the transcriptional level is supported by the ability of actinomycin-D to prevent the AMIO-induced increase in AGT mRNA (Figure B.2), by the failure of AMIO to affect the decay rate of AGT mRNA (Figure B.3) and by the quantitative similarity between inducible AGT mRNA levels measured by realtime PCR (~3.2-fold, Figure B.2) and AMIO-inducible luciferase activity measured through the AGT-LUC reporter system (~2.9-fold, Figure B.4). Upregulation of AGT-LUC expression to the same degree by purified desethyl-amiodarone (Figure B.5), the primary metabolite of amiodarone, is consistent with the role of the P450 system in the degradation of AMIO (Reasor & Kacew 1996) and the known expression of P450 isoforms by AEC both in vivo and in vitro (Johns *et al.* 1983; Rabovsky *et al.* 1986).

Regardless, this report is the first study of the molecular mechanisms regulating AGT expression by pulmonary alveolar epithelial cells. In the liver, the human AGT promoter is well documented to be regulated by glucocorticoids and inflammatory cytokines including TNF-alpha, IL-1 and IL-6 (Brasier *et al.* 1994; Sherman & Brasier 2001). The best studied system, that of transcriptional control of hepatocyte AGT in response to IL-6, has been shown highly dependent on the three STAT binding sites depicted in Figure B.7A. In hepatocytes, these are known to bind STATs –1 and -3 in response to IL-6 stimulation, which upregulates AGT potently. The finding that two of these sites, at –278 and –171, reside within the AMIO-responsive domains in AGT-LUC stimulated initial interest in the possibility that increased STAT-5 binding by AMIO (Figure B.8A) might be exerting control at one or more of these sites. This hypothesis seems highly unlikely, however, in light of the finding that mutation of all three STAT sites, either individually or together, failed to eliminate the response to AMIO (Figure B.7B).

On the other hand, activator protein 1 (AP-1) family transcription factors have been implicated in the induction of apoptosis in a variety of cell types (Hess *et al.* 2004) including AEC. Induction of c-jun and c-fos precede apoptosis of the rat alveolar epithelial cell line RLE in response to donors of nitric oxide or hydrogen peroxide (Janssen *et al.* 1997). Studies of ambient particulate matter (PM) have shown that sublethal concentrations upregulate jun and fra family members in mouse C10 cells, whereas apoptotic concentrations of PM upregulate both fos and jun family members (Timblin *et al.* 2002). Those results were in agreement with investigations of other epithelial cell types such as keratinocytes and hepatocytes, in which the activation of both

jun and fos is proapoptotic (Hess *et al.* 2004).

That model is entirely consistent with the data reported here because the expression of fos and jun together, but not separately, induced AGT-LUC potently (Figure B.10), and AGT by itself induces apoptosis of AEC (Wang *et al.* 1999c). Moreover, AGT expression was blocked by the fos dominant negative construct a-fos (Figure B.9) and by the mutation of the AP-1 site between the TATA box and the transcription initiation site (Figure B.12). These data strongly support the involvement of AP-1 family members in the induction of AGT-LUC by AMIO; they also indicate that the AP-1 site at –15 to –12 is required for the response. On the other hand, the reduction of AMIO-induced AGT-LUC expression by deletion of promoter regions –350 to –260bp and –203 to –46bp (Figure B.6) suggest additional requirements for other cis-acting promoter elements in the response to AMIO. Those elements are likely not AP-1 binding regions, because mutation of the AP-1 sites within the AMIO-responsive promoter domains did not reduce AMIO-induced AGT-LUC activity (Figure B.12). Together, these results suggest the possibility that other transcription factors may interact with AP-1 family members to induce AGT expression in AEC through mechanisms yet to be determined.

Consistent with this interpretation, Yanai *et al.* (Yanai *et al.* 1996) have shown that a ubiquitously expressed nuclear factor termed AGCF1 (human AGT core promoter binding factor 1), which is believed to contain upstream regulatory factor 1 (USF1) as one of its components (Yanai *et al.* 1997a), regulates the responsiveness of the human AGT promoter in HepG2 cells through a cis-acting region located very close (-25 bp) to the proximal AP-1 site mutated in Figure B.12. Interestingly, investigations of the

osteopontin (OP) promoter have shown coordinated transcriptional control of OP by glucose/deoxyglucose or UTP; this control is mediated through USF/AP-1 interactions at binding sites in either close proximity to (Bidder *et al.* 2002) or significantly upstream (Renault *et al.* 2004) from the transcription initiation site. Thus, the possibility exists that AP-1 family members binding to the –15 AP-1 site mutated in Figure B.12 might require interaction with USF, other transcription factors or cis-acting elements located upstream from the TATA box. Although no such USF/AP-1 interactions have yet been demonstrated to control the AGT promoter, this possibility may relate to the presently unknown roles of the required upstream domains at –350 to –260bp and –203 to –46bp (Figure B.6). This issue will be an interesting topic for future study.

The data herein are also consistent with previous demonstrations that antagonists of the angiotensin system can prevent amiodarone-induced lung collagen accumulation in rats (Uhal *et al.* 2003). In that study, six months oral administration of AMIO to Wistar rats (the same rat strain used here for AEC isolation) caused AEC apoptosis and lung collagen accumulation. Both of these effects were significantly inhibited by either the ACE inhibitor captopril or the ANG receptor AT_1 antagonist losartan. Although the synthesis of AGT by AEC in vivo was not one of the endpoints detected in that study, the data of the present paper support the hypothesis that AEC synthesize AGT in response to AMIO in the intact animal as they do in vitro.

In summary, amiodarone (AMIO) induces angiotensinogen (AGT) gene transcription in primary rat AEC and in the human AEC-derived A549 cell line. Upregulation of an AGT promoter/luciferase reporter by AMIO in AEC was independent of STAT binding sites known to be important in hepatocyte AGT expression, but

required promoter domains between –350 to –260bp and –203 to –46bp. AGT-LUC expression was induced by c-fos and c-jun overexpression alone, and a fos dominant negative construct abrogated AMIO-induced AGT-LUC expression. Mutation of one AP-1 binding site between the TATA box and the transcription initiation site was sufficient to eliminate AMIO responsiveness. These data suggest that the induction of AGT expression in AEC by AMIO is mediated by AP-1 family transcription factors, by an AP-1 binding site close to the transcription start site and by additional interactions with upstream promoter elements. The roles of these upstream cis-acting elements are currently under investigation.

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