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Modulation of TGFβ-Induced PAI-1 Expression by Changes in Actin Polymerization in Human Mesangial Cells

Keyur Patel
Old Dominion University

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MODULATION OF TGFβ-INDUCED PAI-1 EXPRESSION BY CHANGES IN ACTIN POLYMERIZATION IN HUMAN MESANGIAL CELLS

by

Keyur Pravinchandra Patel
M.B.B.S., January 1999, Gujarat University

A Dissertation Submitted to the Faculty of Eastern Virginia Medical School and Old Dominion University in Partial Fulfillment of the Requirement for the Degree of

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EASTERN VIRGINIA MEDICAL SCHOOL AND OLD DOMINION UNIVERSITY
May 2006

Approved by:

William F. Glass II (Director)

Pamela Hardina (Member)

Tim Bos (Member)

Julie Kerry (Member)
ABSTRACT

MODULATION OF TGFβ-INDUCED PAI-1 EXPRESSION BY CHANGES IN ACTIN POLYMERIZATION IN HUMAN MESANGIAL CELLS

Keyur Pravinchandra Patel
Old Dominion University, 2006
Director: Dr. William F. Glass II

Chronic renal diseases show increased deposition of extracellular matrix (ECM) in the glomerulus (glomerulosclerosis). Glomerulosclerosis is associated with activation of normally quiescent glomerular mesangial cells into myofibroblast-like cells. The overall objective of this study is to delineate cellular mechanism/s of myofibroblast-differentiation in disease states. In cultured mesangial cells certain characteristics of myofibroblast differentiation (α-smooth muscle actin (α-SMA) and hypertrophy) are associated with an increase in polymeric actin microfilaments (stress fibers). It is likely that other genes are also regulated in an actin cytoskeleton-dependent manner during myofibroblast differentiation. In these studies, we therefore examined the hypothesis that changes in the actin cytoskeleton regulate myofibroblast differentiation of mesangial cells.

In vivo, myofibroblasts play a major role in ECM accumulation and tissue scarring. TGFβ increases ECM deposition by increasing plasminogen activator inhibitor type-1 (PAI-1) expression. PAI-1 inhibits ECM degradation by tissue-(tPA) and urokinase-type (uPA) plasminogen activators. Additionally, the Rho family of GTPases is required for TGFβ-induced PAI-1 expression. Since, regulation of actin cytoskeletal organization is a major function of Rho, the
hypothesis that Rho-mediated changes in actin cytoskeleton modulate PAI-1 expression in glomerular cells was proposed. The effects of modulating the actin cytoskeleton on TGFβ-induced PAI-1 expression were examined in cultured human glomerular mesangial cells. Inhibitors of Rho signaling decreased basal and TGFβ-induced PAI-1 mRNA and protein expression. These effects were mimicked by direct inhibition of actin polymerization by Cytochalasin B (CytB) suggesting that the effects of Rho on PAI-1 expression are mediated through the actin cytoskeleton. CytB also inhibited the activity of a PAI-1 promoter. Conversely, stabilization of actin microfilaments with jasplakinolide (JAS) had the opposite effect on TGFβ-induced PAI-1 expression. These results indicate a bi-directional regulation of PAI-1 by the actin cytoskeleton depending on the state of actin polymerization. In contrast to inhibition of PAI-1, actin-depolymerizing agents increased tPA mRNA expression.

In summary, the changes in the actin cytoskeleton mediate the effects of Rho-GTPases in modulation of PAI-1 and tPA expression in response to TGFβ. Thereby, they may control ECM degradation. Overall, the results suggest that changes in organization of the actin cytoskeleton regulate myofibroblast differentiation and ECM accumulation by coordinately modulating expression of multiple genes.
This thesis is dedicated to my wife, Rupal, and, to my parents, Hansa and Pravinchandra Patel. Your unconditional love, silent sacrifices, continued support and prayers have made my dreams come true.
ACKNOWLEDGMENTS

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she has been a constant source of motivation and personalized guidance. Thank you, Dr. Kerry, for the detailed review of my work.

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I want to thank all four members of my guidance committee for ensuring that my work was scientifically sound and professionally presented. I feel privileged to be your student.

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<td>α-SMA</td>
<td>Alpha-Smooth Muscle Actin</td>
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<td>ADF</td>
<td>Actin Depolymerizing Factor</td>
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<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
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<tr>
<td>ATS</td>
<td>Anti-Thymocyte Serum</td>
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<tr>
<td>CytB</td>
<td>Cytochalasin B</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>ERK</td>
<td>Extracellular Signal Regulated Kinase</td>
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<td>ESRD</td>
<td>End-Stage Renal Disease</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>GAP</td>
<td>GTPase Activating Protein</td>
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<td>GDI</td>
<td>GDP Dissociation Inhibitor</td>
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<td>GDP</td>
<td>Guanidine DiPhosphate</td>
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<td>GEF</td>
<td>Guanidine Nucleotide Exchange Factor</td>
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<td>GRE</td>
<td>Glucocorticoid Response Elements</td>
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<td>GTP</td>
<td>Guanidine TriPhosphate</td>
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<td>HMC</td>
<td>Human Mesangial Cell</td>
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<td>HRE</td>
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<td>JAS</td>
<td>JASplakinolide</td>
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<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
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<td>LAP</td>
<td>Latency Associated Peptide</td>
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<td>LatB</td>
<td>Latrunculin B</td>
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<td>L-NAME</td>
<td>N-Nitro-L-Arginine Methyl Ester (L-NAME)</td>
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<td>ROCK</td>
<td>Rho-Kinase</td>
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<tr>
<td>RT</td>
<td>Reverse Transcription</td>
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<tr>
<td>SAPK</td>
<td>Stress-Activated Protein Kinase</td>
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<td>SEM</td>
<td>Standard Error of Mean</td>
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<td>SERPIN</td>
<td>SERine Protease Inhibitor</td>
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<tr>
<td>SRF</td>
<td>Serum Response Factor</td>
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<td>TBS</td>
<td>Tris-Buffered Saline</td>
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<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
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<td>TPA</td>
<td>Tissue-type Plasminogen Activator</td>
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<tr>
<td>UPA</td>
<td>Urokinase-type Plasminogen Activator</td>
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<tr>
<td>UPAR</td>
<td>uPA Receptor</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral Ureteral Obstruction</td>
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<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cells</td>
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CHAPTER 1
INTRODUCTION

Over 25 million people in the USA suffer from different forms of chronic renal diseases, of which approximately 419,000 have end stage renal disease (ESRD) with an annual increase of 4.6% [1]. Dialysis and organ transplant are the two mainstays of treatment of ESRD, both of which provide a palliative treatment at best and are associated with many limitations [2]. The current preventive measures are only effective if instituted early in the disease process, which is clinically difficult to achieve since early disease is generally asymptomatic [3]. An annual expenditure of $25 billion for treatment of ESRD and lack of definitive treatment warrant further medical research on mechanisms leading to end state renal diseases [1].

The information in this chapter is organized into two parts: The role of activated mesangial cells in pathogenesis of chronic renal diseases and the observations with cultured mesangial cells leading to the central hypothesis are described first (sections 1.1 and 1.2). The second part contains information on actin cytoskeleton and the protein of interest of this study (sections 1.3 to 1.7).

Mesangial cells and glomerulosclerosis

The glomerulus

The renal glomerulus, the primary site of filtration of waste products from
Fig. 1. Normal glomerulus. The renal glomerulus is a highly vascular structure formed by invagination of the afferent arteriole into a double-layered Bowman’s capsule and subsequent branching into capillaries (e) lined by endothelial cells (d). A parietal layer of simple squamous epithelium (a) and a visceral layer of podocytes (b) separated by Bowman’s space (c) form the Bowman’s capsule. The efferent arteriole drains the glomerulus. The point at which the two arterioles penetrate Bowman’s capsule is called the vascular pole and the point at which ultrafiltrate leaves the glomerulus is called the urinary pole. The space in between the capillary loops is called the mesangium (meso= in between, angium= vessels) (f), which consists of the mesangial cells (g) and extracellular matrix laid by them.
the bloodstream, is a highly vascular structure consisting of a network of capillary loops held together by mesangial matrix (Fig. 1) [4]. At the vascular pole, the afferent arteriole invaginates into the double-layered Bowman's capsule consisting of a parietal layer of simple squamous epithelium and a visceral layer of podocytes separated by the Bowman's space (urinary space). The afferent arteriole branches into a tuft of capillaries with multiple loops, collectively called the glomerulus. The glomerulus is drained by the efferent arteriole, which leaves near the point of insertion of afferent arteriole. The space in between the capillary loops is called the mesangium (meso= in between, angium= vessels), which consists of the mesangial cells and extracellular matrix laid by them.

As the blood flows through the capillary loops, some of its constituents including the water, waste products and electrolytes are selectively filtered into the Bowman's space (Fig. 1) [4]. This ultrafiltrate is collected, at the urinary pole, by the proximal convoluted tubules to be further transferred to a downstream system of tubules and ducts. After a complex series of absorption, re-absorption and secretion, the ultrafiltrate is finally converted into the urine.

**Glomerulosclerosis**

Glomerulosclerosis is a major feature of many renal diseases that progress to end stage renal disease (ESRD) [5, 6]. Pathologically, glomerulosclerosis is characterized by increased deposition of extracellular matrix (ECM) and gradual replacement of functioning renal tissue with non-functioning fibrous tissue (Fig. 2). Different underlying mechanisms lead to a
A. Normal

Mesangial Extracellular Matrix

B. Chronic Glomerulosclerosis

ECM deposition and mesangial expansion

C. Global Sclerosis

Replacement of normal glomeruli by ECM

Fig. 2. Glomerular morphology in normal and pathologic states. (A) Glomerulus showing relatively low amounts of extracellular matrix in the mesangium (arrow), (B) chronic glomerulosclerosis showing increased amount of extracellular matrix deposition and resultant expansion of the mesangium (arrow), (C) end-stage renal disease showing complete replacement of the glomeruli by extracellular matrix (arrow) in a process called global sclerosis.

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common pathological feature in the form of scarring of renal tissue [7]. Increased formation of extracellular matrix leading to fibrosis begins in the mesangium, the intercapillary portion of the glomerulus composed of mesenchymal mesangial cells [8, 9]. Expansion of the ECM occurs as a result of deposition of type IV and type I collagen, and fibronectin. In chronic renal diseases, fibrosis results from increased matrix production, decreased matrix breakdown or both. Since filtration and removal of waste products from the blood are the major functions of glomerulus, patients with non-functioning glomeruli develop toxicity due to accumulation of waste products [10].

Renal fibrogenesis

Renal fibrogenesis typically occurs in three distinct phases, with the first two being similar to physiological wound healing, namely the phase of induction, the phase of inflammatory matrix synthesis and the phase of post-inflammatory matrix synthesis [7]. The first phase is associated with an influx of mononuclear cells upon an insult leading to an inflammatory response. The inflammatory cells liberate cytokines and growth factors, which in turn activate fibroblasts [7]. In particular, transforming growth factor β (TGFβ), a pro-fibrotic growth factor, plays a major role in activating normally quiescent mesangial cells [11]. The second phase of inflammatory matrix synthesis is characterized by increased synthesis of ECM and de novo expression of proteins, and reduced degradation of ECM. The structural changes are still potentially reversible throughout this phase [7]. The third phase of post-inflammatory matrix synthesis is marked by autonomous
fibroblast proliferation, synthesis of ECM independent of the presence of primary inflammatory stimulus and perpetuation of the scarring process [7].

The mesangial myofibroblast

During the three phases described above, glomerular mesangial cells contribute to excess matrix deposition in multiple ways. Besides providing structural support to the mesangium, mesangial cells both secrete and respond to growth factors and inflammatory mediators, thereby regulating the glomerular microenvironment [12]. TGFβ activates otherwise quiescent mesangial cells which now start proliferating and expressing proteins that contribute to ECM deposition (Fig. 3). These activated mesangial cells also express α-smooth muscle actin (α-SMA) de novo, which is normally seen in smooth muscle cells, [13-20]. Consequently, these activated mesangial cells with smooth muscle cell-like properties are referred to as myofibroblasts. α-SMA expression and myofibroblast-like differentiation are also seen elsewhere in tissues such as heart, lung, liver and skin [21-24].

Along with α-SMA, activated myofibroblasts start expressing profibrotic plasminogen activator inhibitor-1 (PAI-1) de novo, which in turn inhibits ECM degradation and favors ECM deposition [19, 25]. Thus, TGFβ-mediated myofibroblast-like differentiation is associated with increased PAI-1 expression and excess ECM deposition that eventually leads to glomerulosclerosis and end-stage renal disease (Fig. 3).

The overall objective of this study is to characterize the mechanisms of mesangial myofibroblast-like differentiation in order to obtain useful insights into
Fig. 3. Cascade of events leading to chronic renal diseases. TGFβ is a profibrotic growth factor that increases production of PAI-1 by glomerular mesangial cells. Increased production of PAI-1 leads to decreased degradation and increased deposition of extracellular matrix in mesangium resulting in glomerulosclerosis. Replacement of normal glomeruli by non-functional fibrous tissue in glomerulosclerosis leads to clinical presentation in the form of chronic and end-stage renal diseases, both currently irreversible disease processes.
renal fibrogenesis. The specific aim of this study was to examine regulation of TGFβ-induced PAI-1 expression in human mesangial cells.

**Cultured mesangial cells as a model of myofibroblast phenotype**

Cultured mesangial cells provide a useful *in vitro* model of myofibroblast phenotype. The phases of fibrogenesis can be mimicked in vitro by altering the culture conditions. Cell culture in the presence of serum provides cytokines and growth factors as seen in the phase I to activate mesangial cells (refer to section 1.1.3). Cell culture in the absence of serum mimics phase III, which is marked by the absence of cytokines, development of the myofibroblast-like phenotype (α-SMA expression) and increased ECM production (refer to section 1.1.3). Thus, initial culturing in the presence of serum, followed by serum deprivation, mimics the growth factor and cytokine milieu of myofibroblast activation and differentiation. Primary cultures of human and rat mesangial cells were used in this study to understand mechanisms of myofibroblast differentiation.

**Regulation of α-SMA expression in cultured mesangial cells**

As a part of an overall focus on understanding the myofibroblast-like phenotype, our laboratory previously examined the regulation of α-SMA, expressed *de novo* upon myofibroblast differentiation, in cultured mesangial cells. Stephenson et al showed that serum-deprivation caused mesangial cells to spread, form abundant stress fibers, express more α-SMA and undergo hypertrophy [26]. Likewise, proliferating dermal fibroblasts are initially devoid of microfilaments [24], but their appearance and subsequent disappearance paralleled the expression of α-SMA. The observations of Stephenson et al and
the *in vivo* data regarding dermal fibroblasts, suggested that clearance of growth factors increases actin polymerization thereby controlling myofibroblast differentiation. Since, expression of $\alpha$-SMA paralleled changes in actin cytoskeleton organization, it was hypothesized that changes in actin polymerization regulate $\alpha$-SMA expression and hypertrophy in mesangial cells. The Rho family of small GTPases regulate actin polymerization through the downstream effector, Rho-kinase (ROCK), in a variety of cell types [27]. Inhibition of actin polymerization by agents that inhibit Rho-kinase (Y-2632, HA-1077), as well as by the agents that directly bind to actin and affect its degree of polymerization (CytB, LatB), inhibited $\alpha$-SMA expression and promoter activity [28]. On the other hand, stabilization of actin polymerization by JAS and phalloidin increased $\alpha$-SMA expression and promoter activity. Similarly, mesangial cell hypertrophy was blocked by actin depolymerization. These results confirmed the hypothesis that changes in actin polymerization regulate $\alpha$-SMA expression and hypertrophy in mesangial cells [28].

**Central hypothesis**

Like $\alpha$-SMA expression and hypertrophy, *de novo* expression of PAI-1 is also seen with myofibroblast differentiation [29]. It is possible that $\alpha$-SMA, PAI-1 and multiple other genes are regulated by a common underlying mechanism that regulates overall myofibroblast differentiation. Therefore, a central hypothesis that changes in actin polymerization regulate myofibroblast-like phenotype by simultaneously regulating expression of multiple genes was proposed.
The following sections contain information on the actin cytoskeleton, regulation of its organization, its role in gene expression, introduction to plasminogen activator system, regulation of PAI-1 expression and the specific hypothesis for this study.

**Actin cytoskeleton and gene regulation**

Traditionally, actin cytoskeletal organization is considered important for controlling processes such as cell shape and cell motility [30-32]. At the same time there is increasing evidence suggesting that the actin cytoskeleton and cell shape are important regulators of gene expression and cell function. Early observations of Folkman and Moscona, showing an association between inhibition of cell spreading and decreased DNA synthesis, proposed that cell shape regulates cell function [33]. Over the past 20 years, studies by Ingber have supported the idea that mechanical transduction through cytoskeletal elements affect cell functions [34-38]. Recent studies have shown that networks of actin polymers, also known as stress fibers, provide a highly specific and organized network of fibers for processes such as signal transduction and gene regulation. Localization of signaling molecules at specific cytoskeletal locations allows integration of signaling pathways and effective transmission of a signal [39, 40]. For example, MEKK1 that activates JNK mitogen associated protein kinase is associated with the actin cytoskeleton [41]. During keratinocyte differentiation, cytoskeletal association facilitates coupling of ErbB1 and ERK [42]. Also, expression of genes such as α-SMA seems to be regulated by the state of actin polymerization [28]. Localization of mRNA and protein translation machinery at
highly specific intracellular pockets of actin stress fibers have also been identified [43]. Thus, current evidence points towards regulation of gene expression by actin cytoskeleton.

**The actin cytoskeleton**

The actin cytoskeleton, also known as the microfilament system, is an important structural component of all eukaryotic cells. Actin, a 42-kDa globular polypeptide, is the building block of the microfilamentous cytoskeletal system. Monomeric actin (G-actin) molecules polymerize via non-covalent interactions into filamentous actin also known as F-actin or stress fibers [44]. Actin filaments cross-link with each other and with various cellular and membranous structures to form a highly organized network. The actin filaments traverse the cytoplasm and provide structural support to the cells. They transmit tension and resist deformation of the cells. Other cellular functions regulated by the actin cytoskeleton include but are not limited to cell shape, cell motility, cell division, cell-cell and cell-substrate interaction, transmembrane signaling, endocytosis, secretion and muscle contraction [45, 46].

The actin filaments are very dynamic structures undergoing constant growth in length at one end (barbed end, plus end) and attrition at the opposite end (pointed end, minus end). The rates of growth and attrition determine the amounts of monomeric and polymeric actin in the cell. Actin polymerization is regulated at multiple levels in response to extracellular stimuli or intracellular needs; and involves various cell-signaling pathways, actin binding and associated proteins (Fig. 4) [46].
Fig. 4. Regulation of actin cytoskeleton organization by Rho GTPase. Rho GTPase is a major regulator of actin polymerization. Rho-kinase (ROCK) and mDia are known downstream effectors of Rho that increase polymeric F-actin and decrease monomeric G-actin content by regulating the actions of actin binding proteins such as cofillin and profilin. The hypothesis examined in this thesis proposed that Rho-mediated changes in actin polymerization regulated expression of specific genes through involvement of some yet unknown factors. Agents that increase (Jasplakinolide) or block (Toxin B, HA-1077, Y-2632, Cytochalasin B and Latrunculin B) actin polymerization were used to test the hypothesis by modifying actin polymerization at different levels.
Actin binding proteins

The actin cytoskeleton undergoes different stages of organization such as nucleation, polymerization, cross-linking, folding and movement. As the name implicates, actin-binding proteins bind to actin monomers or polymers during different phases of assembly and regulate the rate and the extent of actin polymerization. The activities of these proteins are in turn regulated by signaling molecules [46]. Major actin-binding proteins are briefly reviewed in Table 1 [47-49].

RhoA and actin cytoskeleton

Rho is a member of Rho family of small GTPases (Rho, Rac and Cdc42) that plays a major role in organizing actin cytoskeleton in the form of stress fibers (Fig. 4) [27, 50, 51]. The Rho GTPases exist in an inactive GDP-bound form complexed with a GDP-dissociation inhibitor (Rho GDI) and an active GTP-bound form. In response to stimuli, guanine nucleotide exchange factors (GEFs) dissociate GDI from Rho and allow Rho to bind to GTP. Activated Rho-GTP in turn activates downstream effecters. GTPase activating proteins (GAP) inactivate Rho by catalyzing GTP hydrolysis [52]. Rho-kinase or ROCK, is the main downstream effector of Rho that inactivates proteins that depolymerize actin such as cofilin and destrin and thereby facilitates actin polymerization. Other effectors of Rho include PIP-5 kinase and mDia, a 140 kDa protein that increases actin polymerization through the actin-binding protein profilin [53-55].
## Table 1. Actin Binding Proteins

<table>
<thead>
<tr>
<th>Actin Binding Protein/s</th>
<th>Effect on Actin Cytoskeleton</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profilin</td>
<td>In resting state, sequesters monomeric actin thereby inhibits actin polymerization, In stimulated state, promotes assembly of actin filaments</td>
</tr>
<tr>
<td>β-thymosin, twinfilin, DNase I</td>
<td>Binds to monomeric actin and sequesters it, thereby inhibits actin polymerization</td>
</tr>
<tr>
<td>Cofilin</td>
<td>Binds and severs actin filaments, Also known as actin depolymerizing factor (ADF)</td>
</tr>
<tr>
<td>Gelsolin, villin, fragmin, adseverin, scinderin</td>
<td>Bind and sever actin filaments</td>
</tr>
<tr>
<td>Fascin, villin, fimbrin, α-actinin</td>
<td>Cross-link actin filaments, Also known as actin-bundling proteins</td>
</tr>
<tr>
<td>Srv2/cyclase-associated protein (CAP) family proteins</td>
<td>Stimulate nucleotide exchange on actin monomers and relieve the inhibitory effects of ADF/cofilin on this exchange</td>
</tr>
<tr>
<td>Wiskott–Aldrich Syndrome Protein (WASP)</td>
<td>Increases nucleation of actin monomers</td>
</tr>
<tr>
<td>Arp 2/3</td>
<td>Binds to actin dimers and increases nucleation of stress fibers</td>
</tr>
</tbody>
</table>
Plasminogen activator system and plasminogen activator inhibitor-1 in chronic renal disease

The plasminogen activator system, which controls activation of plaminogen into plasmin, is the key regulator of matrix degradation (Fig. 5). Activated plasmin, in turn, not only degrades some matrix proteins itself, but also amplifies the fibrolytic response by activating another set of powerful matrix degrading enzymes called matrix metalloproteinases (MMPs) [56]. PAI-1, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are components of the plasminogen activator system (Fig. 5). PAI-1 inhibits matrix breakdown by inhibiting tPA and uPA. Although tPA and uPA have a limited capacity to directly degrade ECM proteins, they trigger activation of a cascade of proteases that lead to ECM degradation (Fig. 5) [56]. The balance between the anti-fibrotic effects of plasminogen activators and the pro-fibrotic effects of PAI-1 determines the rate of matrix turnover and the amount of matrix deposition in the tissue [57, 58]. Transforming growth factor beta (TGFβ) is a major profibrotic growth factor responsible for increased PAI-1 expression and resulting glomerulosclerosis seen in chronic renal diseases [59]. The plasminogen activator system also regulates fibrinolysis in the intravascular space [60].

**tPA**

Tissue-type plasminogen activator (tPA), Mr of 70,000, is secreted in the form of a single or double chain enzyme from endothelial and mesangial cells [61].

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Fig. 5. Regulation of extracellular matrix turnover by the plasminogen activator system. Plasminogen activator system consists of two activators of plasminogen namely tissue-type (tPA) and urokinase-type (uPA) plasminogen activators and their inhibitor called plasminogen activator inhibitor type I (PAI-1). Both tPA and uPA convert inactive plasminogen into active plasmin. Active plasmin, in turn, stimulates extracellular matrix (ECM) degradation mainly by activating matrix metalloproteinases (MMPs) and to a lesser degree by directly degrading ECM. PAI-1 acts as a gatekeeper of matrix degradation cascade by inhibiting tPA and uPA mediated plasminogen activation.
The modular structure of tPA include five domains homologous to those of fibronectin, epidermal growth factor (EGF), plasminogen and trypsin-like protease. The extensive homology of domains of tPA to that of components of ECM, proenzymes and growth factors facilitates the interaction between the components and effective activation of plasminogen. tPA alone activates plasminogen at a very low rate. Binding of tPA to proteins of extracellular matrix, such as thrombospondin and collagen IV, increases the rate of plasminogen activation. Vascular smooth muscle cells (VSMCs), which are phenotypically similar to activated mesangial cells, produce the majority of tPA in vivo and express a tPA binding receptor which not only increases tPA activity but also decreases the inhibition of tPA by PAI-1 [62].

Administration of recombinant tPA provides a protective effect by decreasing glomerular matrix accumulation in an anti-Thy1 model of glomerular matrix expansion [63]. On the other hand, over-activation of tPA can produce harmful effects due to increased MMP activation and tubular basement membrane degradation [64]. Therefore it appears that tPA expression and activity have to be carefully regulated for normal functioning of renal tissue.

uPA

Urokinase-type plasminogen activator (uPA), Mr of 55,000, having trypsin-like protease activity, was first isolated from urine. Like tPA, uPA is also composed of domains that facilitate effective activation of plasminogen. Domains of uPA are homologous to those of EGF, plasminogen and trypsin-like protease.
Many cell types express a cystein-rich 65 kDa receptor for uPA (u-PAR) [62]. uPA binds to N-terminal domain of u-PAR with high affinity. Binding of uPA to uPAR initiates intracellular signal transduction and triggers local proteolysis by decreasing the Km for plasminogen activation by up to 200-300 fold, both of which play a critical role in tissue development. Activated plasmin in turn amplifies the fibrinolytic potential by activating zymogen pro-uPA. Thus an initial activation of small amounts of plasminogen can generate large amounts of plasmin through a positive feedback loop [62]. Mice expressing an inducible uPA transgene or following administration of an adenoviral vector containing a uPA gene had reduced fibrosis [65].

Studies have shown that tPa and uPA are complimentary activators of plasminogen with a high degree of functional overlap. Conventionally, tPA regulates plasminogen activation in intravascular compartment, whereas uPA does so in tissues. [66] Also tPA plays a major role in fibrinolysis, whereas uPA has been associated with cell migration [67].

PAI-1

PAI-1 (Serpin E1) is a member of the SERPIN (SERine-Protease-Inhibitor) family of protease inhibitors [68]. It is a single-chain, 379 amino acid, 52-kDa glycoprotein (~13% carbohydrate) that exists in active, inactive and latent forms. The active form spontaneously converts to a latent form, which can be partially reactivated by denaturing agents [56]. The reactive center of the inhibitor is contained within the exposed “strained loop region” at the carboxy terminal of the molecule. This serves as a pseudosubstrate for the target serine proteases.
PAI-1 is not normally expressed in kidneys but is rapidly induced in a variety of pathological states. Newly synthesized PAI-1 is released into the extracellular space where it is converted into its inactive latent form in a few minutes. Binding of PAI-1 to matrix proteins such as vitronectin increases its half-life >10 times, thus enabling matrix the to resist proteolysis by invading cells [69], [25]. Mesangial cells along with glomerular endothelial and epithelial cells have been identified as a source of PAI-1 expression under different conditions. [25, 70, 71]. The major function of PAI-1 is to regulate extracellular matrix turnover by inhibiting the activation of proteases that break down the matrix. PAI-1 does so by forming a 1:1 stoichiometric reversible complex with tPA and uPA, thereby inhibiting them [56]. PAI-1-plasminogen activator complexes can be endocytosed and subsequently degraded by lysosomes, thereby providing a clearance mechanism for fibrolytic activity [69].

The inhibition of plasminogen activators by PAI-1 is the rate-limiting step in matrix degradation. Not surprisingly, increased PAI-1 expression has been associated with increased matrix deposition and tissue fibrosis. PAI-1 is nearly undetectable in normal kidneys by immunohistochemistry and in situ hybridization. However, increased PAI-1 expression is seen in many human and experimental glomerular diseases including acute (thrombotic microangiopathy, renal vasculitis, proliferative glomerulonephritis and membranous nephritis) and chronic human diseases (diabetic nephropathy, hypertensive nephropathy, focal segmental glomerulonephritis and lupus nephritis) and animal models in different renal cell types including mesangial cells [25]. Studies with knock-out mice
clearly established the role of PAI-1 in fibrosis. PAI-1 knock out mice were resistant to renal injury caused by unilateral ureteral obstruction [71] and pulmonary fibrosis caused by bleomycin (an antineoplastic drug producing pulmonary fibrosis as a side effect) [72]. Similarly, PAI-1 knockout mice were protected against N-nitro-L-arginine methyl ester (L-NAME)-induced perivascular fibrosis [73]. Conversely, over expression of PAI-1 worsened bleomycin-induced fibrosis [72]. By virtue of its action on matrix turnover, PAI-1 also plays an important role in neointima formation, angiogenesis, atherosclerosis, tumor growth and metastasis, and wound healing. PAI-1 may play a role in cell adhesion and migration as a result of its high affinity binding to vitronectin.

PAI-1 acts as a gatekeeper guarding against fibrinolysis by inhibiting unnecessary activation of the plasminogen cascade. Hence it is one of the most highly regulated components of the plasminogen activation system. The human PAI-1 gene is located on the long arm of chromosome 7. It is comprised of nine exons and eight introns, and is ~12.2 kb long. Alternative polyadenylation of PAI-1 mRNA results in two distinct transcripts, one 2.8 kb long and the other 3.2 kb long. The PAI-1 gene and its flanking region contain 12 Alu repeat elements and 5 long poly (Pur) repeat elements. Also, the promoter contains a 4G/5G polymorphism at -675. It is suggested that the presence of 4Gs in the promoter is associated with increased production of PAI-1 [74].

PAI-1 expression is induced by a variety of factors including growth factors, coagulation factors, metabolic factors and hormones such as transforming growth factor-β (TGFβ), epidermal growth factor (EGF), fibroblast
growth factor (FGF), fibrin fragments, thrombin, glucose, insulin, Angiotensin, hypoxia, glucocorticoids and radiation [25]. Regulation is primarily at the transcription level, although cyclic nucleotides may regulate PAI-1 message stability [25, 75]. The PAI-1 promoter has two AP-1 like sites, two SP-1 sites, three SMAD sites, one NF-kB site, glucocorticoid response elements (GRE) and hypoxia response elements (HRE) allowing the regulation of promoter by multiple pathways [76-79]. Indeed, inflammatory mediators such as TGFβ, triglycerides, fatty acids and phorbol esters increase PAI-1 expression. PAI-1 expression is regulated by signal inputs from many intracellular signaling pathways including MAPK, Smad, PKA, PKC, nuclear receptors and Rho signaling [76, 80-84].

PAI-1, in turn, inhibits uPA-plasminogen activation, which normally activates latent TGFβ, a major inducer of PAI-1 expression in mesenchymal cells. Thus TGFβ turns off its own production through induction of PAI-1. The autoregulatory feedback loop between TGFβ and PAI-1 plays an important role in wound healing and in limiting ECM deposition [69]. A detailed understanding of signal transduction involved in TGFβ induced PAI-1 expression is required to identify potential targets for the intervention of TGFβ mediated PAI-1 expression and fibrosis.

In a previous study we have shown that changes in actin cytoskeleton regulate α-SMA gene expression and hypertrophy in mesangial cells [28]. Like α-SMA expression and hypertrophy, de novo expression of PAI-1 is also seen with myofibroblast differentiation [85, 86]. It is possible that regulation of both the genes share common or similar underlying regulatory mechanisms, suggesting
regulation of PAI-1 by actin cytoskeleton. Also, recently Schnaper et al have shown that the inhibition of actin polymerization blocks TGFβ-mediated collagen expression [87]. It is logical to think that changes in actin cytoskeleton may also similarly modulate expression of PAI-1, another TGFβ-dependent gene and a regulator of ECM. Thus two independent lines of evidence suggest regulation of PAI-1 expression by changes in actin polymerization.

**TGFβ in Renal Diseases**

Transforming Growth Factor-β (TGFβ) regulates extracellular matrix production by increasing the production of matrix components such as collagen, fibronectin, laminin and heparan sulfate proteoglycans, and simultaneously decreasing matrix degradation by increasing production of plasminogen activator inhibitor-1 (PAI-1) [88]. The net result is an increase in extracellular matrix production and glomerulosclerosis [89, 90]. TGFβ regulates a range of cellular processes including the cell cycle, differentiation, wound healing, extracellular matrix production and the immune response [91-94]. TGFβ circulates in an inactive form bound with latency-associated peptide (LAP) and is activated locally when LAP binds to integrin αvβ6 and TGFβ is cleaved.

Not surprisingly, TGFβ is present in human glomeruli in several glomerular diseases including diabetic nephropathy [95] and is associated with increased mesangial matrix production [96]. During pathogenesis of these renal diseases, TGFβ signaling causes tubular atrophy, podocyte depletion, loss of capillary endothelial cells, mesangial cell activation and epithelial-to-mesenchymal transdifferentiation. The combination of these multiple pathogenic
events leads to loss of functioning renal tissue [91]. The role of TGFβ in renal fibrosis has also been proven in a variety of animal models [97, 98]. Integrin αvβ6 knockout mice, which are unable to release LAP inhibition of TGFβ, were protected against pulmonary fibrosis [99]. Administration of anti-TGFβ antibody protected against scarring in an animal model of chronic renal disease confirming the role of TGFβ in fibrosis [100].

**Rho and PAI-1 expression**

Rho proteins regulate gene expression by increasing activity of transcription factors such as c-jun, ATF2a, ELK and STAT3 through intracellular signaling pathways such as MAPK and src [101]. Recently, the protective effects of Rho-kinase inhibitors against tissue fibrosis have established the role of Rho in the pathogenesis of tissue fibrosis. The Rho-kinase (ROCK) inhibitor Y-27632 markedly decreased bleomycin-induced pulmonary fibrosis and collagen accumulation in mice [102]. Similarly, Y-27632 protects against hepatic fibrosis [103]. The inhibition of Rho-kinase also protects against tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction [104]. The basis for the protective effects of Rho-inhibition is not fully understood. Inhibition of expression of profibrotic molecules such as PAI-1 appears to be a potential mechanism. Inhibition of Rho by C3 exoenzyme decreases PAI-1 production in rat proximal tubules [105] and aortic endothelial cells [106]. Co-transfection with dominant negative RhoA decreases PAI-1 promoter activity, whereas constitutively-active RhoA increases PAI-1 in chicken atrial cells [107]. ROCK inhibition blocks PAI-1 expression in response to Angiotensin II [108]. Also, ROCK inhibition blocks
PAI-1 promoter activity [81]. Thus, both inhibition of Rho activation and inhibition of signaling downstream of Rho lead to inhibition of PAI-1 expression. Collectively, these facts suggest that inhibition of Rho protects against fibrosis through inhibition of PAI-1.

Although the role of Rho in the regulation of PAI-1 expression is becoming increasingly clear, the mechanism for this effect has not been examined. Since actin polymerization is one of the major functions of Rho-ROCK signaling (Fig. 3), it is plausible that the effect of Rho-inhibition is mediated by depolymerization of actin. Taken together, these observations suggest that Rho-mediated changes in actin cytoskeletal structure modulate PAI-1 expression. Given the diversity of signaling pathways that interact with Rho, it is possible that Rho mediates and integrates the effect of these pathways on PAI-1 expression by modulating actin cytoskeleton organization.

**Hypothesis and aims**

The Central hypothesis is that changes in actin polymerization regulate the myofibroblast-like phenotype of kidney mesangial cells by simultaneously regulating expression of multiple genes.

The overall objective is to characterize the mechanisms of mesangial myofibroblast-like differentiation.

The working hypothesis is that Rho-mediated changes in actin cytoskeletal structure modulate PAI-1 expression in glomerular mesangial cells.

My specific aims are to examine the role of Rho in the regulation of TGFβ-induced PAI-1 expression in human mesangial cells as follows:
Specific Aim 1: To profile the effects of actin cytoskeleton on the expression of multiple genes in human mesangial cells.

Specific Aim 2: To examine the effect of TGFβ on PAI-1 expression.

Specific Aim 3: To examine the role of Rho and actin cytoskeleton in TGFβ-induced PAI-1 expression.

Specific Aim 4: To examine the role of Rho and actin cytoskeleton in TGFβ-induced PAI-1 promoter.

Specific Aim 5: To examine the effect of TGFβ and actin cytoskeleton on plasminogen activators.
CHAPTER 2

MATERIALS AND METHODS

Cell culture

Human mesangial cells (HMCs) were cultured in RPMI 1640 (Mediatech Inc., Herndon, VA) with 16.7% fetal bovine serum (Invitrogen, Carlsbad, CA). from renal glomeruli isolated by the sieving method. HMCs between passages 5 and 9 (P5 -P9) were used for experiments. After plating in serum-containing media, cells were serum-starved at 60-80% confluence. After 24 hours of serum starvation, cells were pre-treated with agents that depolymerize (Cytochalasin B: 1.0 μM, Latrunculin B: 0.1 μM, Toxin B: 10 pM, Y-27632: 10 μM, HA-1077: 20 μM) and/or stabilize actin cytoskeleton (Jasplakinolide: 50 nM) for one hour prior to addition of 10 ng/ml TGFβ for the indicated time periods. Cytochalasin B was purchased from Sigma Chemical Co., St. Louis, MO. The Rho-kinase inhibitor, Y-27632, was purchased from Welfide Corporation, Osaka, Japan. Another Rho-kinase inhibitor, HA-1077, was purchased from AG Scientific Inc., San Diego, CA. Clostridium Difficile Toxin B (Toxin B), Latrunculin B (LatB) and Jasplakinolide (Jas) were purchased from Calbiochem, San Diego, CA. Human recombinant TGFβ was purchased from R&D Systems, Minneapolis, MN.

DNA Array Analysis

The RNA isolation, DNAsse treatment, Poly A+ enrichment, cDNA probe synthesis, hybridization and analysis of scanned arrays were performed as per manufacturer’s protocol [109].
RNA Isolation

Human mesangial cells were plated on five 10 cm dishes per condition. At the end of the desired treatment, cells were trypsinized and spun down at 680xg for 5 minutes. The pellet was resuspended in 1 ml denaturing buffer. A 0.5 ml aliquot was transferred to sterile 1.5 ml tube and incubated on ice for 5-10 min. After vortexing briefly, the homogenate was centrifuged at 13,370xg for 15 min at 4°C to remove cellular debris. The supernatant was transferred to a new 2 ml tube and 1 ml of Tris-EDTA saturated phenol was added to it. The tube was vortexed for 1 min. After a 5 min incubation on ice, 0.3 ml chloroform was added. The sample was vortexed vigorously for 1-2 min and then incubated on ice for 5 min. The homogenate was centrifuged at 13,370xg for 10 min at 4°C. The upper aqueous phase containing RNA was transferred to a new 2 ml tube. A second round of phenol chloroform extraction using 0.8 ml saturated phenol and 0.3 ml chloroform was performed. Next, 1 ml isopropanol was added with incubation on ice for 10 min. The samples were centrifuged at 13,370xg for 15 min at 4°C. The pellet was washed with 0.5 ml of 80% ethanol. The samples were centrifuged at 13,370xg for 15 min at 4°C. The supernatant was discarded and the pellet was air-dried. The pellet was re-suspended in 25 μl RNase-free H₂O.

DNase Treatment of Total RNA

The DNase treatment reaction was set up in a final volume of 200 μl containing 100 μg total RNA, DNase (1 unit/μl) and 10 x DNase buffer (400 mM Tris-HCl (pH 7.5), 100 mM NaCl, 60 mM MgCl₂). After a 30 min incubation at 37°C, the reaction was stopped by adding 20 μl termination mix (0.1 M EDTA (pH
8.0), 1 mg/ml glycogen). The reaction was split into two 1.5 ml tubes. To each tube, 100 μl of saturated phenol and 60 μl chloroform were added followed by centrifugation at 16,170×g for 10 min at 4°C to separate phases. Top aqueous layer was carefully removed and placed in a 2.0 ml tube. To each tube, 1/10 volume of 2 M (10 μl) NaOAc and 2.5 volumes (0.3 ml) of 95% ethanol were added. The mixtures were vortexed thoroughly and incubated on ice for 10 min. The tubes were centrifuged in a microfuge tube at 16,170×g for 15 min at 4°C. The pellets were washed with 100 μl of 80% ethanol followed by centrifugation at 16,170×g for 5 min at 4°C. The supernatant was removed and the pellet was air-dried. The pellets were dissolved in 50 μl RNase-free H₂O.

**Poly A+ RNA Enrichment and Probe Synthesis**

**Streptavidin Magnetic Bead Preparation**

Magnetic beads were re-suspended by inverting and gently tapping the tube. Aliquots containing 15 μl of beads per probe synthesis reaction were transferred into 0.5 ml tubes. Beads were separated on magnetic particle separator. The supernatant was pipetted off and discarded. Beads were washed with 150 μl of 1x binding buffer [109] and separated again on a magnetic particle separator. The supernatant was pipetted off and discarded. The cycle was repeated three times. The beads were resuspended in 15 μl 1x binding buffer per reaction.

**Poly A+ RNA Enrichment**

The PCR thermal cycler was preheated to 70°C. Aliquots of 10-50 μg total RNA were transferred into 0.5 ml tubes. Deionized H₂O was added to make up a
final volume of 45 µl. To each tube, 1 µl Biotinylated Oligo (dT) was added followed by incubation at 70°C for 2 min in the preheated thermal cycler. The reaction was cooled at room temperature for 10 min. To each tube, 45 µl 2x binding buffer was added. The washed beads were resuspended by pipetting up and down. Fifteen µl of beads were added to each RNA sample and mixed on a vortexer or shaker for 30 min at room temperature. Beads were separated using the magnetic separator. The supernatant was pipetted off and discarded. Beads were resuspended in 50 ul 1x wash buffer. Beads were again separated and washed once. Beads were resuspended in 6 µl dH2O.

cDNA Probe Synthesis

A master mix containing 5X reaction buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂), 10x dNTP mix (5 mM each dCTP, dGTP, dTTP), [α-³²P] dATP (3000 Ci/mmol, 10 µCi/µl, 5 µl/reaction) and 10 mM DTT was prepared. One µl cDNA synthesis primer mix [109] was added to the resuspended beads. The beads and primer mix were incubated in the preheated thermal cycler at 65°C for 2 min. Meanwhile, 2 µl MMLV reverse transcriptase per reaction was added to master mix. To each reaction tube, 13.5 µl master mix was added followed by thorough mixing. Tubes were incubated at 50°C for 25 min. The reactions were stopped by adding 2 µl 10x termination mix (0.1 M EDTA [pH 8.0], 1 mg/ml glycogen).

Column Chromatography

The probe synthesis reaction mixture was diluted to a final volume of 200 µl with Buffer NT2 [109]. The sample was added to a NucleoSpin Extraction Spin
Column and centrifuged at 16,170xg for 1 min. The NucleoSpin column was inserted into a fresh 2-ml collection tube and 400 μl Buffer NT3 [109] was added to the column. After centrifugation at 16,170xg for 1 min, the collection tube and the flow-through were discarded. The wash with Buffer NT3 was repeated twice. The NucleoSpin column was transferred to a clean 1.5-ml microcentrifuge tube. One hundred μl of Buffer NE [109] was added to the column for 2 min followed by centrifugation at 16,170xg for 1 min to elute purified probe. The radioactivity of the probe was checked by scintillation counting.

**Hybridization Procedure**

The hybridization solution (BD ExpressHyb + sheared salmon testes DNA heated at 95°C for 5 min) was prewarmed at 68°C. The membrane was prehybridized for 30 min with continuous agitation at 68°C. To prepare the probe for hybridization, 5 μl Cot-1 DNA [109] was added to the entire pool of labeled probe followed by incubation in a boiling (95–100°C) water bath for 2 min. Next, the probe was incubated on ice for 2 min. The probe was added to the membrane and hybridized overnight with continuous agitation at 68°C.

The next day, the array membrane was washed with continuous agitation at 68°C with Wash Solution 1 (2X SSC, 1% SDS; 3 X 30 min washes) and Wash Solution 2 (0.1X SSC, 0.5% SDS; 1X 30 min wash). The final 5-min wash was performed in 200 ml of 2X SSC with agitation at room temperature. The blot was wrapped with plastic and exposed to phosphorimager plates at room temperature.
Analysis

The phosphor imager plates were scanned after a 3 day exposure to radio-active blots. Atlas Image software (Clontech, Palo Alto, CA) was used to analyze the scanned blots. According to manufacturer's instructions, the signals from cDNA spots were normalized to a panel of three house-keeping genes (GAPDH, cytoplasmic β-actin, 60S ribosomal protein L13A) on the same blot. The software takes into account the changes in the level of multiple house-keeping genes and corrects the test gene signals accordingly. This provides a more accurate normalization of test genes as compared to using a single house-keeping gene for normalization. Three housekeeping genes that were relatively unaffected by the treatment were selected to increase the accuracy of normalization. The ratios of normalized gene-expression values for CytB treated and untreated control were prepared. Arbitrarily, either more than a 2-fold increase or more than 50% inhibition in gene-expression were considered significant. The genes that failed to show a signal in either of the blots or showed a near-background signal were excluded from the analysis.

Western blot analysis

Western blot analysis was performed using alkaline phosphatase or enhanced chemiluminescence detection as described below.

Electrophoresis

Since PAI-1 is secreted upon synthesis, conditioned media in which the cells were cultured was used to detect changes in PAI-1 protein. Equal amounts of conditioned media were analyzed. After a 1:10 dilution of conditioned media in
1X sample buffer (0.12M Tris 1.51 g, 2% SDS, 10% glycerol, pH 6.8), 20 µl of diluted samples per lane were electrophoresed on reducing polyacrylamide gels at 4°C. Proteins were separated first on 4% stack gel at 50 V for 30 min and then on 11% resolving gel at 200 V for 90 min. The gels were run in tank buffer (Tris: 3.04 g/l, Glycine 14.4 g/l, SDS 1 g/l) at 4°C. An HRP-labeled molecular weight marker, MagicMark (Invitrogen, Carlsbad, CA) was run on each gel along with the samples. The samples were transferred onto PVDF overnight at 15 V in cooled (4°C) Western blot buffer (Tris 3 g/l, Glycine 14.5 g/l, methanol 20%).

**Immunodetection**

**Alkaline-Phosphatase Detection**

After overnight transfer, membranes were washed five times with dH₂O to remove excess methanol. Membranes were blocked overnight with 5% milk in high salt-TBS (Tris 20 mM, NaCl 500 mM). After two 10-min washes with TBS-Tween (Tris 20 mM, NaCl 500 mM, Tween 20 0.05%, pH 7.5), membranes were incubated one hour each at room temperature, first with goat anti-PAI-1 antibody (American diagnostica, Stamford, CT) diluted 1:500 in TBS/Tween and then with a biotinylated secondary anti-goat antibody (Pierce, Rockford, IL) diluted 1:500 in TBS-Tween. Between and after incubations with antibodies, membranes were washed four times for 15 min each with TBS-Tween. After washing, PAI-1 antigen was detected using the Vectastain ABC system (Vector laboratories, Burlingame, CA). Blots were incubated for one hour with Vectastain ABC reagent for alkaline phosphatase detection. Blots were washed twice for 15 min each wash with high salt TBS-Tween and once for 15 min with high salt TBS. After
briefly washing membranes with BCIP-NBT buffer (Tris 0.1 M, MgCl2 0.5 mM, pH 9.5), BCIP-NBT detection reagent (Bromo Chloro Indolyl Phosphate-Nitrosozym Blue in BCIP-NBT buffer) was added to the membrane. After a sufficient amount of signal was obtained, blots were washed with dH2O and air-dried.

**Enhanced Chemiluminescence Detection**

After overnight transfer, membranes were washed five times with dH2O to remove excess methanol. Membranes were blocked for one hour with NAP-Sure Blocker (Geno Technology, St. Louis, MO). Membranes were incubated one hour each at room temperature, first with Goat Anti-PAI-1 antibody (American diagnostica, Stamford, CT) diluted 1:1000 in TBS/Tween and then with horse radish peroxidase (HRP)-labeled secondary anti-goat antibody (Jackson Immunoresearch, West Grove, PA) diluted 1:20,000 in TBS/Tween. Anti-tPA antibody (PAM-3 clone, American diagnostics, Stamford, CT) was used at 1:200 dilution and anti-uPA antibody (American diagnostics, Stamford, CT) was used at 1:1000 dilution. HRP-labeled secondary antibodies were used at 1:20,000 dilution. Between blocking and incubations with antibodies, membranes were washed once for 15 min and twice for 5 min with TBS/Tween (Tris 20 mM, NaCl 500 mM, Tween 20 0.05%, pH 7.5) At the end of the incubation with the secondary antibody, membranes were washed once for 15 min and four times for 5 min with TBS/Tween. Substrate buffer from Enhanced Chemiluminescence Western blotting analysis system (Amersham Biosciences, Piscataway, NJ) was added to the blots for one minute to detect HRP-signal on the membrane. X-ray
films were exposed to the blot for varying amounts of time to detect optimum signal from the blot.

**Analysis**


**Northern Blot Analysis**

Northern blot analysis for PAI-1 mRNA was performed using partial a PAI-1 cDNA as probe (Clontech, Palo Alto, CA).

**Electrophoresis**

Ten μg total mRNA was incubated with 5.4 μl 6M glyoxal, 16 μl DMSO, 3.0 μl 0.1M Sodium Phosphate at 50°C in heat block for 1 hr. Samples were cooled to 0°C on ice. After adding 4 μl loading dye, samples were run on a 1.2 % agarose gel with ethidium bromide in running buffer (1.9 ml 1M NaH₂PO₄, 6.1 ml 1M Na₂HPO₄, 990 ml DEPC H₂O) at 50 V for 30 min to allow all the samples to migrate out of the well and into the gel. Next, the gel was run at 75 V for approximately 3 hours with buffer circulation. A digital photograph of the gel was taken using Eagle Eye II (Stratagene, LA Jolla, CA) under UV light to document 18S/28S loading in each lane. RNA was transferred onto positively-charged nylon membrane (Roche, Indianapolis, IN) overnight in 1X TAE (Tris 4.84 g/l, Glacial Acetic Acid 1.14 g/l, EDTA 0.744) at 25 V and 4°C.
UV Cross linking

After overnight transfer, the membranes were air dried for 10 min. RNA was cross-linked to membranes using UV cross-linker 1800 (Stratagene, La Jolla, CA) at 120 m Joules. Hybridization was then performed or the membrane was stored at −20°C for probing at a later time.

Hybridization

The blots were incubated at 42°C for 1 hr to overnight in pre-hybridization solution (5% Formamide, 5X SSPE, 5X Denhardt’s, 0.1 mg/ml Salmon Testes DNA, 0.5% SDS) in a Techne Hybridizer HB-2D hybridization oven (Burlington, NJ). After sufficient pre-hybridization, 25-50 ng/2 μl PAI-1 cDNA probe (0.5 μl in case of 18S fragment) (Clontech, Mountain View, CA) was brought up to 15 μl using DEPC H2O. Probe was denatured in boiling H2O for 15 min. While probe was denaturing, the following reagents from Random Primer DNA Labeling System (Invitrogen, Carlsbad, CA) were combined in a final volume of 49 μl on ice: 2 μl dATP, 2 μl dGTP, 2 μl dTTP, random primer buffer solution (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM MgCl2, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD260 units/ml oligodeoxyribonucleotide primers, pH 6.8), 32P-dCTP (approximately 50 μCi or 20 μCi for the 18S probe). The denatured probe was immediately transferred to ice and the probe added to the preassembled labeling mix. To the probe mix, 1 μl Klenow fragment (3U/μl) was added and mixed gently. Probe synthesis was allowed at 37°C for 30 min and then stopped with 5 μl stop buffer (0.5 M EDTA, pH 8.0). Radio-labeled probe was denatured by
boiling at 100°C for at least 10 min. While the probe was being labeled, the pre-
hybridization solution was replaced by 10 ml hybridization solution per blot (50% 
Formamide, 5X SSPE (NaCl 43.8 g/l, Na phosphate (mono) 6.9, EDTA 1.9 g/l, 
pH 7.4), 5X Denhardt's (Ficoll 1 g/l, polyvinylpyrrolidone 1 g/l, BSA 1 g/l), 0.11 
mg/ml Salmon Testes DNA, 0.5% SDS, 4% Dextran Sulfate). Radio-labeled 
probe was carefully added to the tube and the probe was hybridized with the blot 
in hybridization oven overnight at 42°C.

Washing
After overnight hybridization, blots were washed twice for 10 min each with 100 
ml 5X SSPE (NaCl 43.8 g/l, Na phosphate (mono) 6.9, EDTA 1.9 g/l, pH 7.4) and 
0.5 % SDS to remove non-specific binding. Blots were washed for 5 min at 65°C 
in 0.5% SSPE and 1% SDS. If the blots were still radioactive at the edges, an 
additional wash with 0.1X SSPE and 1% SDS was performed for 5 min at 65°C. 
The blots were wrapped in saran wrap and exposed to a phosphor-imaging 
cassette (Molecular Dynamics-Amersham, Piscataway, NJ). For PAI-1 mRNA 
detection, the phosphorimager plates were scanned after 2-3 days.

The membranes were stripped before probing for 18S. The blots were 
washed in 50-100 ml nearly boiling DEPC H2O for 15 min 2-3 times.

18S Hybridization
The membranes were probed with radio-labeled 18S fragment using the 
Northern blot protocol described above. The blots were exposed to phosphor 
imager plates for 30 min to few hours.
Analysis

The blots were scanned using phosphor imager scanner. The densitometric analysis for PAI-1 bands was performed using Image Quant Software (Molecular Dynamics-Amersham, Piscataway, NJ).

Immunocytochemistry

Adherent cells on coverslips were immunofluorescently stained using a two-day protocol. For PAI-1 staining, cells were plated on serum-coated dishes in serum-free media.

At the end of desired period of treatment, cells were washed 3 times with 2 ml Hank’s balanced salt solution. Cells were fixed with 2 ml 3.7% paraformaldehyde in PBS (monobasic sodium phosphate 0.5 g/l, dibasic sodium phosphate 2.3 g/l, NaCl 8.5 g/l) at room temperature for 10 min. Paraformaldehyde was washed off with 3x 2 ml washes with TBS. Cells were permeabilized with 2 ml 0.5% Triton X-100 in TBS (Tris 25 mM, NaCl 0.9%, pH 7.5) for 5 min at room temperature, after which they were washed 3x with 2 ml TBS. Cells were incubated with 5% blocking milk (centrifuged at 13,370 G for 1 hour) in TBS for at least one hour at room temperature. Coverslips were incubated with primary goat anti-PAI-1 antibody diluted 1:100 in milk-TBS overnight at 4°C in a humidified chamber.

After overnight incubation, coverslips were washed 1x with milk and 2x with TBS for 10 min each. Coverslips were incubated with secondary antibody Biotinylated anti-Goat diluted 1:100 in milk-TBS for 2 hr at 37°C in a humidified box. Excess antibody was washed off once with milk and twice with TBS for 10 min.
min each. Coverslips were incubated with mixture of FITC-avidin and Texas red phalloidin diluted 1:200 in TBS at 37°C for 1 hr. Cells were washed 3x with TBS briefly and then once with 1 μM TBS-Biotin (10 ml TBS/ 100μl 1mM biotin). Finally after washing once with dH2O, excess water was drained off. Coverslips were air dried and mounted on glass slide with 25 μl gelvatol.

Images were captured using a Spot digital camera attached to a fluorescent microscope.

**Quantitative Real-time Polymerase Chain Reaction (PCR)**

**Principle of Quantitative real-time PCR**

In real-time PCR, amplification of DNA is monitored during each cycle allowing accurate quantitative estimation of nucleic acid concentration. The cycle number at which the signal starts to increase exponentially over the background is called the crossover point. Comparison of crossover points of samples with those of known concentrations of standard cDNAs gives the concentration of a particular template in the sample. Relative quantification with external standards as a method for comparing gene expression was used in this study. In this method of quantification, a reference, typically a house-keeping, gene is used to normalize the concentration of the target gene. First, the concentrations of target and reference genes are obtained by using an external standard curve of known amounts of nucleic acid. The results are expressed as target/reference ratio. The use of an endogenous control, corrects for the factors affecting PCR efficiency. Following is the protocol used in this study.
RNA Isolation and Quantification

Total cellular RNA was isolated using TRIzol method as described earlier and quantified using a UV spectrophotometer.

DNAse treatment

The protocol for real time PCR analysis was modified from a previous protocol [110]. Briefly, 1 µg total RNA sample was DNAse treated and subsequently reverse transcribed using specific anti-sense primers for PAI-1 and Ubiquitin (UBC, reference gene) in the same reaction (Table 2). In a typical reaction, a total of 4 µg mRNA was treated with 4 µl of 1 U/µl DNAse I (Invitrogen, Carlsbad, CA) in the presence of 2 µl of 10 x reaction buffer in a total reaction volume of 20 µl. After a 15-minute incubation at room temperature, DNAse treatment was stopped by incubating the mixture at 65°C for 10 min with 2 µl of 25 mM EDTA. DNAse-treated total RNA was stored at -80°C until further use in the reverse transcription (RT) reaction.

RT reaction

DNAse treated mRNA was reverse-transcribed using a thermocycler (MJ Research, Reno, NV). The test gene and housekeeping gene were reverse-transcribed simultaneously using their specific anti-sense primers in the same reaction. Performing RT for both genes, the gene of interest and the reference gene, in the same reaction minimizes the error due to the efficiency of the RT reaction and hence provides a good way of normalizing for a reference gene in the RT reaction. 1 µg of DNAse treated RNA (1 µg/5 µl) was reverse transcribed in a final reaction volume of 25 µl. The reaction mix also contained RT buffer
(Promega, Madison, WI), 60 U RNAse inhibitor, 3 μM anti-sense primer for sample gene, 3 μM anti-sense primer for Ubiquitin, 10 μM DTT and 500 μM dNTPs. Reverse transcription reaction was performed at 37°C for one hour in a thermocycler followed by heat inactivation of the enzyme at 70°C for 10 min. RT products were immediately used for real-time PCR analysis on the Roche lightcycler PCR instrument.

**Preparation of cDNA standards for real time PCR**

The standards for the quantification were prepared as described previously [110], by amplifying 10-fold serial dilutions of known concentrations of templates for each gene. Briefly, cDNA was prepared for each gene from 1 μg human mesangial cell total RNA using specific anti-sense primers and amplified using a PCR reaction for 35 cycles. PCR products from 5 identical reactions for the same gene were pooled in a single tube. An aliquot of PCR products was run on 8% acrylamide to confirm the product size. PCR products were purified using “High pure PCR products purification kit” (Roche Diagnostics, Indianapolis, IN). The concentration of purified PCR products was measured using picoGREEN dsDNA quantification kit (Molecular probes, Eugene OR) [111]. Briefly, a 2 μg/ml stock solution of lambda dsDNA standard was prepared in TE (10 mM Tris-HCl, 2 mM EDTA, pH 7.5). The stock solution was diluted in duplicate vials with TE to provide 0, 1.25, 2.5, 6.25, 12.5, 18.75 and 25 μg/μl concentration of standard. Duplicate vials containing 1 μl or 2 μl of test sample in a final volume of 1000 μl TE were prepared. To each vial 1 ml of PicoGreen dsDNA quantitation reagent [111] was added for 5 min. The vials were incubated in dark. After incubation,
fluorescence from the standards and the samples were measured using a fluorometer using an excitation wavelength of 480 nm and emission wavelength of 520 nm. The values for standard concentrations were plotted on a graph. The concentration values for samples (µg/µl) were derived using the linear graph equation.

After fluorometric measurement, the concentration of purified product was adjusted to 5000 pg/µl. Ten-fold serial dilutions of PCR products were prepared to cover a range from 0.001 pg to 1 ng for the standard curve. These serial dilutions were amplified on lightcycler using specific parameters for each gene. Melting curve analysis was performed at the end of the PCR run to ensure the purity of the product and to eliminate the possibility of primer-dimers. Roche Lightcycler software was used to prepare a standard curve. The identities of PAI-1 and ubiquitin cDNAs were confirmed by sequencing (Midland Sequencing, Midland, TX).

Real-time PCR

After diluting RT products 1:10 in dH2O, 2 µl of diluted sample was amplified using the LightCycler (Roche, Indianapolis, IN). Each gene of interest and the reference gene were analyzed in separate glass capillaries. The reactions were set up in the capillaries in a final volume of 20 µl, which contained 2 µl of standard or diluted sample, MgCl2 (4 mM for PAI-1 or uPA, 1 mM for tPA), 1 µM each of both the primers (table 2) and 10 µl of 2x SYBR green PCR mix (Qiagen, Valencia, CA). The hot start method was used to initiate PCR reaction.
Table 2. Primer sequences for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer (5'-3')</th>
<th>Anti-Sense Primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1</td>
<td>TGCTGGTGGAATGCCCTCTACT</td>
<td>CGGTCATTCCCAGGTTCCTCTA</td>
</tr>
<tr>
<td>UBC</td>
<td>ATTTGGTGTCGCGGTCTTG</td>
<td>TGCCCTTGACATTTCTCGATGGT</td>
</tr>
<tr>
<td>TPA</td>
<td>CCCAAGCAGATTTGCAC</td>
<td>GCCTTTCGACTGCTTTCTGAA</td>
</tr>
<tr>
<td>uPA</td>
<td>CACGCAAGGGGAGATGAA</td>
<td>ACAGCATTGGTGCTGACTT</td>
</tr>
</tbody>
</table>

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by heating the samples at 95° C for 15 min prior to PCR cycling. The following cycling parameters were used for the amplification: denaturation at 95° C for 15 s, annealing at 60° C (PAI-1, uPA) or at 56° C (tPA) for 5 s, and extension at 72° C for 18 s. Samples were amplified for 35 (PAI-1, tPA) or 40 cycles (uPA). Gene amplification was monitored in real-time with SYBR green dye. The crossing points of sample genes were compared against the crossing points of known standards to determine the absolute concentration of a gene in a particular sample. Values for the gene of interest were normalized to Ubiquitin amplified from the same sample. At the end of PCR cycling, the melting curve of the products was analyzed to ensure the specificity of the reaction. PCR products from representative experiments were run on the gel and visualized by ethidium bromide staining to further ensure the specificity of the reaction.

Table 2 shows the primers used for real-time PCR analysis of PAI-1, tPA, uPA and Ubiquitin. The primers for PAI-1, uPA and Ubiquitin were purchased from a commercial vendor (Midland Reagents, Midland, TX) using previously published primer sequences [110]. The protocol for tPA detection was developed in our lab using the primers designed and synthesized by TIB Molbiol LLC (Adelphia, NJ).

**Melting curve analysis**

Melting curve analysis was performed at the end of each run to ensure specific amplification of a single product in three steps:

a. Rapid heating to 95° C to denature all the DNA present in the mixture
b. Cooling to below annealing temperature to allow formation of double stranded DNA.

c. Gradual heating to $95^\circ C$ to melt double stranded DNA

Melting curve analysis showed a single peak suggesting a pure population of PAI-1, tPA, uPA and UBC cDNA in PCR products. The PCR products were run on a gel to confirm the size of the product and to detect the purity of the reaction. Subsequently, products from representative experiments were run on a gel to ensure amplification of single template of expected size.

**Cloning of PAI-1 promoter**

**PCR cloning of the promoter**

The PAI-1 promoter containing major regulatory elements as listed in chapter 6 was cloned from human mesangial cells using specific primers. Human genomic DNA was isolated from primary culture human mesangial cells using a DNA isolation kit (Clontech, Palo Alto, CA). Primers for a 1106 bp promoter (-973 to +133) were purchased from a commercial vendor (Midland Reagents, Minland, TX) as published by Grenette et al [112].

Forward primer: 5'-CGATCGGTACCTAAAAGCACACCCTGCAAAC-3'

Reverse primer: 5'-CGATCAGATCTCAGAGGTGCCTTGCGATTG-3'

A total of three reactions were set up with 2 µg genomic DNA, 4 µg DNA or no DNA. The reactions were set up in a 100 µl reaction mix containing 150 pmol of sense and anti-sense primers, 1.5 mM MgCl$_2$ and 1 µl platinum taq polymerase. PCR reaction was performed for 30 cycles using the following parameters:
denaturation at 94°C for 45 seconds, primer annealing at 58°C for 45 seconds and primer extension at 72°C for 45 seconds. An additional 5-min incubation at 72°C was performed to complete the reaction. PCR products from all three reactions were run on a 1% agarose gel in TAE buffer (Tris 4.84 g/l, Glacial Acetic Acid 1.14 g/l, EDTA 0.74 g/l) to ensure the product size. Bands were seen at the expected sizes of 1100 bp. No bands were seen in the negative control, which contained no DNA.

**Ligation**

Next, pGL3 basic vector (restriction sites at 5 and 36) and PCR products (restriction sites in cloning primers) were cut with KpnI and Bgl II restriction enzymes. Digested fragments were cleaned with geneclean spin kit (Qbiogene, Carlsbad, CA). The approximate concentration of digested fragments was determined by comparing the densities of bands with the density of ladder. Sticky ends of PAI-1 promoter were ligated into pGL3 basic vector at an insert: vector ratio of 1:3 using either 2 µl or 4 µl DNA ligase (1 U/µl) in a final volume of 15 µl at room temperature overnight. Top 10 competent E. coli were transformed with pGL3 vectors to select the plasmid containing the PAI-1 promoter. The transformed Top 10 competent cells were grown on ampicillin plates overnight. Representative colonies of bacteria were inoculated into 10 ml of LB media (Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 5 g, dH₂O 1 L, pH 7.0) containing 125 µg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. The tubes were incubated on ice for 30 minutes. Bacteria were harvested by centrifugation at 1540 G for 15 min at 4°C. The pellet was washed.
in 1 ml ice-cold STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) followed by centrifugation at 2810xg for 15 min at 4°C. The pellet was resuspended in 300 μl of Buffer P1 (50 mM Tris, 10 mM EDTA, pH 8.0). Next, 300 μl of Buffer P2 (200 mM NaOH, 1 % SDS) was added with gentle mixing. Finally, 300 μl Buffer P3 (2.55 M Potassium Acetate adjusted to pH to 4.8 with acetic acid) was added with gentle mixing. The tubes were centrifuged at 15,000xg for 15 min at 4°C. The supernatant was transferred to a fresh, sterile microfuge tube. Plasmid DNA was precipitated by incubation with 800 μl of 100% isopropanol at room temperature for 30 minutes. The tubes were centrifuged at 16,000 G for 10 min at 20°C. The pellet was washed twice with 70% ethanol followed by centrifugation at 16,000xg for 10 min at 20°C. The pellet was dried under vacuum and resuspended in 100 μl of sterile dH2O containing 1 μl of RNAse A (1 μg/μl). The plasmid concentration was measured by measuring 260/280 absorbance with a UV spectrophotometer.

A restriction enzyme digest of the isolated plasmid with KpnI and BglII was set up to ensure that the isolated plasmid contained the desired PAI-1 promoter. The digested products were run on the gel. The preparation that showed a band corresponding to expected size of PAI-1 promoter (1106 bp) was presumed to be the successful cloning of PAI-1 promoter. Sequencing of the insert in this plasmid confirmed successful cloning of 1100 bp human PAI-1 promoter in pGL3 basic vector.
Transient Transfection

Human and rat mesangial cells were co-transfected with 1 µg pGL3 vector containing PAI-1 promoter and 1 µg renilla luciferase vector per well (control for transfection efficiency). Control cells were co-transfected with empty pGL3 basic vector and renilla luciferase vectors. Fugene 6 (Roche, Indianapolis, IN) was used to transfct cells in 6-well plates with PAI-1 promoter. Cells were transfected at 60-80% confluence with 1 µg PAI-1 promoter-containing plasmid and 1 µg renilla luciferase plasmid. The cells were serum-starved at the time of transfection. First, 4 µl Fugene 6 per µg DNA was incubated with 100 µl serum-free media/well for 15 minutes. Next, 2 µg DNA/well was added to the tube. The resulting transfection mixture was incubated at room temperature for 30 min. Meanwhile, the cells were washed and the media was changed to 1 ml fresh serum-free media. To each well 100 µl Fugene-DNA complexes were added drop wise. After three hours of transfection, CytB was added to appropriate wells. After 24 hours, cells were treated with 10 ng/ml TGFβ for 6 hours.

Luciferase Reporter Assay

Upon completion of the experiments, transfected cells were washed 3 times with ice-cold PBS and 150 µl 1X passive lysis buffer (Promega, Madison, WI) was added to each well. Plates were wrapped in Parafilm and frozen at -80°C for at least 1 hour. Cells were scraped after thawing the plates at room temperature for 3 min. Lysates were centrifuged at 13,370xg for 1 min at 4°C to remove cellular debris. After equilibrating to room temperature, 20 µl of supernatant was added quickly to 100 µl of reporter assay reagent containing
firefly luciferase substrate. Luciferase activity was measured by using TD 20/20 luminometer (Turner Diagnostics, Sunnyvale, CA) set for dual luciferase detection mode. The sensitivity of the detection was maintained at manufacturer's default level (50.9%) for all the assays. The samples were read after a delay of 2 seconds to allow luciferase activity to begin. The samples were read for a total of 15 seconds. Stop n Glo buffer (Promega, Madison, WI) was added to stop the firefly luciferase activity and to provide substrate for the renilla luciferase activity. The activity of renilla luciferase was measured on the luminometer using the same parameters as firefly luciferase on luminometer. In rat cells, the values for firefly luciferase activity for each sample were normalized to the values for renilla luciferase for the same sample. In human cells, the values for renilla luciferase were not detected above the baseline and hence a different method of normalization was necessary. For human cells, protein concentration of each sample was determined by BCA protein assay kit (Pierce, Rockford, IL). Luciferase activity was normalized for the protein concentration of each sample to get the activity in counts/mg protein for each sample. For each treatment, cells were transfected at least in duplicates.

Development of chronic glomerulonephritis in experimental animals

The following protocol was approved by the institutional animal care and use committee of Eastern Virginia Medical School under the title “Prevention of glomerulosclerosis by rho-kinase inhibition” (Approval # 02-010).
Production of injury

Repeated Injections of anti-thymocyte serum (ATS) were used to induce chromic glomerulonephritis in experimental rats. In three experimental groups, six male Sprague-Dawley rats were injected with 25 mg/kg sheep anti-thymocyte serum (30 mg/ml) through tail vein injections. Four weekly injections of ATS were given to the animals. The ATS injections were immediately followed by injection of 1.2 ml normal sheep serum as a source of complement. The doses of ATS were adjusted each week according for the weight gain. A control group (Group 1) of six animals was injected with equal volumes of normal saline.

Treatment

Out of the three groups that received ATS-complement, one group did not receive any treatment and hence served as the injury group (Group 2). The remaining two of the three ATS-complement groups received 30 mg/kg/day HA-1077 in drinking water. The concentration of the drug in the drinking water was determined according to the average weight of the animal and average daily water consumption. Within the two treatment groups, one group (Group 3) was treated from one day before the first injection to see if the treatment prevents the onset of the injury, whereas the other group (Group 4) was treated one week after the injury to see if the progression of the disease was altered. The animals were fed normal rat diet and were allowed to drink ad lib. After four weekly injections, animals were treated and observed for 6 more weeks (total of 4+6=10 weeks) to allow progression of the chronic disease. At the end of the tenth week, animals were killed and samples were collected for analyses.
Study groups

1: Normal saline injection alone

2: ATS-complement injection

3: ATS-complement+ HA-1077 treatment started 1 day before the first injection

4: ATS-complement+HA-1077 treatment started 1 week after the first injection

Sample collection

Urine

After 24-hour acclimatization in metabolic collection chambers, urine samples representing next 24-hour urine collection were collected one day before the first injection and at the end of 2, 4, 6, 8, 10 weeks. The first two collections showed contamination by food particles and hence, the food was removed from the chambers for the later set of collections.

Blood pressures

The tail-cuff method was used to measure blood pressure at the end of the study.

Serum

At the end of the study animals were anesthetized with intraperitoneal injection of sodium pentobarbital (Nembutal). Blood was collected from abdominal aorta for serum preparation.
Tissue samples

Kidneys were harvested in ice-cold Hanks's balanced salt solution. Animals were killed by removal of the heart. One kidney from each animal was pooled with others from the same group for isolation of glomeruli through differential sieving method. Most of the glomeruli (90%) were suspended in Trizol for isolation of RNA and the remaining glomeruli were suspended in electrophoresis sample buffer for SDS-PAGE and Western Blot analysis. The second kidney was bisected and one half was cut into thin slices, some of which were fixed in paraformaldehyde (4% in PBS) overnight, and then snap frozen for immunohistochemistry. The remaining slices were fixed in 10% buffered formalin for paraffin section preparation. The other half was cut into multiple thin slices and snap frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) for immunohistochemistry or laser capture microdissection (LCM).

Histology and Immunohistochemistry

The sections from formalin-fixed tissue were stained with mason trichrome staining to visualize ECM deposition. The sections from snap frozen tissue were stained with α-SMA antibody as described in immunocytochemistry section.

Statistical Analysis

The statistical significance of the results was determined using Student's paired t-test. A p-value of <0.05 was considered statistically significant. Experimental variation is reported as standard error of the mean in the bar graphs (except where indicated differently).
CHAPTER 3

PROFILING THE EFFECTS OF ACTIN CYTOSKELETON ON EXPRESSION OF MULTIPLE GENES

Introduction

As described in chapter 1, studies in this lab showed that changes in actin polymerization regulate α-SMA expression and hypertrophy in cultured mesangial cells suggesting that the actin cytoskeleton may regulate myofibroblast differentiation [28]. The role of the cytoskeleton as an important regulator of gene expression has also been supported by many other studies. Studies by Folkman and Moscona on cell-spreading [33], and Ingber on mechanical transduction and cell functions [34, 113, 114] provided early evidence for the role of actin cytoskeleton in the gene regulation. More recently specialized actin cytoskeleton locations have been identified for integration of signaling such as JNK signaling [39-42]. The importance of localization of mRNA in effective protein translation has also become evident [43]. The collective evidence suggests that the actin cytoskeleton is a highly specialized network of fibers that precisely regulate multiple cellular responses. But the precise regulatory pathways and mechanisms have not been characterized. Also the list of genes that are affected by actin polymerization is far from complete.

The regulatory effect of actin cytoskeleton on α-SMA expression in myofibroblast differentiation and the evidence from the literature suggested that
other genes could also be regulated in a similar manner. Also, the inhibition of hypertrophy suggested that multiple genes could be simultaneously regulated by the actin cytoskeleton. It was therefore hypothesized that other genes associated with the myofibroblast phenotype are also regulated by changes in actin polymerization. The aim of the next experiment was to identify the effect of the actin cytoskeleton on a panel of genes related to myofibroblast differentiation.

**Experimental model**

Recently, DNA arrays consisting of multiple genes spotted on a glass coverslip or a nylon membrane have become available to facilitate rapid and high throughput analysis of large populations of genes. Commercially available arrays contain functionally related genes on the same array allowing a highly relevant, focused, and time- and cost-effective analysis of gene expression.

Since myofibroblasts are largely responsible for extracellular matrix production in fibrotic diseases, one such array (cell interaction array) containing genes related to cell-interaction and extracellular matrix was used to study the role of actin cytoskeleton in myofibroblast differentiation. The Cell Interaction array (Clontech, Palo Alto, CA) contains a total of 265 cDNAs immobilized on a nylon membrane. The genes on this Cell-Interaction array include extracellular matrix proteins; cell-cell adhesion receptors; matrix adhesion receptors; growth factors, chemokines, cytokines and their receptors; neuropeptides; cell surface antigens; extracellular transporters and carrier proteins; metalloproteinases; serine proteases; protease inhibitors, oncogenes and tumor suppressor genes; intracellular transducers, effectors and modulators; G proteins; cell-cycle related
proteins and house-keeping genes covering a broad range of relevant gene population. A detailed list of these genes can be found on the internet at http://www.clontech.com/clontech/atlas/genelists/7746-1_HuCellInt.pdf. The gene expression profile of cells with depolymerized actin cytoskeleton (CytB treated cells) was compared with that of cells containing intact actin cytoskeleton (untreated serum-starved cells) as described in Chapter 2.

Results

Actin depolymerization affects expression of multiple genes

Between control and CytB-treated cells a total of 74 genes were detected on a 265-gene cell-interaction array, out of which 65 genes were detected in both the conditions and 9 genes were detected under only control conditions (Fig. 6). Using an arbitrarily defined criteria of either more than a 2-fold increase or more than 50% inhibition as a significant change, 10 genes were decreased and 4 genes were increased upon CytB treatment. All 9 genes expressed only under control conditions were excluded from the final analysis since their expression was very close to the base-line. Table 3 shows the ratio of signal intensities in CytB treated cells and untreated control cells in serum-free media. The genes that decreased after CytB treatment, mainly represented extracellular matrix components or other molecules that favor increased matrix deposition. In contrast, 3 of the 4 genes that increased after CytB treatment, decrease matrix deposition either by direct fibrolysis (tPA, MMP11) or by inhibiting pro-fibrotic growth factors (decorin). These changes suggest that the overall effect of actin
Fig. 6. DNA array analysis of effect of actin depolymerization on gene expression. Serum-starved human mesangial cells were treated with 1.0 µM CytB for 24 hours in serum-free media. $^{32}$P-labeled gene-specific probes prepared from cellular poly A mRNA were hybridized with the membrane overnight. After washing, membranes were exposed to phosphorimager plates for 3 days. Scanned blots were analyzed by AtlasImage software for differences in gene expression (n=1). The top blot represents gene expression profile of cells in serum-free media, whereas the bottom panel represents gene expression profile of cells treated with 1.0 µM CytB. The panel on left shows house-keeping genes and negative control spots. The spots corresponding to PAI-1 (oval) and tPA (rectangle) genes are highlighted for comparison on both the blots.
Table 3. Summary of changes in gene expression on DNA array analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>CytB:S- ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Genes showing decreased expression after Cytochalasin B treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Endothelial plasminogen activator inhibitor-1 precursor (PAI-1)</td>
<td>0.093</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 5 precursor (IGFBP5)</td>
<td>0.210</td>
</tr>
<tr>
<td>Fibronectin receptor alpha subunit (FNRA)</td>
<td>0.310</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 3 precursor (IGFBP3)</td>
<td>0.314</td>
</tr>
<tr>
<td>Tumor metastatic process associated protein (NM23)</td>
<td>0.326</td>
</tr>
<tr>
<td>Fibronectin precursor (FN)</td>
<td>0.339</td>
</tr>
<tr>
<td>Hypoxanthine-guanine phosphoribosyltransferase (HPRT)</td>
<td>0.370</td>
</tr>
<tr>
<td>Collagen 3 alpha 1 subunit precursor (COL3A1)</td>
<td>0.382</td>
</tr>
<tr>
<td>Collagen 1 alpha 2 subunit precursor (COL1A2)</td>
<td>0.393</td>
</tr>
<tr>
<td>Collagen 4 alpha 2 subunit precursor (COL4A2)</td>
<td>0.397</td>
</tr>
<tr>
<td><strong>B. Genes showing increased expression after Cytochalasin B treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Tissue-type plasminogen activator precursor (tPA)</td>
<td>2.840</td>
</tr>
<tr>
<td>Bone proteoglycan II precursor (PGS2), decorin (DCN)</td>
<td>3.250</td>
</tr>
<tr>
<td>Junction plakoglobin (JUP); desmoplakin 3 (DP3)</td>
<td>3.875</td>
</tr>
<tr>
<td>Matrix metalloproteinase 11 (MMP11), stromelysin 3</td>
<td>9.000</td>
</tr>
</tbody>
</table>
depolymerization favors increased matrix degradation and decreased matrix accumulation. The net effect may be a decrease in extracellular matrix.

**Confirmation of DNA array results using Northern and Western Blotting**

The results obtained by the initial screening experiments required both confirmation and more accurate quantification of the changes using conventional methods such as Northern blot analysis. The changes in protein expression were also examined to confirm similar effects on mRNA and protein expression. Out of 14 genes identified by the array experiment, PAI-1 and tPA, the members of plasminogen activator system, are particularly relevant to the overall objective of understanding mechanisms of myofibroblast-like differentiation and regulation of ECM accumulation. PAI-1 is a natural inhibitor of the plasminogen activation by tPA. CytB not only decreases PAI-1 expression, but also increases tPA expression. These changes may result in increased and unopposed actions of tPA leading to more activation of plasminogen into plasmin. Activated plasmin in turn degrades ECM. Consequently, the balance between tPA and PAI-1 determines the rate of ECM turnover. A relative increase in tPA leads to increased degradation of ECM. In contrast, a relative increase in PAI-1 leads to inhibition of ECM degradation and increased ECM deposition as seen in renal fibrosis. Such an increase in PAI-1 expression and resulting ECM deposition are associated with myofibroblast-like activation of mesangial cells. Thus, changes in tPA and PAI-1 expression on gene array are directly pertinent to myofibroblast differentiation and fibrosis.
Therefore, the changes in PAI-1 and tPA expression seen on DNA array were confirmed first using conventional methods such as Northern blot and Western blot analyses. mRNAs from cells treated with actin depolymerizing agents CytB (direct inhibitor of actin polymerization) and Y-27632 (inhibitor of signal-mediated actin polymerization) were analyzed. The Northern blot analysis showed two PAI-1 transcripts at 2.8 kb and 3.2 kb positions resulting from two polyadenylation sites in the PAI-1 gene [115]. The results showed that in presence of serum (S+ conditions), inhibition of actin polymerization with CytB decreased PAI-1 mRNA expression in a time and dose dependent manner (Fig. 7). The effect of CytB was apparent at 24 hours and continued at 48 and 72 hours (Fig. 7A). The dose response shows that the minimum dose of CytB required to produce a visible effect on PAI-1 mRNA was 0.4 μM at 24 hours. CytB caused >50% inhibition of PAI-1 mRNA expression at 1 μM concentration (Fig. 7B). We used 1 μM (0.5 μg/ml) CytB for the rest of the study. In serum-starved cells, inhibition of Rho-signaling by 10 μM Y-27632 also inhibited PAI-1 mRNA expression in a time-dependent manner (Fig. 7C). The effect was evident as early as 4 hours and continued at 8, 24 and 48 hours. Similar inhibitory effects of actin depolymerization on PAI-1 protein expression were confirmed in the laboratory by other investigators. An increase in tPA expression by actin depolymerization as shown in the array experiment was also confirmed at mRNA and protein levels by Northern blot and Western blot analyses respectively in the lab.
Fig. 7. Effect of actin depolymerization on PAI-1 mRNA expression in human mesangial cells. (A) Time-course of effect of 1.0 µM CytB (8, 24, 48 and 72 hours) on PAI-1 mRNA expression in serum-fed cells by Northern blot analysis (n=1). CytB treatment blocks PAI-1 expression at 24, 48 and 72 hours. (B) Dose-response analysis of effect of CytB (0.1, 0.2, 0.4, 1 and 2 µM) on PAI-1 mRNA expression in serum-fed cells by Northern blot analysis (n=1). CytB concentrations of 0.4, 1 and 2 µM block PAI-1 mRNA expression. (C) Time-course of effect of 10 µM Y-27632 (8, 24, 48 and 72 hours) on PAI-1 mRNA expression in serum-starved cells by Northern blot analysis (n=1). Y-27632, an inhibitor of Rho-kinase that blocks Rho-mediated actin polymerization, blocks PAI-1 mRNA expression at 24, 48 and 72 hours.
In summary, the DNA array analysis showed that actin depolymerization coordinately regulated cell-interaction and ECM-related genes in human mesangial cells. The inhibition of PAI-1 and increase in tPA expression were confirmed by Northern blot and Western blot analyses. To further investigate the mechanisms of actin cytoskeleton mediated regulation of PAI-1 and tPA expression in pathophysiological context, TGFβ treatment was used as a model system as described in the next chapter.
CHAPTER 4
EFFECT OF TGFβ ON PAI-1 EXPRESSION

Introduction

Results of the array experiment and subsequent confirmation with Northern and Western blot analysis showed that actin depolymerization changed PAI-1 and tPA in opposite directions. PAI-1 was studied first and in more detail than tPA because PAI-1 expression has been correlated with myofibroblast differentiation [19, 25]. Moreover, PAI-1 acts as the gatekeeper of the fibrolytic cascade regulating the activity of plasminogen activators including tPA. Therefore, functionally changes in PAI-1 expression are more directly related to tissue fibrosis.

Since transforming growth factor β (TGFβ) is the major regulator of PAI-1 expression in disease, the ability of actin depolymerization to antagonize its effect on PAI-1 expression was examined. Also, TGFβ was chosen for further studies particularly since it plays a central role in regulating myofibroblast differentiation and tissue fibrogenesis [116-120]. In the kidney, expression of TGFβ by mesangial cells increases extracellular matrix (ECM) production and inhibits matrix degradation [88, 89, 91, 121]. The net result is an increase in ECM deposition and glomerulosclerosis [90, 122]. Consequently, inhibition of TGFβ signaling was shown to protect against renal fibrosis [88, 123]. It was therefore important to understand the pathways and mechanisms involved in
TGFB-induced fibrosis. Experimentally, serum-free culture conditions were selected to allow the study of TGFB without other serum-factors.

The overall experimental approach was to induce PAI-1 expression by TGFB and to determine the effects of changes in actin polymerization on PAI-1 expression in basal as well as TGFB-induced states. Since the effects of growth factors can vary according to cell-type and cell-culture conditions; the effective dose, effective time period and the magnitude of effect of TGFB on PAI-1 expression in human mesangial cells were determined first.

**Experimental model**

Primary cultures of human mesangial cells between passages 5 and 9 (P5-P9) were used in the study. Initially, the cells were cultured in RPMI media containing 16.7% fetal bovine serum (FBS). Cells were serum-starved at 60-80% confluence. After 24 hours of serum starvation, TGFB was added to the cells. The effects on PAI-1 expression were examined at the indicated time-points. Serum-free media, being free of other growth factors, allows the study of effects of TGFB alone. Also, serum starvation increases the stress fiber content of the cells which is consistent with myofibroblast-like phenotype [26]. The effects of inhibition of actin polymerization can be easily compared to untreated control cells that have abundant stress fibers. A culture in serum-free media helps in studying the regulation of basal expression of PAI-1, where as treatment with TGFB allows the study of induced PAI-1 expression.

To determine the optimum dose of TGFB and the optimum duration of treatment, human mesangial cells were treated with increasing doses of TGFB
for 0, 1, 2, 4, 8, 24 and 48 hours. At each time point, supernatants (conditioned media) were stored for detection of PAI-1 protein and the cells were lysed in TRIzol for RNA isolation. PAI-1 protein was detected from conditioned media using Western blot analysis with enhanced chemiluminescence detection, whereas PAI-1 mRNA was detected using quantitative real-time RT-PCR.

Results

Quantification of PAI-1 mRNA using real-time PCR analysis

PAI-1 mRNA from cultured human mesangial cells was successfully detected by a reverse transcription reaction in a thermal cycler (MJ Research, Reno, NV) followed by a quantitative real-time PCR analysis on Roche Lightcycler (Roche Diagnostics, Indianapolis, IN). The Lightcycler instrument measures fluorescence emitted by SYBR Green I, a double-stranded DNA binding dye, at the end of each cycle during the log-linear phase of the PCR and generates an amplification curve by plotting changes in fluorescence intensities against cycle number (Fig. 8A, Fig. 9A). The cycle at which the fluorescence signal rises above the background signal is called the crossing point, which is directly proportional to the concentration of the target DNA. The crossing point for an unknown sample can be compared to crossing points of known standards for quantification of sample concentration. The values for PAI-1 concentration were derived in picograms, which were further normalized to similarly obtained values for a reference gene, ubiquitin (UBC), in each sample. Ubiquitin was selected as the reference or house-keeping gene because it is expressed constitutively in the cells and its level did not change upon the experimental treatments (refer to table...
Fig. 8. PAI-1 standard curve for quantitative real-time PCR analysis. (A) Amplification of PAI-1 plotted as fluorescence vs. cycle number. Each sample is represented in a uniquely colored line on the real-time PCR software, allowing tracing of amplification of individual samples. (B) PAI-1 standard curve plotted as cycle number vs. log concentration showing a linear standard curve ($r = -1$). (C) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing only one PCR product corresponding to PAI-1. (D) Melting curve analysis data plotted as changes in fluorescence signal over time ($dF/dT$) vs. temperature. Presence of only one melting peak in this reaction corresponding to melting temperatures of PAI-1 (~85.5°C) indicates specificity of the amplification reaction and absence of primer-dimers.
4). Also it is expressed at relatively lower levels, which is a desired property for real-time PCR analysis allowing detection of smaller changes in the expression unlike genes that are too abundant in the cells such as 18S.

Standard curves for PAI-1 and UBC were developed by performing real-time PCR on known concentrations of purified cDNA (Fig. 8, Fig. 9). For every experiment, one purified standard cDNA (0.01 pg for PAI-1 and 100 pg for UBC) with similar crossing point as a representative experimental sample was also run with the samples. The sample concentrations were calculated by the software in a two-step process. First, the software compared the crossing point of the known standard in each experiment with the previously saved standard curve to account for the PCR efficiency. Next, the concentrations of the unknown samples were derived by comparing the sample crossing point to the standard crossing point.

Following completion of the PCR, a melting curve analysis of PCR products was performed by the instrument to confirm the purity of the reaction. Melting curve analysis measures changes in fluorescence intensities as the temperature is gradually increased. Separation of double stranded DNA with increasing temperature caused an initial gradual fall in the fluorescence signal followed by a precipitous fall (Fig. 8C, Fig. 9C). When these changes in fluorescence intensity with time were plotted against the temperature, a melting peak corresponding to the maximum changes in signal intensity (dF/dT) at melting temperature (Tm) was seen (Fig. 8D, Fig. 9D). The number of melting peaks in each reaction indicates the number of target sequences being amplified. A unique peak at the melting temperature of the target sequence indicated
**Fig. 9. UBC standard curve for quantitative real-time PCR analysis.** (A) Amplification of UBC plotted as fluorescence vs. cycle number. Each sample is represented in a uniquely colored line on the real-time PCR software, allowing tracing of amplification of individual samples. (B) UBC standard curve plotted as cycle number vs. log concentration showing a linear standard curve ($r = -1.00$, Slope = -3.54). (C) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing only one PCR product corresponding to UBC. (D) Melting curve analysis data plotted as changes in fluorescence signal over time ($dF/dT$) vs. temperature. Presence of only one melting peak in this reaction corresponding to melting temperatures of UBC (~82°C) indicates specificity of the amplification reaction and absence of primer-dimers.
absence of non-specific amplification and primer-dimers in the reaction. As an added quality control measure to ensure the size of the product and to rule out non-specific amplification, the PCR products from standard curve samples and representative experimental samples were run on 8% agarose gel (Fig. 10, Fig. 11D). Gel electrophoresis was used as a quality control tool only to visualize the number and size of products in each reaction. It was not used for quantification of mRNA.

Figures 8 and 9 show standard curves for real-time PCR analysis of PAI-1 and UBC respectively. Figure 10 shows gel electrophoresis of PCR products from PAI-1 and UBC PCR standard curves.

**TGFβ dose-response on PAI-1 mRNA expression**

The effect of TGFβ on PAI-1 mRNA were studied using real-time quantitative PCR analysis with lightcycler PCR instrument. Figure 11 shows a representative real-time monitoring of PAI-1 and UBC amplification at the end of each cycle in panel A. Panels B and C represent melting curve analysis of PAI-1 and UBC at the end of PCR cycling showing a single peak each for both the genes. Panel D show gel electrophoresis of PCR products showing a single product for each gene correlating with the expected band size.

To determine the effective dose of TGFβ, human mesangial cells were serum-starved at 60-80% confluence. After a 24-hour serum-starvation, 1, 2, 5, 7.5 or 10 ng/ml TGFβ was added to serum-free media for 8 hours. Total cellular RNA was isolated using TRIzol method. The changes in PAI-1 mRNA expression were detected using quantitative real-time RT-PCR analysis. In two separate
Fig. 10. Gel-electrophoresis of standard curve PCR products. Aliquots of PCR products (2 µl out of 20 µl total) were run on 8% acrylamide gel followed by ethidium bromide staining. (A) PAI-1 and (B) UBC PCR products show a single band at expected base-pair location confirming specific amplification of respective templates.
Fig. 11. Representative images from quantitative real-time PCR of PAI-1 and UBC from experimental samples. (A) Amplification of PAI-1 and UBC plotted as fluorescence vs. cycle number. Each line is represented in different color on the real-time PCR software, allowing tracing of amplification of individual samples. The crossing points for both PAI-1 and UBC fall within the bars marking approximately cycles 15 and 22. (B) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing two PCR products corresponding to PAI-1 and UBC. (C) Melting curve analysis data plotted as changes in fluorescence signal over time (dF/dT) vs. temperature. Presence of only two melting peaks in this reaction corresponding to melting temperatures of UBC (~82°C) and PAI-1 (~85.5°C) indicate specificity of the amplification reaction and absence of primer-dimers. (D) Gel electrophoresis of PCR products from representative experimental samples show a single PCR product of expected base-pair size in each reaction.
experiments, treatment with 1, 2, 5, 7.5 and 10 ng/ml TGFβ increased average PAI-1 mRNA expression to 381.9%, 323.1%, 443.3%, 485.1% and 287.9% of untreated control respectively (Fig. 12A).

**TGFβ dose-response on PAI-1 protein expression**

To determine the effective dose of TGFβ on PAI-1 protein expression, human mesangial cells were serum-starved at 60-80% confluence in 2-wells per condition in 6-well plates. After 24-hour serum-starvation, 1, 2, 5, 7.5 or 10 ng/ml TGFβ was added to serum-free media for 24 hours. Conditioned media was analyzed for changes in PAI-1 protein using Western blot analysis. In a single experiment, treatment with 1, 2, 5, 7.5 and 10 ng/ml TGFβ changed average PAI-1 protein expression to 125.9%, 100.9%, 79.3%, 133.6% and 283.6% of untreated control respectively (Fig. 12B). The maximum increase in PAI-1 protein was seen with 10 ng/ml TGFβ. Although TGFβ showed an effect at lower concentrations, it was inconsistent. Fan et al. previously showed that renal tubular epithelial-myofibroblast transdifferentiation in vitro required TGFβ at concentrations between 10 to 50 ng/ml [119]. Others have also shown 10 ng/ml TGFβ as an effective dose [124, 125]. The effectiveness of higher concentration of TGFβ correlates with high serum TGFβ levels seen in patients with chronic and end-stage renal disease [126].

Since 10 ng/ml TGFβ showed consistent effects on both mRNA and protein expression, that dose was used in the remaining experiments. Previously, Motojima et al. also reported 10 ng/ml TGFβ to be the most effective dose for the induction of PAI-1 in rat mesangial cells [82].
**TGFB time-course of PAI-1 mRNA and protein expression**

To determine the optimum length of time of TGFβ-treatment for maximum PAI-1 mRNA and protein expression, human mesangial cells were serum-starved at 60-80% confluence and after 24-hours of serum starvation, 10 ng/ml TGFβ was added to serum-free media for 1, 2, 4, 8, 24 and 48 hours. At each time point, conditioned media were saved for protein analysis and cells were used for RNA analysis. In three separate experiments, treatment with 10 ng/ml TGFβ for 1, 2, 4, 8, 24 and 48 hours changed PAI-1 mRNA expression to 180.4±22.8%, 186.0±30.7%, 191.0±23.5%, 100.1±13.5% and 90.6±8.7% of respective time-matched controls (Fig. 13A). At the same time, the basal expression of PAI-1 mRNA at 2 and 4 hours decreased to 90.9±10.9% and 59.7±9.4% (p<0.05) of 0-time point control. At 8, 24 and 48 hours, basal PAI-1 mRNA expression changed to 105.0±7.5%, 134.7±14.7% and 146.2% of 0-time point control.

In the same set of experiments, the effect on PAI-1 protein expression was examined using Western blot analysis at 8, 24 and 48 hours in conditioned media. PAI-1 expression could not be detected at 8 hours. At 24 hours TGFβ increased PAI-1 protein to 424 ± 104.6% of time-matched control (Figure 13B). At 48 hours, the increase in PAI-1 protein was 181.9% and 131.7% in two separate experiments (average 158%). Thus, 10 ng/ml TGFβ increased PAI-1 mRNA expression.
Fig. 12. Dose-response analysis of TGFβ induction of PAI-1 expression.
Serum-starved human mesangial cells were treated with 0, 1, 2, 5, 7.5 and 10 ng/ml TGFβ for 8 (mRNA) or 48 (protein) hours (A) PAI-1 mRNA expression measured by quantitative real-time PCR analysis shows an increase with TGFβ treatment. Each bar represents the average increase at corresponding concentration normalized to untreated control (n=2). The ends of error bars show value for each experiment. (B) PAI-1 protein expression was measured in conditioned media using Western blot analysis with chemiluminiscence detection. Treatment with 10 ng/ml TGFβ increased PAI-1 protein expression to 283.6% of untreated control (n=1).
expression maximally and consistently at 8 hours, whereas it increased PAI-1 protein expression maximally at 24 hours. The maximum increase in PAI-1 protein expression at 24 hours is consistent with previous studies by Matsumoto et al [127].

In summary, the results of time-course and dose-response analysis of TGFβ on PAI-1 expression showed 10 ng/ml TGFβ as the effective dose and, 8-hour and 24-hour as effective treatment duration for mRNA and protein studies respectively. The remaining studies were performed using these time points.
Fig. 13. Time-course analysis of TGFβ induction of PAI-1 expression.
Serum-starved mesangial cells were treated with or without 10 ng/ml TGFβ for indicated lengths of time. (A) Changes in PAI-1 mRNA were detected using quantitative real-time PCR analysis (n=3 except 48 hours: n=2). (B) Changes in PAI-1 protein expression were measured in conditioned media using Western blot analysis with chemiluminescence detection at 24 (n=3) and 48 (n=2) hours. The vertical bars and the error bars represent the mean PAI-1 values and SEM respectively. The value of either 0-hour (A) or time-matched (B) untreated control is set at 100%. *: p<0.05 compared to time-matched control. †: p<0.05 compared to 0-hr time-point.
CHAPTER 5
EFFECT OF CHANGES IN ACTIN POLYMERIZATION ON TGFβ-INDUCED PAI-1 EXPRESSION

Introduction

The results of earlier chapter confirmed induction of PAI-1 by TGFβ in human mesangial cells. The role of actin cytoskeleton in this induction was examined next. Previous studies in this lab showed that changes in actin cytoskeleton regulate α-SMA gene expression, a marker of myofibroblast differentiation, and hypertrophy in mesangial cells [28]. Like α-SMA expression, de novo expression of PAI-1 is also seen with myofibroblast differentiation. It is possible that the expression of PAI-1 is also regulated in a cytoskeleton-dependent manner similar to that of α-SMA.

The Rho family of small GTPases controls organization of the actin cytoskeleton [27]. Rho activates Rho-kinase, a serine-threonine kinase that acts through downstream kinases to stabilize actin polymers e.g. stress fibers and adhesion. The small GTPases, Rho and Rac are both involved in the regulation of PAI-1 expression. Inhibition of Rho by the C3 exoenzyme decreased PAI-1 production in rat proximal tubules [105] and aortic endothelial cells [106]. In chicken atrial cells, co-transfection with dominant-negative RhoA decreased PAI-1 promoter activity, whereas dominant-active RhoA increased promoter activity [107]. In smooth muscle cells, Y-27632, a Rho-kinase inhibitor, attenuated expression of PAI-1 in response to Angiotensin II (AngII) [81]. These suggest a
role of Rho GTPases as intracellular mediators of diverse stimuli leading to PAI-1 expression. Rho has also been shown to regulate TGFβ-mediated gene expression. In human mesangial cells, inhibition of Rho and its downstream effector Rho-kinase blocked TGFβ-mediated expression of α1 collagen, a component of ECM [87]. It is possible that Rho may play a similar role in regulating TGFβ-mediated expression of PAI-1.

Since regulation of actin polymerization is a major function of the Rho family, the working hypothesis that Rho-mediated changes in actin polymerization modulate TGFβ-induced PAI-1 expression was examined. If Rho-signaling worked through actin cytoskeleton for regulation of PAI-1 expression, inhibition of actin cytoskeleton would produce similar results as the inhibition of Rho.

Experimental model

Pharmacologic agents that stabilize or destabilize the polymeric actin cytoskeleton by acting at different levels (Fig. 4) were used to test the hypothesis that Rho GTPase regulates TGFβ-induced PAI-1 expression through changes in actin polymerization. The treatment conditions included (a) inhibition of signal-mediated actin polymerization by inhibiting Rho GTPase (Clostridium difficile Toxin B, 10 pM) or its downstream effector Rho-kinase (HA-1077 (20 μM), Y-27632 (10 μM)) [87, 128, 129]; (b) inhibition of actin polymerization by agents directly binding to actin polymers (Cytochalasin B, 1 mM) or monomers (Latrunculin B, 0.1 μM) [130, 131]; and (c) increase in polymeric actin content by
stabilizing the actin polymers (Jasplakinolide, 50 nM) [132]. The final outcome was either an increase or a decrease in the actin polymers in the cells.

**Results**

**Actin depolymerization inhibits TGFβ–induced PAI-1 mRNA expression**

First, the effect of actin depolymerizing agents on TGFβ-induced PAI-1 mRNA expression was examined (Fig. 14). Either Toxin B (Fig. 14A), a direct inhibitor of Rho, or Y-27632 (Fig. 14B) and HA-1077 (Fig. 14B), both inhibitors of a downstream effector of Rho, Rho kinase (ROCK), were used to inhibit Rho-mediated actin polymerization. ToxinB, Y-27632 and HA-1077 decreased basal PAI-1 mRNA expression to 19.5±5.6%, 52.8±5.4%, 59.3±17.9% of untreated control respectively, and decreased TGFβ–induced PAI-1 expression to 30.1±7.6%, 51.1±6% and 55.8±15.6% of TGFβ-treated control respectively (Fig. 14A-C). Direct inhibition of the actin polymerization by CytB and LatB decreased basal PAI-1 expression to 29.9±3.3% and 45.3±16% of untreated control respectively, and decreased TGFβ–induced PAI-1 expression to 35.2±6.5% and 49.7±16.5 of TGFβ–treated control respectively (Fig. 14D, E). Northern blot analysis (Fig. 14G) confirmed these results. This observation, that the effects of Rho-inhibition are mimicked by direct actin depolymerization, suggests that the effects of Rho on PAI-1 expression are mediated by the changes in actin cytoskeleton.
**Fig. 14. Effect of actin polymerization on TGFβ-induced PAI-1 mRNA expression.** Serum-starved mesangial cells were treated with (A) 10 pM Toxin B, (B) 10 μM Y-27632, (C) 20 μM HA-1077, (D) 1 mM CytB, (E) 0.1 μM LatB or (F) 50 nM JAS for 1-hour prior to 8-hour treatment with 10 ng/ml TGFβ (n=3). The changes in PAI-1 mRNA were measured using real-time PCR analysis (A-F) and Northern blot analysis (G). The vertical bar and the error bars represent the mean PAI-1 mRNA values and SEM for the experimental condition. The value for untreated control is set at 100%. The value for untreated control is set at 100%. *:p<0.05, **:p<0.01 and ***:p<0.005 compared to S- control. †: p<0.05 and †††: p<0.005 compared to TGFβ treated control. (G) Representative Northern blot showing inhibition of basal and TGFβ-induced PAI-1 expression with actin depolymerization (n=2).
Actin depolymerization inhibits TGFβ-induced PAI-1 protein expression

To ensure that the changes in PAI-1 mRNA expression were reflected at the level of protein expression, the effect of the actin-depolymerization on PAI-1 protein expression was examined using Western blotting.

The effect on cellular PAI-1 protein was examined first (Fig. 15A). Cell-lysates scraped in 1x sample buffer were used to detect changes in PAI-1 protein. Serum-starved mesangial cells were pre-treated with CytB for one hour before 24 or 48 hour treatments with TGFβ. TGFβ had no apparent effect on PAI-1 expression at 24 or 48 hours. This is possibly due to saturation of PAI-1 binding sites (vitronectin) on the substratum during prior culture [133]. CytB blocked PAI-1 expression in both basal and TGFβ-treated conditions. At 24 hours, CytB blocked basal and TGFβ-treated PAI-1 expression to 14.4% and 38.8% of their respective controls. At 48 hours, CytB blocked basal and TGFβ-treated PAI-1 expression to 4.2% and 46.1% of their respective controls.

Active PAI-1 is rapidly secreted from the cells upon synthesis [25]. Therefore, the conditioned media was examined for changes in PAI-1 protein expression in response to TGFβ. In the same experiment, changes in PAI-1 levels were clearly apparent in conditioned media (Fig. 15B). At 24 hours, TGFβ increased PAI-1 expression to 273% of control. CytB blocked basal and TGFβ-induced PAI-1 expression to 7.9% and 20.7% of their respective controls. At 48 hours, TGFβ increased PAI-1 expression to 217% of control. CytB blocked basal and TGFβ-induced PAI-1 expression to undetectable levels.
Fig. 15. Effect of TGFβ and actin depolymerization on PAI-1 protein expression. Serum starved mesangial cells were pre-treated with 1 mM CytB prior to 24-hour treatment with 10 ng/ml TGFβ. Cell-lysates (A) and Conditioned media (B) were analyzed for PAI-1 detection using Western blot analysis (n=1). An increase in PAI-1 upon TGFβ treatment is seen in conditioned media (B) but not in cell lysates (A). CytB blocks PAI-1 expression, under basal and TGFβ-treated conditions, in both cell lysates and conditioned media.
Next, the effect of a panel of actin cytoskeleton inhibitors on TGFβ-induced PAI-1 expression was examined using conditioned media (Fig. 16). The changes in PAI-1 protein expression were consistent with the changes seen in mRNA. Densitometric analysis of the Western blots showed that TGFβ maximally stimulates PAI-1 expression to 304.3±31.4% of control at 24 hours. Toxin B, Y-27632, HA-1077, CytB and LatB inhibited basal PAI-1 expression to 31.7±4.4%, 42.2±7.8%, 31±3.7%, 37.3±5.6% and 44.4±7.8% of control respectively, and inhibited TGFβ-induced PAI-1 expression to 26.8±7%, 38.6±4.9%, 33.6±6.9%, 21.1±4.1% and 31±8.3% of TGFβ-treated control respectively. A representative Western blot is shown in Fig. 16A. These results suggest that agents with actin depolymerizing activity blocks TGFβ-induced PAI-1 expression regardless of their mechanism of action.

**Immunocytochemical analysis of effect of actin depolymerization on TGFβ–induced PAI-1 expression**

In culture, secreted PAI-1 binds to vitronectin on the pericellular substratum and shows characteristic intracellular and extracellular distribution [134]. Indirect immunofluorescence microscopy was used to visualize the effects of Y-27632 and CytB on PAI-1 deposition on the substratum (Fig. 17). These cells were plated and stained at relatively low density. Thus the substratum is not saturated with PAI-1 and it is possible to visualize the effect of TGFβ on PAI-1 protein expression.

Cells were counterstained with Texas Red-phalloidin to allow concurrent examination of filamentous actin (F-actin) content. PAI-1 staining was seen
Fig. 16. Effect of Actin depolymerization on TGFβ-induced PAI-1 protein expression. Serum-starved mesangial cells were treated with 1 mM CytB, 0.1 μM LatB, 10 μM Y-27632 or 20 μM HA-1077 for 1-hour prior to 24-hour treatment with 10 ng/ml TGFβ. The effect of actin depolymerization on PAI-1 protein expression was measured in conditioned media using Western blot analysis (n=3). (A) A representative Western blot showing increase in PAI-1 expression with TGFβ treatment in serum-free media. (B) Inhibition of actin polymerization by agents using different mechanisms blocks basal and TGFβ-induced PAI-1 expression. The vertical bars and the error bar represent mean values for PAI-1 protein and SEM respectively. The value for untreated control is set at 100%. *: p<0.05 compared to S- control, **: p <0.005 compared to S- control, †: p <0.05 compared to TGFβ treated control.
Fig. 17. Effect of Actin depolymerization on TGFβ-induced PAI-1 expression by immunocytochemistry. Serum-starved mesangial cells were treated with 1 mM CytB (E, F) or 10 μM Y-27632 (G, H) before 24-hour treatment with 10 ng/ml TGFβ (C-H). Cells were stained with Texas-Red phalloidin to visualize stress fibers (arrow) (A, C, E, G) and PAI-1 antibody (arrowhead) (B, D, F, H). Phalloidin staining shows abundant stress fibers (red staining) in S- control (A) and TGFβ treated (C) cells. TGFβ treated cells show more intense staining for PAI-1 (green staining) (D) compared to S- control (B). Loss of stress fibers with cytoskeleton inhibitors (E, G) correlates with loss of staining for PAI-1 (G, H) (n=1).

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intracellularly and on the substratum. TGFβ increased staining for PAI-1 after 24-hour treatment. Y-27632 and CytB reduced stress fiber content and staining for PAI-1 in both basal and TGFβ-stimulated conditions (Fig. 17). Thus PAI-1 expression changes in parallel with changes in actin polymerization.

**Stabilization of actin cytoskeleton increases TGFβ-induced PAI-1 expression**

The results showed that inhibition of actin polymerization inhibited basal as well as TGFβ-induced PAI-1 expression. If the actin cytoskeleton controls PAI-1 expression, then an increase in actin fibers may increase PAI-1 expression. To test the possibility that stabilization of actin polymers can increase PAI-1 expression, serum-starved cells were pre-treated for 1 hour with Jasplakinolide (JAS), an agent that increases the F-actin content of cells by directly binding to actin polymers and increasing nucleation. Cells were treated with 10 ng/ml TGFβ for indicated time periods, with and without JAS. In contrast to the actin depolymerizing agents, JAS increased basal and TGFβ-mediated PAI-1 mRNA expression to 154.3±14% and 142.7±10.3% of respective controls as determined by quantitative real-time RT PCR analysis (Fig. 14F). To ensure that the effects of JAS were mediated by increased F-actin content, cells were co-treated with JAS and actin depolymerizing agents in the presence or absence of TGFβ (Fig. 18). Co-treatment with CytB, Y-27632 or Toxin B depolymerized actin cytoskeleton and antagonized the JAS-mediated increase in PAI-1 mRNA to 24.6±4.6%, 52.6±8.9% and 39.1±8.7% of their respective JAS-treated controls. CytB, Y-27632 or Toxin B also blocked JAS-mediated increase in TGFβ-induced
Fig. 18. Effect of changes in actin polymerization on TGFβ-induced PAI-1 mRNA expression. Serum-starved mesangial cells were treated with 50 nM JAS and, 1 mM CytB (A), 10 µM Y-27632 (B) or 10 pM Toxin B (C) for one hour prior to 8-hour treatment with 10 ng/ml TGFβ (n=3). PAI-1 mRNA expression was measured using quantitative real-time PCR analysis. The vertical bars and the error bars represent mean PAI-1 mRNA values and SEM respectively. The value for untreated control is set at 100%. *: p<0.05 compared to S-, **: p<0.01 compared to S-, ***: p<0.005 compared to S-, †: p<0.05 compared to TGFβ, †††: p<0.005 compared to TGFβ, ‡: p<0.05 compared to JAS, #: p<0.05 compared to TGFβ+JAS, ###: p<0.005 compared to TGFβ+JAS.
Fig. 19. Effect of JAS and Toxin B mediated changes in actin polymerization on TGFβ-induced PAI-1 protein expression. Serum-starved mesangial cells were treated with 50 nM JAS and 10 pM Toxin B for one hour prior to 24-hour treatment with 10 ng/ml TGFβ. PAI-1 protein was measures in conditioned media using Western blot analysis. (A) Representative Western blot analysis of conditioned media for PAI-1 detection using Western blot analysis (n=3). (B) The vertical bars and the error bars represent mean PAI-1 protein values and SEM respectively. The value for untreated control is set at 100%. *: p<0.05 compared to S, †: p<0.05 compared to TGFβ, ‡: p<0.05 compared to JAS, #: p<0.05 compared to TGFβ+JAS.
increase in TGFβ-induced PAI-1 mRNA expression; decreasing PAI-1 mRNA to 17.9±1.1%, 51.3±14.8% and 19.1±10.5% of TGFβ+JAS- treated controls.

To confirm the effect of JAS on PAI-1 protein expression under the same conditions, cells were treated with combinations of TGFβ, JAS and actin depolymerizing agents for 24 hours. Western blot analysis of conditioned media showed JAS increased basal and TGFβ-induced PAI-1 protein to 148.5±5% and 148.1±7.1% of respective control at 24 hours. Inhibition of actin polymerization by Toxin B (Fig. 19), CytB (Fig. 20) and Y-27632 (Fig. 20) inhibited JAS-mediated increase in PAI-1 protein to 28.7±4.9%, 19.3±6.2% and 46.4±11.3% of their respective JAS-treated controls (Figs. 19, 20). At the same time, Toxin B, CytB and Y-27632 blocked JAS-mediated increase in TGFβ-induced PAI-1 protein expression to 18.9±3.4%, 13.1±2.9% and 29.1±7.6% of their respective TGFβ+JAS-treated controls (Figs. 19, 20).

Table 4 shows representative calculation of the changes in PAI-1 cDNA concentration by real-time PCR analysis. The software automatically calculates the concentration based on the crossing point of each sample. The values for PAI-1 expression were normalized to the values for UBC, which remained relatively unaffected by the experimental conditions.

In summary, the bi-directional changes in actin polymerization were followed in the same direction by the changes in TGFβ-mediated PAI-1 expression. An increase in stress fiber content of the cells caused an increase in PAI-1 expression, whereas inhibition of actin polymerization caused inhibition of
Fig. 20. Effect of changes in actin polymerization on TGFβ-induced PAI-1 protein expression. Serum-starved mesangial cells were treated with 50 nM JAS and, 1 mM CytB or 10 μM Y-27632 for one hour prior to 24-hour treatment with 10 ng/ml TGFβ. PAI-1 protein expression was measured in conditioned media using Western blot analysis. (A) Representative Western blot analysis of conditioned media for PAI-1 detection using Western blot analysis (n=3). (B) The vertical bars and the error bars represent mean PAI-1 protein values and SEM respectively. The value for untreated control is set at 100%. *: p<0.05 compared to S-, †: p< 0.05 compared to TGFβ, ‡: p<0.05 compared to JAS, #: p<0.05 compared to TGFβ+JAS.
### Table 4. Calculation of PAI-1 cDNA concentration by real-time PCR

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<tr>
<th>CONDITION</th>
<th>PAI-1</th>
<th>UBC</th>
<th>PAI-1 / UBC</th>
<th>% CONTROL</th>
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<tr>
<td></td>
<td>Cr.</td>
<td>Calc.</td>
<td>Cr.</td>
<td>Calc.</td>
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<tr>
<td></td>
<td>Pt.</td>
<td>(pg)</td>
<td>Pt.</td>
<td>(pg)</td>
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<td>0.0118</td>
<td>20.2</td>
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<tr>
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<td>19.9</td>
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<td>237.7</td>
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<tr>
<td>TGFβ + JAS</td>
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<td>0.0501</td>
<td>19.9</td>
<td>288.5</td>
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<tr>
<td>CytB</td>
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<td>19.8</td>
<td>306.4</td>
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<tr>
<td>TGFβ + CytB</td>
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<td>20.0</td>
<td>278.0</td>
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<td>0.0087</td>
<td>19.8</td>
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<tr>
<td>UBC standard</td>
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<td>-</td>
<td>21.5</td>
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Abbreviations are: Cr. Pt, crossing point; Calc. Con, calculated concentration.
PAI-1 expression. The inhibition of actin polymerization also prevented actin stabilization by JAS.
CHAPTER 6

EFFECT OF ACTIN CYTOSKELETON ON THE PAI-1 PROMOTER

Introduction

The results discussed in previous chapter showed that the changes in actin polymerization regulated PAI-1 mRNA and protein expression. The mechanism of this regulation was examined next.

The expression of PAI-1 is tightly regulated by a variety of cytokines, growth factors, hormones and other agonists [78]. These agents include TGFβ, Angiotensin II, thrombin, glucocorticoids, tumor necrosis factor-α and endotoxin [78, 135]. The regulation of PAI-1 mRNA expression is primarily at the level of transcription [136, 137]. Functional analysis of the regulatory region of the PAI-1 promoter shows the presence of multiple regulatory elements that respond to diverse stimuli listed above [79]. Schematic representation of selected consensus sequences in human PAI-1 promoter is shown in fig. 21 [69, 76, 115]. The human PAI-1 promoter has at least 3 AP-1 sites in its distal promoter region (-800 to -636) and one in the proximal promoter region (-81 to -75) [78]. These AP-1 sites are critical for induction of PAI-1 by the agents listed above [78, 138]. The regulation of PAI-1 promoter by TGFβ has been extensively studied [135, 78, 139]. Two TGFβ-inducible, cis-acting regulatory elements were first identified in the 5’ flanking DNA of the human PAI-1 gene; a relatively strong element between -791 and -328, and a weaker element between -328 and -187.
Fig. 21. Schematic representation of PAI-1 promoter. -RE, response element; TGFβ, transforming growth factor; VLDL, very low density lipoprotein; CTF, CCAAT box binding transcription factor; NF1, nuclear factor 1; PMA, phorbol 12-myristate 13-acetate; AP, activator protein; TRE, tetradecanoyl phorbol acetate response element; GRE, glucocorticoid response element. Used with permission from Binder et al [69].
Later, three SMAD-binding sites were identified between -794 and -532 that were sufficient for induction by TGFβ [140]. PAI-1 promoter also contains NF-1 like sequences, one p53 binding site between -139 and -160 [141], two glucocorticoid response elements (GREs) between -100 and +75, and -800 and -549 [76], CRE, five Hypoxia Responsive Elements (HREs) [142] and two Sp1 binding site-like sequences at -72 to -67 and -45 to -40 [143]. The rat PAI-1 gene shares many structural similarities with the human PAI-1 gene and contains similar regulatory elements in the promoter [144]. For example, the rat PAI-1 promoter contains binding sites for AP-1 and Sp1, glucocorticoids response elements and CRE. Although the rat and human PAI-1 promoter are similar, the number of each type of regulatory elements may be different. For example, the rat PAI-1 promoter contains five GREs as compared to two contained in the human PAI-1 promoter. Also, the rat PAI-1 promoter contains two regions that have at least 80% sequence similarities to its human counterpart. The first is between -91 and TATA box, and the second between -800 and -549.

The serum response factor (SRF), a transcription factor, has been shown to mediate effects of changes in actin cytoskeleton on expression of some genes [145]. AP-1-dependent genes are direct targets of SRF. For example, inhibition of actin polymerization by inhibitors of direct and Rho-mediated polymerization blocked inducible nitric oxide gene expression in human epithelial cells [145]. Similarly, inhibition of Rho by C3 toxin blocked activation of AP-1 in rat-1 cells [146]. TGFβ-mediated SMAD 2/3 activation also required Rho signaling in human breast carcinoma cells [147]. Thus, the evidence from the literature suggests a
role for Rho and actin cytoskeleton in regulation of genes through similar regulatory elements as contained in PAI-1 promoter. The hypothesis that Rho-mediated changes in actin polymerization regulate TGFβ-induced PAI-1 expression at the promoter level was therefore examined.

**Experimental Model**

To examine if PAI-1 expression was at the transcriptional level, the human PAI-1 promoter, spanning -973 to +133 nucleotides, was cloned into a luciferase vector as described in chapter 2. Human and rat mesangial cells were serum-starved and transfected with human PAI-1 promoter vector for 24 hours. Cells were then pre-treated with CytB to inhibit actin polymerization followed by treatment with TGFβ. The effect on promoter activity was determined by luciferase assay.

**Results**

**TGFβ induces PAI-1 promoter activity in rat mesangial cells**

The initial time-course analysis of the effect of TGFβ on PAI-1 promoter activity was conducted in rat mesangial cells due to their better transfection efficiency. Rat mesangial cells (p27) were co-transfected with PAI-1 promoter vector and renilla luciferase vector in serum-free media. After 24 hours, the cells were treated in triplicate wells with 10 ng/ml TGFβ (dose shown to be effective on PAI-1 mRNA and protein expression) for varying amounts of time to identify the time-point for the maximum increase in PAI-1 promoter activity. In a single experiment, TGFβ-treatment for 2, 4, 6, 8 and 24 hours increased PAI-1 promoter activity to 108.4±3.7% 124.2±7.7%, 128.5±3.1%, 109.0±3.9% and
Fig. 22. Time-course analysis of TGFβ on PAI-1 promoter activity. Rat mesangial cells were co-transfected with PAI-1 promoter plasmid and renilla luciferase plasmid in serum-free media. After 24 hours, 10 ng/ml TGFβ was added to the media for the indicated times. Duplicate wells of control cells in serum-free media and triplicate cells of TGFβ-tread cells were used for the experiment. Luciferase activities in samples were measured using dual luciferase assay (n=1). Each bar represent average luciferase activity for PAI-1 promoter normalized to renilla luciferase activity in the same sample. The normalized Luciferase activity of time-matched untreated control is set as 100%.
116.6±4.2% of their respective time-matched controls. The values for renilla luciferase activities were above background and hence were used to normalize for transfection efficiency.

The effect of TGFβ was maximum at 6-hours with the minimum variation. In the following set of experiments, cells were treated with TGFβ for 6 hours. **Inhibition of actin polymerization blocks TGFβ-induced PAI-1 promoter activity**

In rat mesangial cells, the effect of TGFβ on PAI-1 promoter (128.5%) was much smaller than the effects on PAI-1 mRNA (~250%) and protein (~250%) in human mesangial cells. Therefore the remaining experiments were performed in human cells. Serum-starved mesangial cells were transfected with PAI-1 and renilla luciferase plasmid as described above. After 24 hours, cells were pre-treated with 1 μM CytB followed by 10 ng/ml TGFβ for six hours. Since, co-transfection with renilla luciferase was not successful, luciferase counts were normalized to the protein concentration. Previously, Owens et al showed normalization for protein concentration as an acceptable method for normalization for transfection efficiency [148, 149]. The treatment with TGFβ increased PAI-1 promoter to 293.6±36.5% of control. CytB inhibited basal as well as TGFβ-induced PAI-1 expression to 45.1±6.1% and 34.6±12.7% of their respective controls. The effects of other cytoskeleton inhibitors could not be determined due to limited transfection efficiency.
Fig. 23. The effects of TGFβ and actin depolymerization on human PAI-1 promoter activity. Human mesangial cells were co-transfected at 60-80% confluence with 1 μg human PAI-1 promoter-containing plasmid and 1 μg renilla luciferase plasmid. The cells were serum-starved at the time of transfection. After three hours of transfection, CytB was added to appropriate wells. After 24 hours, cells were treated with 10 ng/ml TGFβ for 6 hours. Luciferase activity was normalized for the protein concentration of each sample to get the activity in counts/mg protein for each sample. For each treatment, cells were transfected at least in duplicates (n=3). Each bar represent average luciferase activity for PAI-1 promoter normalized to cellular protein concentration. The normalized Luciferase activity of untreated control is set as 100%. *: p<0.05 compared to S-, †: p< 0.05 compared to TGFβ.
CHAPTER 7

EFFECTS OF TGFβ AND ACTIN CYTOSKELETON ON PLASMINOGEN ACTIVATORS

Introduction

The effects of TGFβ and actin cytoskeleton on PAI-1 expression were determined as shown in chapters 4 to 6. Next, the effects of cytoskeleton disruption on two other components of plasminogen activator system namely tissue- (tPA) and urokinase- (uPA) type plasminogen activators were examined.

Two activators (tPA, uPA) and one inhibitor (PAI-1) of plasminogen activation constitute the plasminogen activator system, which regulates ECM degradation. The mesangial extracellular matrix determines the physical, mechanical and functional properties of glomerulus in normal and pathological conditions [57]. Plasminogen activators convert inactive plasminogen into active plasminogen. Plasmin activates matrix metalloproteinases (MMPs), which then digest ECM. Plasmin itself has some matrix digesting activity. PAI-1 binds to tPA and uPA, and blocks the activation of plasminogen into plasmin. Thus, tPA and uPA favor ECM degradation whereas PAI-1 favors ECM deposition. The balance between plasminogen activators and PAI-1 thus determines the rate and the amount of ECM deposition.

The DNA array analysis showed a 2.8-fold increase in tPA expression upon actin depolymerization (Table 3, Chapter 3), suggesting a coordinated regulation of the plasminogen activator system by the actin cytoskeleton. The
effects on uPA still remained to be examined. Moreover, treatment with TGFβ is shown to have varying effects on the expression of plasminogen activators depending on the cell-type [61, 124, 150-152]. The following set of experiments was therefore performed to understand the role of TGFβ and the actin cytoskeleton on the regulation of plasminogen activator system as a whole.

**Experimental Model**

Serum-starved human mesangial cells were treated with 10 ng/ml TGFβ for 4, 8, 24 and 48 hours to determine optimum treatment duration. The changes in tPA and uPA mRNA expression were examined by real-time quantitative PCR analysis. In subsequent experiments cells were pre-treated with CytB, LatB or Y-27632 for one hour to inhibit actin polymerization, followed by 8-hour treatment with TGFβ. These treatment conditions were identical to those used to examine the effects on PAI-1 expression in earlier chapters. The effect of TGFβ and actin depolymerization on tPA and uPA expression was determined at the mRNA level. Real-time PCR analysis was used to detect changes in tPA and uPA mRNA expression. The changes in tPA and uPA protein expression were examined in conditioned media at 24 hours to match the experimental conditions used for PAI-1 protein using Western blot analysis.

**Results**

**Quantification of tPA and uPA mRNA expression by real-time PCR analysis**

The existing literature show conflicting evidence about production of tPA and uPA by human mesangial cells and the regulation of plasminogen activator system by TGFβ [61, 89, 152-158]. Therefore, the expression of uPA and tPA...
mRNA by serum-starved mesangial cells was confirmed first prior to examining the effect of TGFβ on tPA and uPA expression. Using real-time PCR detection, both uPA and tPA mRNAs were successfully detected in human mesangial cells. Briefly, a reverse transcription reaction on thermocycler (MJ Research, Reno, NV) was followed by a quantitative real-time PCR analysis on Roche lightcycler (Roche Diagnostics, Indianapolis, IN). First, standard curves for tPA (Fig. 24) and uPA (Fig. 25) cDNA were generated using known concentrations of cDNA templates as described in chapter 2. The products of standard curve polymerase chain reactions were analyzed by gel electrophoresis to ensure amplification of a single, specific product (Fig. 26).

Subsequently, one known concentration of standard (0.001 pg for tPA and 0.0001 pg for uPA) was amplified along with experimental samples. The values for tPA and uPA concentrations were derived in picograms, which were further normalized to similarly obtained values for a reference gene, Ubiquitin (UBC), in each sample. The sample concentrations were calculated by the software by comparing the crossing point of experimental sample with that of the known standard normalized to previously saved standard curve as described in chapters 2 and 4. Ubiquitin was used as the reference gene to normalize the concentrations for tPA and uPA. Figures 27 and 28 show amplification (panel A), melting curve analysis (panel B), melting peak analysis (panel C) and gel electrophoresis (panel D) of tPA and uPA respectively from representative experimental samples.
Fig. 24. tPA standard curve for real-time PCR analysis. (A) Amplification of tPA cDNA plotted as fluorescence vs. cycle number. Each sample is represented in a uniquely colored line on the real-time PCR software, allowing tracing of amplification of individual samples. (B) tPA standard curve plotted as cycle number vs. log concentration showing a linear standard curve ($r = -1$). (C) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing only one PCR product corresponding to tPA. (D) Melting curve analysis data plotted as changes in fluorescence signal over time ($dF/dT$) vs. temperature. Presence of only one melting peak in this reaction corresponding to melting temperatures of tPA (~85.5°C) indicates specificity of the amplification reaction and absence of primer-dimers.
Fig. 25. uPA standard curve for real-time RT-PCR analysis. (A) Amplification of uPA plotted as fluorescence vs. cycle number. Each sample is represented in a uniquely colored line on the real-time PCR software, allowing tracing of amplification of individual samples. (B) uPA standard curve plotted as cycle number vs. log concentration showing a linear standard curve ($r = -1$). (C) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing only one PCR product corresponding to uPA. (D) Melting curve analysis data plotted as changes in fluorescence signal over time ($dF/dT$) vs. temperature. Presence of only one melting peak in this reaction corresponding to melting temperatures of PAI-1 (~83.5°C) indicates specificity of the amplification reaction and absence of primer-dimers.

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Fig. 26. Gel-electrophoresis of tPA and uPA standard curve PCR products. Aliquots of PCR products (2 μl out of 20 μl total) were run on 8% acrylamide gel followed by ethidium bromide staining. (A) tPA and (B) uPA PCR products show a single band at expected base-pair location confirming specific amplification of respective templates.
Fig. 27. Representative images from real-time PCR of tPA and UBC from experimental samples. (A) Amplification of tPA and UBC plotted as fluorescence vs. cycle number. Each line is represented in different color on the real-time PCR software, allowing tracing of amplification of individual samples. The crossing points for both tPA and UBC fall within the bars marking approximately cycles 15 and 22. (B) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing two PCR products corresponding to tPA and UBC. (C) Melting curve analysis data plotted as changes in fluorescence signal over time (dF/dT) vs. temperature. Presence of only two melting peaks in this reaction corresponding to melting temperatures of UBC (~82°C) and tPA (~85.5°C) indicate specificity of the amplification reaction and absence of primer-dimers. (D) Gel electrophoresis of PCR products from representative experimental samples show a single PCR product of expected base-pair size in each reaction.
Fig. 28. Representative images from real-time PCR of uPA and UBC from experimental samples. (A) Amplification of uPA and UBC plotted as fluorescence vs. cycle number. Each line is represented in different color on the real-time PCR software, allowing tracing of amplification of individual samples. The crossing points for uPA and UBC fall within the bars marking approximately cycles 23-28 and 15-19 respectively. (B) Real-time monitoring of melting curve analysis of PCR products plotted as fluorescence vs. cycle number showing two PCR products corresponding to uPA and UBC. (C) Melting curve analysis data plotted as changes in fluorescence signal over time (dF/dT) vs. temperature. Presence of only two melting peaks in this reaction corresponding to melting temperatures of UBC (~82°C) and uPA (~83.5°C) indicate specificity of the amplification reaction and absence of primer-dimers. (D) Gel electrophoresis of PCR products from representative experimental samples show a single PCR product of expected base-pair size in each reaction.
**TGFβ has opposite effects on tPA and uPA mRNA expression**

In previous experiments, the effective dose of TGFβ was determined to be at 10 ng/ml (chapter 4). In a time-course analysis 10 ng/ml TGFβ treatment increased uPA mRNA levels to 117.8%, 207.2%, 137.9% and 35.4% of respective time-matched control at 4, 8, 24 and 48 hours (n=1) (Fig. 29). Whereas, 10 ng/ml TGFβ inhibited average tPA mRNA to 89.3%, 69.5%, 70.65 and 28.8% of respective time-matched control at 4, 8, 24 and 48 hours (n=2) (Fig. 29). Thus, TGFβ increased uPA mRNA expression maximally at 8 hours. On the contrary, TGFβ inhibited tPA mRNA expression at most of the time points examined. Maximum increase in PAI-1 mRNA was seen at 8-hours (chapter 4) which corresponds to maximum increase in uPA expression and consistent inhibition of tPA expression at 8 hours. Therefore 8 hours was determined to be an optimum time-point for TGFβ treatment.

**Actin depolymerization regulates tPA and uPA mRNA expression**

The rate of plasminogen activation depends on the balance between plasminogen activators and PAI-1. Therefore the effects of inhibiting actin polymerization on expression of the plasminogen activators was examined first in order to understand the potential net effect on plasminogen activation. The samples from the same experiments in which actin depolymerization had shown inhibition of TGFβ-induced PAI-1 expression were examined for tPA and uPA mRNA expression using quantitative real-time PCR analysis. In three separate experiments, TGFβ decreased tPA mRNA expression slightly, but significantly, to 80.7 ± 5.5% of untreated control (p<0.05), consistent with the profibrotic effects
Fig. 29. Time-course analysis of TGFβ-treatment on tPA and uPA mRNA expression. Human mesangial cells at 60-80% confluence were serum starved for 24 hours. Cells were treated with 10 ng/ml TGFβ for 4, 8, 24 and 48 hours. At each time point, the effect of TGFβ treatment on tPA (A) and uPA (B) mRNA was measured by real-time PCR analysis. (A) TGFβ inhibited tPA mRNA at all the time-points examined (n=2). Each bar represent mean value for tPA expression. The ends of error bars represent the value for each of the two experiments. (B) TGFβ treatment maximally increased uPA mRNA at 8 hours (n=1).
of TGFβ (Fig. 30). Actin depolymerization by Y-27632, CytB and LatB increased basal tPA expression to 232 ± 22.3%, 195.4 ± 26.7% and 288.9 ± 29.8% of untreated controls. Actin depolymerization also effectively reversed the inhibitory effects of TGFβ on tPA expression. Y-27632, CytB and LatB increased tPA mRNA to 260.9 ± 22.6%, 208.3 ± 33.8% and 244.9 ± 39% of their respective TGFβ-treated controls. This suggests that actin depolymerization coordinately regulates the plasminogen activator system. Actin depolymerization simultaneously inhibits PAI-1 expression and increases tPA expression, the net result of which favors activation of plasminogen activation and degradation of extracellular matrix. In contrast, TGFβ increased uPA expression by 246.7±61.4% of control (p<0.05). Inhibition of actin polymerization by CytB and Y-27632 changed uPA mRNA expression to 259.1 ± 119% and 115.6 ± 24.5% of respective controls under serum-free conditions. In the presence of TGFβ, CytB and Y-27632 changed uPA mRNA expression to 95.8 ± 37.7% and 77.7 ± 16.3% of TGFβ-treated control respectively. The effect of CytB and Y-27632 were not statistically significant (Fig. 31). In summary, TGFβ increased uPA mRNA expression, whereas actin depolymerization did not have a statistically significant effect on the same.

Effect TGFβ and actin depolymerization on plasminogen activators protein expression

Since tPA and uPA are secreted upon synthesis, changes in tPA and uPA were determined by analyzing conditioned media using Western blot analysis. Neither tPA nor uPA proteins could be detected in conditioned media using
Fig. 30. The effects of TGF-β and actin depolymerization on tPA mRNA expression. Human mesangial cells at 60-80% confluence were serum starved for 24 hours. Cells were then pre-treated for one hour with 1.0 mM CytB, 0.1 μM LatB or 10 μM Y-27632 to inhibit actin polymerization. TGF-β (10 ng/ml) was added to the media for next 8 hours. At the end of 8-hour treatment with TGF-β, mRNA was isolated and analyzed by quantitative real-time PCR analysis for tPA mRNA expression (n=3). The vertical bars and the error bars represent mean tPA mRNA value and SEM respectively. The value for untreated control is set as 100% *: p<0.05 compared to S-, †: p<0.05 compared to TGF-β, †††: p<0.005 compared to TGF-β.
Fig. 31. The effects of TGFβ and actin depolymerization on uPA mRNA expression. Human mesangial cells at 60-80% confluence were serum starved for 24 hours. Cells were then pre-treated for one hour with 1.0 mM CytB or 10 μM Y-27632 to inhibit actin polymerization. TGFβ (10 ng/ml) was added to the media for next 8 hours. At the end of 8-hour treatment with TGFβ, mRNA was isolated and analyzed by quantitative real-time PCR analysis for uPA mRNA expression (n = 5). The vertical bars and the error bars represent mean uPA mRNA value and SEM respectively. The value for untreated control is set as 100%. *: p<0.05 compared to S-. 

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Western blot analysis. Multiple optimization efforts including using smaller amounts of media for incubation (to reduce dilution of secreted tPA and uPA), using larger amounts of media for detection, concentrating the media using spin concentrators and using varying amounts of primary antibodies were unsuccessful in detecting tPA and uPA proteins in conditioned media. Previously uPA was successfully detected in this lab in a preparation of substratum attached extracellular matrix and membrane proteins called adhesion plaques but not in conditioned media [159, 160].

In summary, the results show an increase in tPA mRNA expression by actin depolymerization. PAI-1 expression was inhibited under similar experimental conditions, suggesting a coordinated regulation of these two genes with opposite functions by actin cytoskeleton.
Mesangial myofibroblast activation as a disease model

Diverse cells and tissues react to injury with similar responses, including myofibroblast differentiation and formation of scar tissue from increased extracellular matrix accumulation [22-24, 29, 161-166]. In the event of tissue injury, native fibroblasts or mesenchymal cells with fibroblast-like properties can transdifferentiate into activated myofibroblasts. De novo expression of genes, possibly required to cope with demands of injury and tissue repair, is seen upon myofibroblast differentiation [19, 119, 167]. For example, the activation of α-SMA expression, a property associated with smooth muscle phenotype and hence the name myofibroblast, is a common feature of myofibroblasts regardless of the tissue affected [168]. In the skin, dermal fibroblasts transdifferentiate into myofibroblast during the process of wound healing and start expressing α-SMA de novo [11]. Similarly, in the kidneys, glomerular mesangial cells, mesenchymal cells that hold the capillary tufts of glomeruli together, do not normally express α-SMA. But in the event of injury, the activated mesangial cells transdifferentiate into myofibroblasts and start expressing α-SMA [19]. They also undergo hypertrophy to meet the functional demands in disease states [168]. Hypertrophic cells also produce more ECM proteins leading to scar tissue formation. Indeed, myofibroblast differentiation is associated with excess ECM deposition in glomeruli in chronic glomerulosclerotic diseases [165]. The association of the
myofibroblast differentiation with ECM deposition and scarring suggests that the knowledge of the mechanisms that regulate myofibroblast differentiation may provide new insights into the cellular processes that control tissue scarring. The overall objective of the current study was to identify the mechanisms regulating myofibroblast differentiation. In this study, the role of actin cytoskeleton in the regulation of gene expression and phenotype changes in glomerular mesangial cells was examined.

**Cultured mesangial cells as the experimental model of myofibroblast differentiation**

Previously, Glass et al showed that cultured mesangial cells mimic in vivo changes during myofibroblast activation[26]. Initial culture in presence of serum causes mesangial cell proliferation similar to that seen in early stages of glomerular injury. Whereas, in the absence of serum, the mesangial cells stop proliferating, spread more, become hypertrophic, increase their stress fibers, and express more α-SMA [26]. The phenotype seen during the serum-deprivation closely resembles that of myofibroblasts seen in the later stages of glomerular injury. Thus, the cultured mesangial cells share similarities both in phenotypes and in the sequence of phenotype changes with in vivo myofibroblasts. Therefore, cultured mesangial cells serve as a good model system to study the myofibroblast phenotype in vitro.

Primary culture human mesangial cells between passages 5 and 9 were therefore used in the current study to understand the regulation of myofibroblast differentiation. The serum-free media was used as the basal culture condition to
allow the mesangial cells to acquire myofibroblast-like phenotype. The use of already activated mesangial cells resembles the clinical scenario in chronic glomerular diseases, where a majority of the patients present with already established glomerular lesions containing activated mesangial cells. Also, the role of individual growth factor can be studied when added to the serum-free media used as the basal culture condition.

**Actin cytoskeleton as a regulator of gene expression**

The concordant increase in stress fiber formation with \( \alpha \)-SMA expression and hypertrophy in serum-starved mesangial cells lead to the hypothesis that the actin cytoskeleton regulates \( \alpha \)-SMA expression and hypertrophy in mesangial cells. In a further study, the use of agents that modulate actin cytoskeleton showed that the changes in actin cytoskeleton regulated both hypertrophy and \( \alpha \)-SMA expression in serum-starved mesangial cells [28]. The \( \alpha \)-SMA expression was regulated at the level of transcription and message stability. These findings were consistent with the regulation of \( \alpha \)-SMA mRNA by the actin cytoskeleton in different cell types [169, 149]. These results showed the evidence for the role of actin cytoskeleton in gene regulation and myofibroblast-differentiation in mesangial cells.

Since cellular hypertrophy is associated with an increase in global protein synthesis, it was hypothesized that the actin cytoskeleton regulates myofibroblast phenotype by simultaneously regulating the expression of multiple genes. Recent availability of DNA arrays containing multiple genes with related functions on one array provides opportunity to study the expression of multiple genes.
simultaneously. Since the myofibroblast differentiation is associated with increased ECM, the effects of actin cytoskeleton on a DNA array containing genes related to ECM and cell-interaction were examined.

The array experiment way performed only once, but the results were consistent with the hypothesis that cytoskeletal organization coordinately regulates genes involved in myofibroblast function and sclerosis. Also the results were confirmed, in particular, for PAI-1 and tPA. The expression of 74 out of 265 genes was detected on the array (Chapter 3). Cell-type specific and/or cell-culture conditions could account for the detection of limited number of genes. Alternatively, optimization of the protocol could help detect some of the genes not detected in this study. Also, repeating the experiment with the same and other actin depolymerizing agents could show the consistency of changes in pattern of gene expression.

A noticeable pattern of gene expression was observed among the detected genes. Out of ten down-regulated genes, four were precursors of extracellular matrix components, namely procollagen 3 α1 subunit, procollagen 1 α2 subunit, collagen 4 α2 subunit and fibronectin. Endothelial plasminogen activator inhibitor-1 (PAI-1) precursor also decreased. Since the role of PAI-1 is to favor ECM deposition, a decrease in PAI-1 would also decrease ECM. Thus actin depolymerization decreases both ECM components as well as profibrotic regulators of ECM turnover. The collective effect is a decrease in ECM deposition. Thus, actin depolymerization correlates with decreased PAI-1 expression and ECM deposition. Since, PAI-1 expression is an attribute of
myofibroblast differentiation and the ECM deposition is an outcome of such differentiation, these findings support the overall hypothesis that changes in actin polymerization regulate the myofibroblast phenotype of mesangial cells.

At the same time, out of four genes that increased, three favor degradation of ECM. These include tissue-type plasminogen activator (tPA), decorin (DCN) and matrix metalloproteinases 11 (MMP 11). tPA converts inactive plasminogen into active plasmin with fibrinolytic properties [56]. MMP11, although not a major fibrinolytic enzyme, digests ECM under certain conditions [170]. Decorin inhibits profibrotic TGFβ and thereby prevents deposition of ECM [122, 171]. Together these genes lead to increased removal of ECM.

The cut-off ratios for increase (>2-fold) or decrease (>50%) in gene expression set during the DNA array analysis were arbitrarily selected, requiring further validation of the findings. Also, the experiment was performed only once. Thus, further validation of the changes in gene expression seen on DNA array was required. Therefore, the changes in the expression of two genes, relevant to myofibroblast differentiation and ECM regulation, seen on DNA arrays were confirmed by Northern blot and Western blot analysis. The components of plasminogen activator system, PAI-1 and tPA, were selected for the confirmation first since they are the major regulators of fibrinolysis and are directly related to ECM turnover. Also they both were regulated in opposite directions by actin cytoskeleton consistent with their opposite functions on ECM. The findings on PAI-1 and tPA were confirmed at mRNA and protein levels, providing strong evidence for coordinated gene regulation by the actin cytoskeleton. Indeed,
inhibition of actin depolymerization by CytB and Y-27632 inhibited PAI-1 mRNA and protein expression, whereas CytB increased tPA protein expression. Thus the findings of DNA array experiments were reflected at the protein level. These experiments were set under different cell-culture conditions, some in the presence of serum and some in the absence of serum, making the comparison of results difficult. The aim of the next set of experiments was to understand the significance of regulation of PAI-1 and tPA in the context of their pathophysiological regulation using a uniform cell culture condition.

**TGFβ induction of PAI-1 expression**

Since PAI-1 is a major mediator of the ability of TGFβ to cause fibrosis [172] and it acts as a gate-keeper of the plasminogen activation cascade by inhibiting both tPA and uPA, the regulation of PAI-1 expression was studied first and more extensively than that of tPA or uPA. The cells were treated with TGFβ, a major regulator of PAI-1, plasminogen activator, ECM turnover and myofibroblast differentiation; to resemble in vivo regulation of plasminogen activator system. The effect of actin cytoskeleton on TGFβ-mediated expression of the plasminogen activator system was studied in human mesangial cells. The use of TGFβ as an agonist provides a model that mimics in vivo regulation of plasminogen activators, and helps to understand myofibroblast differentiation and ECM regulation.

The dose-response analyses of TGFβ on PAI-1 mRNA and protein expression were performed first (Chapter 4). Although treatment with 1, 2, 5 and 7.5 ng/ml TGFβ increased average PAI-1 mRNA expression to 381.9%, 323.1%,
443.3% and 485.1% of control in two different experiments, the effects on PAI-1 protein were inconsistent at these lower doses. At 10 ng/ml, TGFβ increased both PAI-1 mRNA and protein expression to 287.9% and 283.6% of respective controls. Additionally, this concentration is very commonly used in published studies by numerous investigators. In a similar dose-response analysis, Motojima et al also found 10 ng/ml as the most effective dose of TGFβ for PAI-1 induction in rat mesangial cells [82]. Other researchers in this lab have also used TGFβ successfully at 10 ng/ml for studying effect on COX-2 expression (Harding et al, unpublished observations). Also, this concentration of TGFβ is pathological which makes the experiments more relevant to an actual pathological state [126]. The failure to show an increase with lower doses of PAI-1 expression was difficult to interpret since the experiment was performed only once. It may result from inefficient translation and/or secretion of PAI-1, inactivation of lower doses of TGFβ in the treatment media at 24-hour treatment or lack of activation of co-stimulatory pathways.

The time-course analysis showed that TGFβ increased PAI-1 mRNA maximally to a value of 191.0 ± 23.5% at 8 hours (Chapter 4). PAI-1 mRNA decreased to 100.1 ± 13.5% and 90.6 ± 8.7% of time-matched controls at 24 and 48 hours respectively suggesting either loss of TGFβ activity or inhibition of TGFβ activity at those time points. Although not tested directly at protein level, the array experiment did show presence of decorin mRNA, a known inhibitor of TGFβ [122]. The analysis for PAI-1 protein showed maximum activation at 24 hours. At earlier time-points PAI-1 could not be detected in the conditioned
media. Since PAI-1 is a secreted protein, there may be a lag period before PAI-1 is synthesized and secreted in sufficient amounts to reach the level of detection in the culture media. Even using a more sensitive detection method such as enzyme-linked immunosorbent assay (ELISA), Matsumoto et al found 24 hr treatment with TGFβ to maximally induce PAI-1 protein expression [127]. Therefore, the use of more sensitive detection method may still show 24 hours as optimum duration of treatment. Although PAI-1 is a rapidly secreted protein, protein synthesis takes longer than mRNA synthesis, partially accounting for the differences in the time-frame of their detection. Therefore, PAI-1 mRNA reached a peak at 8 hours and protein reached a peak at 24 hours. At 48 hours, PAI-1 protein was still elevated (146%), whereas mRNA returned to the control levels (90.6 ± 8.7%). Alternatively, this sustained increase at 48 hours in protein expression could represent accumulation in the media. Since a consistent and comparable increase was seen in PAI-1 mRNA at 8 hours and protein at 24 hours, these time points were used for subsequent experiments.

**Inhibition of actin cytoskeleton on TGFβ–induced PAI-1 expression**

*In vivo* and *in vitro* evidence suggest that Rho and the actin cytoskeleton regulate PAI-1 expression and matrix deposition. The small GTPase, Rho, is known to regulate expression of PAI-1 [81, 108, 173, 174]. Also, inhibition of Rho signaling prevents fibrosis in animal models, an effect consistent with PAI-1 inhibition [102-104]. Since one of the major functions of Rho is to regulate actin polymerization, the effects of Rho on PAI-1 expression could be mediated through the actin cytoskeleton. Secondly, activated mesangial cells or...
myofibroblasts express α-SMA and PAI-1 de novo in various forms of renal fibrosis. Recently this lab showed that Rho-mediated changes in actin polymerization regulate α-SMA expression and hypertrophy, both attributes of myofibroblast differentiation, in mesangial cells [28]. Although the injurious agents and cytokines involved are diverse, it is likely that common cellular mechanisms underlie the regulation of myofibroblast differentiation and increased ECM deposition common to sclerotic diseases. PAI-1, another attribute of myofibroblast differentiation, could also be similarly regulated by the changes in actin polymerization. Thus two separate lines of evidence suggest the role of actin cytoskeleton in the regulation of PAI-1 expression. Therefore the hypothesis that Rho GTPase regulates PAI-1 expression by modulating actin polymerization was examined.

To test the hypothesis, the effects of either inhibition of Rho-mediated signaling or direct inhibition of actin polymerization was examined on TGFβ-induced PAI-1 mRNA and protein expression (Chapter 5). The inhibition of Rho blocked both basal and TGFβ-induced PAI-1 mRNA expression in human mesangial cells. Similarly, direct inhibition of actin polymerization with CytB and LatB also inhibited basal and TGFβ-induced PAI-1 mRNA expression. The results seen by real-time PCR were comparable to those seen by conventional methods such as Northern blot analysis. These confirmed the utility of real-time quantitative PCR in detecting changes in mRNA expression. Since the effects of Rho inhibition on PAI-1 mRNA expression are mimicked by direct inhibition of
actin polymerization, it suggests that the effects of Rho on PAI-1 mRNA expression are mediated by changes in actin polymerization.

Surprisingly, TGFβ did not show any effect on PAI-1 expression in cell-lysates. Since PAI-1 is rapidly secreted upon synthesis, the induction by PAI-1 may only be reflected in secreted PAI-1. The secreted PAI-1 binds to the substratum on tissue culture dishes and saturates the binding sites on the substratum. The cell-lysates were prepared by scraping adherent cells. The lysates generated in this manner include both intracellular and substrate-bound extracellular PAI-1 and hence may not show a true representation of changes in protein expression. The Western blot showed specific inhibition of the PAI-1 band with CytB treatment, whereas the other non-specific bands seen on the blot remained constant. This suggests that CytB is not affecting the secretion of PAI-1 in which case an increased intracellular PAI-1 would have been seen. However, since the cell scrapings contain both intracellular and the substratum-bound PAI-1, which is generally saturated at the cell density used in this study, these differences were masked on cell-scrappings. CytB treatment blocked PAI-1 expression in both cell-lysates and conditioned media suggesting that the inhibition was at the level of protein synthesis or earlier at the mRNA level. Subsequently, the conditioned cell culture media containing the secreted proteins was used to detect the changes in PAI-1 protein expression. The changes in PAI-1 protein expression upon treatment with agents that increase or decrease actin polymerization were consistent with the changes in PAI-1 mRNA expression under similar treatment conditions.
The changes in actin cytoskeleton upon treatment under the experimental conditions examined above were visualized by staining with Texas-red labeled phalloidin, an agent that binds polymerized F-actin fibers (Chapter 5). Treatment with Y-27632 and CytB showed loss of abundant stress fibers seen under control conditions. Simultaneous immunostaining of the same cells for PAI-1 antigen showed inhibition of PAI-1 expression in cells with less F-actin staining. This experiment confirmed simultaneous decrease in F-actin content and PAI-1 expression upon treatment with inhibitors of actin polymerization.

**Stabilization of actin cytoskeleton on TGFβ-induced PAI-1 expression**

If the hypothesis that the changes in actin cytoskeleton regulate PAI-1 expression is true and decreased stress fiber content of the cells blocks PAI-1 expression, then an increase in stress fibers should increase PAI-1 expression. Jasplakinolide stabilizes the stress fibers thereby increasing the cellular stress fiber content. Jasplakinolide increased basal PAI-1 expression and augmented the stimulatory effect of TGFβ on PAI-1 mRNA and protein expression (Chapter 5). Treating the cells with agents that increase the stress fiber content tested the possibility that enhanced actin cytoskeletal assembly has an effect opposite to that of disassembly on PAI-1 expression and essentially rules out the possibility that the observed effects of actin depolymerization are artifacts of the use of pharmacologic inhibitors. The increase in stress fiber content caused by JAS could not be visualized since it binds to the F-actin fibers and therefore interferes with binding of phalloidin, the agent that helps to visualize F-actin [175].
To ensure that the increase in PAI-1 expression upon JAS treatment was associated with the stabilization of actin cytoskeleton, the cells were pre-treated with inhibitors of actin polymerization prior to JAS treatment (Chapter 5). Cotreatment with actin depolymerizing agents such as CytB, Toxin B and Y-27632 blocked the stress fiber formation and reversed the effects of Jasplakinolide on PAI-1 expression. Thus, the changes in actin polymerization correlate with changes of PAI-1 expression. Taken together, these results indicate that Rho modulates the ability of TGFβ to regulate PAI-1 expression by controlling the state of actin polymerization and that the actin cytoskeleton mediated changes in TGFβ-induced PAI-1 expression depend upon the degree of its organization.

The findings of this study are based on pharmacological agents. The effects of pharmacological inhibitors may show non-specific effects. However, multiple agents using unique mechanisms of action showed a similar effect suggesting that the effects are specific to the treatment effect. Still, the use of constitutively active or dominant negative mutants may provide further confirmation of the observations.

The opposing effects of actin depolymerization and stabilization on PAI-1 expression are consistent with similar effects on α1 (I) collagen, a component of ECM, by Schnaper et al [87]. They showed inhibition of TGFβ-mediated expression of α1 (I) collagen by actin depolymerization [87]. In the same study they showed that stabilization of stress fibers with JAS antagonized the inhibitory effect of actin depolymerization. These results also show that the degree of organization of actin cytoskeleton correlates with net ECM deposition. Since
collagen and PAI-1 both contribute to ECM accumulation, it is plausible that both the genes are regulated by similar or coordinated mechanisms. Studies have shown that Rho-kinase inhibition also inhibits PAI-1 expression in response to other agonists such as Ang II [81]. This is consistent with the hypothesis that changes in actin polymerization may act as a final common pathway that modulates the effect of multiple extracellular stimuli on gene expression.

**The effect of actin cytoskeleton on PAI-1 promoter activity**

Once the role of actin cytoskeleton in regulation of PAI-1 expression was confirmed, the subsequent studies aimed to determine the mechanism of this regulation. Since PAI-1 expression is mainly regulated at the level of transcription, the effects of actin cytoskeleton on PAI-1 transcription were examined using human PAI-1 promoter (Chapter 6).

The mechanism of PAI-1 mRNA regulation by cytoskeleton was investigated by examining the effects of actin depolymerization on PAI-1 promoter activity. First, an 1100 bp human PAI-1 promoter containing major regulatory elements was cloned into a luciferase vector. The identity of the cloned promoter was confirmed by sequencing. Due to difficulties in transfecting primary cultured human mesangial cells, initial experiments were performed in rat mesangial cells. The rat and human PAI-1 promoter contain similar regulatory elements, albeit in different numbers. Dual luciferase assay system containing firefly luciferase under the control of PAI-1 promoter and renilla luciferase under minimal thymidine kinase promoter was used to transfect rat mesangial cells. The renilla luciferase was used to normalize for transfection efficiencies. In the
initial time-course experiment with TGFβ, no changes in renilla luciferase were obtained upon different treatments. Normalized PAI-1 values show that TGFβ increases PAI-1 promoter activity marginally in rat mesangial cells. The efforts to reproduce similar experiment in human mesangial cells failed to show consistent counts for renilla luciferase activity. Consequently, luciferase values from human mesangial cells were normalized to the protein concentration of the sample. The luciferase values were represented as luciferase count/µg protein. Studies with human mesangial cells show ~250% increase in human mesangial cells. This effect is consistent with previous studies with the same PAI-1 promoter [112]. This increase is also consistent with the increase in PAI-1 mRNA and protein expression seen upon TGFβ treatment. Since the fold-increase in PAI-1 promoter and mRNA are similar, the regulation of mRNA seems to be at the transcriptional level. These findings are consistent with previous reports suggesting PAI-1 mRNA is mainly regulated at the level of transcription [136, 137]. Treatment with CytB blocked basal as well as TGFβ-induced PAI-1 promoter activity suggesting critical requirement of organized actin cytoskeleton for PAI-1 expression.

Although it is not completely known how changes in actin polymerization regulate PAI-1 promoter activity, there are several possible mechanisms that can explain this regulation. Since PAI-1 promoter is rich in regulatory elements, it is possible that actin depolymerization alters the binding of transcriptional factors to the regulatory elements in PAI-1 promoter. Alternatively, the binding to yet uncharacterized PAI-1 promoter elements may be altered by actin depolymerization. As described previously in the thesis, there are potential
similarities in the regulation of PAI-1 and α-SMA. The promoter region of α-SMA contains CArG elements, which are sensitive to changes in actin polymerization [176]. Hautmann et al have shown cooperation between TGFβ-responsive elements and CArG elements for TGFβ-induced expression of α-SMA [177]. Although, CArG elements have been shown only in uPA promoter so far, other yet unidentified cytoskeleton-responsive elements may drive PAI-1 promoter in concert with TGFβ-response elements [178]. Another possible mechanism may involve Nuclear Factor Yin Yang 1 (YY1). In myofibroblasts YY1 represses expression of α-SMA and TGFβ-responsive genes such as PAI-1 [179]. Ellis et al have shown that YY-1 competes with serum response factor (SRF) for binding to serum response elements (SRE) in the α-SMA promoter. F-actin inhibits this repressive effect of YY1 on α-SMA expression, thereby increasing α-SMA expression [180]. Conversely, G-actin inhibits α-SMA expression by facilitating repression by YY1. Similarly, YY1 competes with SMAD for binding to the PAI-1 promoter and blocks TGFβ-induction of PAI-1 promoter [179]. Although, it is a potential mechanism of regulation of PAI-1 expression by changes in actin cytoskeleton, no direct evidence linking actin cytoskeleton and YY1-binding to PAI-1 promoter has been shown so far. In future studies, this can be examined by performing an electrophoretic gel mobility-shift assay on the nuclear extracts from the cells treated with actin depolymerizing or stabilizing agents. Given the facilitation of YY1 binding to promoter elements by G-actin, an increase in G-actin content upon actin depolymerization is expected to result in the displacement of SMAD binding to SMAD-binding sites by YY1, and vice versa by
increase in F-actin content. Alternatively chromatin immunoprecipitation (ChIP) assay can be used to identify interaction of YY1 or other potential regulatory factors to PAI-1 promoter elements [181].

The effect of actin cytoskeleton on plasminogen activators

Having confirmed the regulation of PAI-1 expression by the actin cytoskeleton, the regulation of other members of plasminogen activators system was examined next. The effects of TGFβ and actin cytoskeleton on tPA and uPA expression were examined in the same set of samples that were used to study PAI-1 expression.

An eight hour treatment with TGFβ caused a small but statistically significant inhibition of tPA mRNA expression (Chapter 6). This effect is consistent with the role of TGFβ as a profibrotic growth factor. Previously, however, Baricos et al did not see any significant effect of TGFβ on tPA protein in cultured human mesangial cells at 72 hours [89]. The effect of TGFβ observed on tPA mRNA expression in the current study was small and remains to be confirmed at the protein level. This small effect could represent the variability among different mesangial cell isolates or the effect of differences between the treatment periods. At the same time, inhibition of actin polymerization increased basal tPA mRNA expression and reversed the inhibition exerted by TGFβ. This finding is particularly interesting in that it shows coordinated regulation of the two genes by the actin cytoskeleton. Simultaneous inhibition of PAI-1 expression and upregulation of tPA allows tPA to act unopposed by PAI-1 resulting in a fibrolytic state. These results are in agreement with previous studies showing increased
tPA expression and fibrolytic activity by simvastatin, an HMG CoA reductase inhibitor that inhibits Rho activation in human vascular smooth muscle cells, endothelial cells and peritoneal mesothelial cells [173, 182, 183]. These studies provide indirect evidence that inhibition of Rho activation increases tPA expression. The current study provides direct evidence that inhibition of Rho-mediated actin polymerization increases tPA expression in human mesangial cells and that there is a coordinated regulation of the plasminogen activation system by the actin cytoskeleton. If the effect seen at the mRNA levels is sustained functionally, it favors increased breakdown of ECM by tPA upon treatment with actin depolymerizing agents, a potential utility in breaking down ECM from sclerosed glomeruli of diseased kidneys.

The coordinated regulation of PAI-1 and tPA may result from the extensive homology between 5' -flanking regions of PAI-1 and tPA genes [115]. Bosma et al observed only six gaps during alignment of 521 positions of 5' sense strand of PAI-1 (non-coding) with 5' antisense strand of tPA (coding). The presence of extensive nucleotide homology, although on opposite strands, may allow common regulatory factors to drive the expression of PAI-1 and tPA coordinately. Accordingly, changes in actin cytoskeleton may change the binding of regulatory factor to PAI-1 and tPA coordinately. In fact, a coordinated increase in the expression of expression of PAI-1 and tPA has been shown in vivo in association with stress, surgery and myocardial infarction; and in vitro by endothelial cells in response to thrombine, histamine and glucocorticoids [115].
On the other hand, uPA expression was increased by TGFβ (Chapter 7). The increase seen in uPA by TGFβ is consistent with a similar increase shown by Baricos et al. [89]. However, they showed that although TGFβ increased both uPA and PAI-1 expression, the net effect was inhibition of plasminogen activation by PAI-1, suggesting that the increase in PAI-1 overcomes the increase in uPA in human mesangial cells. Therefore, the TGFβ-mediated increase in uPA mRNA may not be functionally significant. In the current study, the net effect of TGFβ on ECM turn over was not examined. The effects of actin depolymerization on uPA mRNA did not show statistical significance possibly because of too much data variability.

Human mesangial cells produce smaller amounts of uPA and may not respond consistently to TGFβ-treatment [155]. Alternatively, increasing the sensitivity of the mRNA detection protocol may minimize the data variation. Overall, the observation that the actin cytoskeleton regulates tPA and PAI-1 but not uPA in mesangial cells is consistent with the conclusions of Baricos et al. that tPA has a more important role in ECM turnover. They reported that loss of uPA had no effect on plasminogen activation in mesangial cells from uPA knock out mice. In contrast, tPA null mesangial cells showed defective plasminogen activation [61].

The tPA and uPA proteins could not be detected from the conditioned media using Western blot analysis despite multiple optimization attempts. The amounts of tPA and uPA expression are much smaller as compared to PAI-1. Using conditioned media from cultured human mesangial cells, Baricos et al
showed a large molar excess of PAI-1 \((1.2 \pm 0.1 \times 10^{-9} \text{ M})\) over uPA \((1.2 \pm 0.1 \times 10^{-12} \text{ M})\) and tPA \((0.19 \pm 0.04 \times 10^{-9} \text{ M})\) [155]. The expression levels for PAI-1 mRNA \((0.01 \text{ pg range})\) in the current study were also at least 10 fold higher than those for tPA and uPA \((0.001 \text{ pg range})\). These low levels of tPA and uPA protein expression may be below the level of detection for Western blot analysis. In future, more sensitive detection methods such as ELISA may be helpful in detecting tPA and uPA from conditioned media.

Taken together, the results of tPA and uPA analysis show that TGFβ inhibited tPA mRNA and increased uPA mRNA expression. Actin depolymerization increased tPA expression under basal and TGFβ-treated conditions. As discussed earlier, the PAI-1 expression is inhibited under similar culture conditions. The coordinated regulation of tPA and PAI-1 by actin cytoskeleton could be useful therapeutically in countering fibrosis.

In summary, this study supports the concept that actin cytoskeleton can modulate gene expression by regulating intracellular signal transduction. It provides initial evidence that the inhibition of actin polymerization may coordinately block the expression of profibrotic genes (such as PAI-1) and increase the expression of anti-fibrotic genes (such as tPa and uPA). Agents that inhibit actin polymerization can be potentially beneficial in the treatment of sclerotic diseases.
CHAPTER 9
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In summary, TGFβ increased PAI-1 expression in human mesangial cells. Depolymerization of actin cytoskeleton using inhibitors of Rho and Rho-kinase inhibited PAI-1 expression. The effects of Rho-inhibition were mimicked by direct inhibition of actin cytoskeleton suggesting that the effects of Rho-inhibition were mediated through actin cytoskeleton. On the contrary, stabilization of actin cytoskeleton increased basal and TGFβ-mediated PAI-1 expression. Thus bidirectional changes in the organization of actin cytoskeleton were followed by changes in the PAI-1 expression suggesting modulation of gene expression by the actin cytoskeleton. Inhibition of actin polymerization also blocked PAI-1 promoter activity suggesting transcriptional regulation of PAI-1 expression by the actin cytoskeleton. In contrast to the inhibition of PAI-1 expression, tPA expression was increased by actin depolymerization by both direct inhibitors and inhibitors of Rho-signaling. Thus, changes in actin cytoskeleton mediate the effects of Rho-GTPases in modulating PAI-1 and tPA expression in response to TGFβ. The opposite regulation of PAI-1 and tPA suggests a coordinated gene regulation by the actin cytoskeleton whereby they may control ECM degradation. The results of the current studies with PAI-1 and tPA combined with the previous work on α-SMA and hypertrophy suggest that changes in organization of the...
actin cytoskeleton regulate myofibroblast differentiation and ECM accumulation by coordinately modulating expression of multiple genes.

Among the number of interesting questions that arise from these observations, two particularly interesting ones were addressed first to explore future directions. (1) What intracellular signaling pathways are regulated by changes in actin polymerization during the modulation of TGFβ-induced PAI-1 expression? (2) Can agents that inhibit actin polymerization, including inhibitors of Rho, protect against fibrosis by inhibiting PAI-1 expression in vivo in animal models? The first question was the logical next step in characterizing the mechanism of cytoskeleton mediated gene regulation. The second question addressed the clinical usefulness of the findings of this study.

**Role of MAPK in TGFβ-induced PAI-1 expression**

**Introduction**

Results discussed in chapters 3 through 7 showed that the Rho-mediated changes in actin cytoskeleton modulate induction of PAI-1 in response to extracellular stimulus such as TGFβ. The intracellular signaling pathways involved in this regulation are not known. SMAD proteins, being the primary intracellular mediators of TGFβ, are potential candidates. However Tsuchida et al recently showed that SMAD4, essential in SMAD-dependent target genes responses, regulates only the early (2 hours) TGFβ-mediated PAI-1 response and not the sustained PAI-1 response in mesangial cells [184]. Thus, it appears that non-SMAD intracellular signaling pathways may also regulate TGFβ-induced PAI-1 expression. This observation is supported by findings that the effects of
SMAD and mitogen-activated protein kinases (MAPKs), a group of serine-threonine kinases consisting of three sub-families of intracellular signal transducers, are additive in achieving maximum TGFβ-dependent responses in human mesangial cells [185]. TGFβ-induced PAI-1 expression has been shown to require MAPK in a cell-type specific manner [186-190]. TGFβ-mediated PAI-1 expression requires extracellular signal regulated kinase (ERK) in astrocytes and renal epithelial cells [188, 189], p38 in hepatocytes and fibroblasts [186, 190], and c-jun-NH2 terminal kinase (JNK) in Mv1Lu epithelial cells [187]. The requirement of MAPK for TGF-β mediated PAI-1 expression has not been examined yet in human mesangial cells.

Recently, Schnaper et al have showed that TGFβ-induction of collagen gene expression requires ERK activation [191]. Also, actin depolymerization blocks TGFβ-induced collagen expression [87]. Taken together these studies suggest that actin depolymerization blocks TGFβ-induced collagen expression by inhibiting ERK activation. Similarly, TGFβ may be activating MAPK to induce PAI-1 in an actin cytoskeleton dependent manner. The hypothesis that the actin cytoskeleton modulates TGFβ-induced PAI-1 expression by modulating MAPK activation was proposed. As a first step towards examining this hypothesis, the requirement of MAPKs in TGFβ-induced PAI-1 expression was examined in human mesangial cells.

**Working hypothesis**

TGFβ-induced PAI-1 expression in human mesangial cells requires activation of MAPK pathway in a cytoskeleton dependent manner.
Experimental model

To examine the requirement of MAPK in TGFβ-mediated PAI-1 expression, pharmacological inhibitors of MAPK were used to block MAPK signaling. The efficacies of reported effective concentrations of MAPK inhibitors were confirmed in human mesangial cells in the lab prior to the use. The literature indicated that the effective doses were 30 μM for PD98059, the ERK inhibitor [192], 5 μM for SB203580, the p38 inhibitor [193] and 10 μM for SP600125, the JNK inhibitor [194]. These kinase inhibitors were relatively specific at the doses used in the study. PD98059 and SB203580 were found specific inhibitors of ERK and p38 respectively with minimal inhibition of other kinases at doses higher than those used in this study [195]. SP600125 was found >20-fold selective inhibitor of JNK at IC₅₀ of 10 μM, the same dose as used in this study [196].

To examine if one or more of the three MAPKs were required for TGFβ-induced PAI-1 activation in human mesangial cells, serum-starved mesangial cells were pre-treated with MAPK inhibitors followed by TGFβ-treatment. The effects on PAI-1 mRNA and protein expression were examined.

Results

Since TGFβ activates different MAPK modules depending on the cell type and the target gene, the requirement of each of the three MAPK for TGFβ mediated PAI-1 expression in human mesangial cells was examined first. Serum-starved human mesangial cells were pre-treated for one-hour with 30 μM PD98029, 5 μM SB203580 and 10 μM SP600125 to inhibit ERK, p38 and JNK.
respectively. Cells were then treated with 10 ng/ml TGFβ for 8 hours (mRNA analysis) or 24 hours (protein analysis). Real-time PCR analysis showed that inhibition of ERK, p38 and JNK changed PAI-1 mRNA expression in serum-free media to 94.6 ± 10.8%, 87.2 ± 6.6% and 82.7 ± 14.0% of untreated control respectively. None of the effects were statistically significant. However, ERK and JNK inhibition blocked TGFβ-induced PAI-1 expression to 64.7 ± 14.7% (p=0.06) and 52.7 ± 4.7% (p<0.005) of TGFβ-treated control respectively. Inhibition of p38 increased PAI-1 expression to 114.9 ± 5.7% of TGFβ-treated control (p=0.05) (Fig. 32).

Western blot analysis of conditioned media (n=3) showed that ERK and JNK inhibition inhibited basal PAI-1 expression to 70.6 ± 13.2% and 64.3 ± 14.5% of untreated control respectively (p<0.05). Inhibition of p38 had no significant effect on basal or TGFβ-induced The PAI-1 protein expression (96.6 ± 12.4%). However, ERK and JNK inhibition blocked TGFβ-induced PAI-1 expression to 49.5 ± 12.6% (p<0.005) and 44.4 ± 12.6% (p<0.001) of TGFβ-treated control respectively. Inhibition of p38 changed PAI-1 expression to 90.7 ± 5.0% of TGFβ-treated control (Fig. 33).

From these results both ERK and JNK appear to be required for TGFβ-induced PAI-1 expression whereas p38 is not required for basal or TGFβ-induced PAI-1 expression.

**Discussion**

The main focus of this study was to identify which, if any, of the MAPK was being activated by TGFβ for PAI-1 regulation. Inhibitors of ERK and JNK
Fig. 32. Effect of MAPK inhibition on PAI-1 mRNA expression. Serum-starved human mesangial cells were pre-treated for one-hour with 30 μM PD98029, 5 μM SB203580 and 10 μM SP600125 to inhibit ERK, p38 and JNK respectively. Cells were then treated with 10 ng/ml TGFβ for 8 hours followed by quantitative real-time PCR analysis of mRNA. The vertical bars and the error bars represent the mean value for PAI-1 mRNA and SEM respectively. The value for untreated control is set at 100%. *: p<0.005 compared to S-, †: p=0.06 compared to TGFβ, ††: p=0.05 compared to TGFβ, †††: p<0.05 compared to TGFβ (n=3).
Fig. 33. Effect of MAPK inhibition on PAI-1 protein expression. Serum-starved human mesangial cells were pre-treated for one-hour with 30 μM PD98029, 5 μM SB203580 and 10 μM SP600125 to inhibit ERK, p38 and JNK respectively. Cells were then treated with 10 ng/ml TGFβ for 24 hours followed by Western blot analysis of conditioned media (n=3). Representative blot is shown in panel A. Panel B shows graphical representation of three separate experiments. The vertical bars and the error bars represent the mean value for PAI-1 protein and SEM respectively. The value for untreated control is set at 100%. *: p<0.05 compared to S-, **: p<0.005 compared to S-, †: p<0.01 compared to TGFβ, ††: p<0.005 compared to TGFβ.
blocked TGFβ-induced PAI-1 mRNA and protein expression suggesting their involvement in TGFβ signaling. The requirement of ERK is consistent with studies showing involvement of ERK in Angiotensin, vascular endothelial growth factor and platelet derived growth factor induced PAI-1 expression [81, 197, 198]. Involvement of JNK is consistent with regulation of thrombin-induced PAI-1 expression by JNK [199]. Overall, the findings are also similar to the results of Schnaper et al showing activation of ERK and JNK but not p38 by TGFβ in human mesangial cells [191]. The requirements for ERK and JNK may result from their roles in regulating AP-1 dependent transcription of PAI-1. In fact, a study published during the preparation of this thesis showed a requirement of MAPK/AP-1 activation for TGFβ-induced PAI-1 expression in rat mesangial cells [200]. This published result confirmed the original hypothesis proposed in this lab.

The treatment with ERK and JNK inhibitors in serum-free media blocked basal PAI-1 protein expression but did not change PAI-1 mRNA expression. This finding suggests that under basal condition, post-transcriptional regulation of PAI-1 may contribute to the reduction in protein levels by ERK and JNK inhibitors. Conversely, inhibition of p38 resulted in a small but significant increase in TGFβ-induced PAI-1 mRNA expression. The increase may result from increased activity of ERK and JNK upon p38 inhibition as shown by Ohashi et al [201].

In summary, the results confirm a requirement for ERK and JNK in TGFβ-induced PAI-1 expression in human mesangial cells. However, the specificity of the effects of pharmacological inhibitors has to be ultimately confirmed using
dominant negative mutants of each of the three MAPKs. Although the study is focused mainly on identifying the role of MAPK as the intracellular mediator of TGFβ signaling, it is appreciated that TGFβ signaling may involve other intracellular signaling molecules such as protein kinase A (PKA) and protein kinase C (PKC) for the regulation of PAI-1 expression. The activation of ERK and JNK upon TGFβ-treatment can be examined to show a direct evidence for the role of MAPK in TGFβ signaling. The role of actin cytoskeleton in modulation of ERK and JNK activation can be studied by examining the changes in ERK and JNK activation upon inhibiting or stabilizing the actin cytoskeleton. The confirmation of the role of actin cytoskeleton in modulating MAPK activation in TGFβ-induced PAI-1 expression will explain the mechanism of action of cytoskeleton modifying agents. Overall, the elucidation of specific mechanism of gene regulation by the actin cytoskeleton will provide avenue for future research into identifying more specific targets of actin cytoskeleton in the regulation of intracellular signaling and gene expression.

**Effect of Rho-kinase inhibition on sclerosis in chronic mesangiosclerotic glomerulonephritis**

**Introduction**

The results of cell culture experiments showed coordinated regulation of two members of plasminogen activator system, PAI-1 and tPA, by the Rho-mediated changes in actin cytoskeleton (Chapters 3 through 7). The functional significance of these changes in plasminogen activator system was determined next by examining the effect on ECM degradation *in vivo.*
Regulation of ECM turnover is one of the major functions of the plasminogen activator system [56]. Increased intraglomerular deposition of ECM proteins resulting in glomerulosclerosis is a major feature of chronic renal diseases. [202]. Increased ECM deposition may result from inhibition of ECM breakdown by TGFβ-induced PAI-1 expression in chronic glomerulosclerotic renal diseases [59]. The in vitro results of this study show that inhibition of Rho-mediated actin polymerization inhibits TGFβ-induced PAI-1 expression. In vivo inhibition of ROCK by Y-27632 is also shown to protect against tubulointerstitial fibrosis in a unilateral ureteral obstruction model [104]. These observations suggested the possibility that if Rho-kinase inhibitors had similar inhibitory effect on PAI-1 expression in vivo, it could protect against ECM deposition and glomerulosclerosis.

**Working Hypothesis**

Rho-kinase activation increases expression of PAI-1 compared to tPA and uPA, and thus causes mesangial matrix expansion. Treatment of rats with Rho-kinase inhibitor, HA-1077, will block these changes and provide a protective effect against glomerulosclerosis

**Experimental model**

A single injection of anti-Thy 1.1 antibody, that reacts with a Thy 1-like antigen on the surface of glomerular mesangial cells, leads to a disease characterized by mesangial proliferation and, deposition of collagen and fibrin in mesangium [203]. However, this injury is self-limited and resolves as early as 6
weeks [204]. Repeated injections of anti-Thy 1.1 antibody have been reported to produce a more severe form of glomerulonephritis with unresolved glomerulosclerosis akin to the lesions seen in chronic glomerulonephritis [202, 205]. This model system was used to examine effectiveness of ROCK-inhibitor in prevention and treatment of ECM accumulation in chronic renal diseases.

In order to cause mesangioasclerotic glomerulonephritis rats were given four weekly injections of Thy 1.1 antiserum (ATS) containing anti-Thy 1.1 antibody as described in the chapter 2. Each ATS injection was followed by injection of goat serum to provide complement for fixation of the antibody. Also, after four weekly injections, the animals were observed/treated for six more weeks to produce progressive injury [206]. The Rho-kinase inhibitor HA-1077 was used in this study to treat the animals because of its specificity at the dose used in the study, water-solubility for ease of administration and lower expense. Moreover, it is already under clinical trials for the use in human patients suffering from stroke suggesting its potential use in humans [207, 208]. The glomerulosclerotic injury was visualized by histology and immunohistochemistry. Twenty-four hour urinary protein excretion and serum creatinine levels were measured as indicators of glomerular injury.

Results

Histopathologic grading of glomerular ECM deposition and injury

The histological sections were stained by Masson trichrome method. The slides were coded before the analysis. Sclerosis was graded on a scale of 0 for normal to 3+ for diffusely increased trichrome-positive (blue) mesangial matrix
material. Scores of 2+ and 3+ correspond to focally positive or intermediate staining intensity of trichrome-positive glomeruli. A practicing renal pathologist performed grading of trichrome-stained sections. The code was broken only after completion of the grading. The average score of control rats was 0.8 ± 0.3 (s.e.) while the group with ATS administration had average score of 1.7 ± 0.6. Rats treated with HA-1077 had an average score of 1.0 both whether the therapy was initiated before (s.e. 0.5) or after (s.e. 0.3) the first ATS injection (Table 5). While these results suggest that the therapy with HA-1077 may have been beneficial, none of these differences achieved statistical significance at a confidence level of p < 0.05.

The sections were also stained for smooth muscle α-actin (α-SMA) to examine myofibroblast differentiation in mesangial cells. For each animal, 100 glomeruli were sequentially analyzed at 40X objective. The glomeruli showing clear non-hilar staining for α-SMA were counted as positive. The results were similar to those seen with trichrome staining. The percentage of positive glomeruli were: 0.8% ± 0.5% in normal control rats, 3.8% ± 2% in ATS-treated rats and, 2.5% ± 0.6% in rats treated with HA-1077 before and after the ATS injection (Table 5). Again, none of these results showed any statistically significant difference between treated and untreated groups.

Effect on urinary protein excretion

Urinary protein results at the end of the study were consistent with production of mild glomerular injury (Table 5). The control rats excreted 14.6 ± 1.7 mg protein/24 h, ATS-treated rats excreted 35.0 ± 11.0 mg protein/24 h (p<0.05).
Fig. 34. Effect of Rho-kinase inhibition on sclerosis in a rat model of chronic glomerulonephritis. Animals were divided into four groups as follows: Group 1: Normal saline injection alone (A), Group 2: ATS-complement injection (B), Group 3: ATS-complement+ HA-1077 treatment started 1 day before the first injection (C), Group 4: ATS-complement+HA-1077 treatment started 1 week after the first injection (D). Trichrome staining of representative glomeruli show increased mesangial sclerosis in group 2 (B) and, a relative decrease in groups 3 (C) and 4 (D) suggesting a protective effect of Rho-kinase inhibition. The arrowheads mark mesangial ECM deposition stained blue by trichrome staining.

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Table 5. Effect of Rho-kinase inhibitor on experimental glomerulonephritis

<table>
<thead>
<tr>
<th>Group</th>
<th>24-hour urinary protein (mg)</th>
<th>Serum creatinine (mg/dL)</th>
<th>Histological grading (0-3)</th>
<th>α-SMA staining (% positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>14.6 ± 1.7</td>
<td>1.18 ± 0.16</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>2. Disease, no treatment</td>
<td>35.0 ± 11.0 (p&lt; 0.05)</td>
<td>1.41 ± 0.2</td>
<td>1.7 ± 0.6</td>
<td>3.8 ± 2.0</td>
</tr>
<tr>
<td>3. Disease, Pre-treatment</td>
<td>19.0 ± 2.1 (p=0.09)</td>
<td>1.07 ± 0.14</td>
<td>1.0 ± 0.5</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>4. Disease, post-treatment</td>
<td>24.42 ± 3.9 (p=0.12)</td>
<td>1.21 ± 0.18</td>
<td>1.0 ± 0.3</td>
<td>2.5 ± 0.6</td>
</tr>
</tbody>
</table>
Rats started on therapy with HA-1077 before the first ATS injection excreted 19.0 ± 2.1 mg protein/24 h (p=0.092 as compared to untreated group), whereas rats started on therapy one week after the first injection had 24.42 ± 3.9 mg protein/24 h (p=0.193 as compared to untreated group). SDS-PAGE analysis of urinary proteins showed that albumin was the major urinary protein.

Effect on serum creatinine levels

Serum creatinine measurement at the end of the study followed the same trends as urinary proteins, but again failed to reach statistical significance for the treatment effect. These values were: control 1.18 ± 0.16 mg/dl, ATS only 1.41 ± 0.2 mg/dl, HA-1077 started before ATS; 1.07 ± 0.14 mg/dl; and, HA-1077 started after ATS; 1.21 ± 0.18 mg/dl (Table 5).

Determination of drug efficacy

HA-1077 solutions used in the study effectively inhibited actin polymerization in cultured human mesangial cells throughout the treatment period suggesting that the drug was stable in water.

There were no significant differences between groups in kidney to body weight ratio, blood pressure, daily water consumption and daily urine output.

Discussion

The results of the histologic scoring, α-SMA staining, urinary protein and serum creatinine values all suggest the same conclusion, that the Rho-kinase inhibitor, HA-1077 had a beneficial effect on glomerular injury in this chronic model of mesangial sclerotic glomerulonephritis (Table 5). Although none of these results individually achieved statistical significance, these studies provide
initial evidence that a Rho-kinase inhibitor could be useful in the treatment of glomerulosclerosis. In future optimization of the disease model may yield statistically significant results. At this stage, this chronic model is difficult to reproduce (personal communication between Dr. Glass, the principle investigator, and other investigators in the field).

Several factors may contribute to the inability of the results to achieve statistically significant differences between the study groups. First, the degree of mesangial sclerosis was mild even in the most severely affected rats. Thus a model with more severe mesangial injury will be necessary. Border et al. allowed 18 weeks (instead of 10 weeks as used in this study) for the progression of the disease in their study [206]. Alternatively, more experimental models with more invasive procedures such as surgical renal ablation could be used to produce severe injury [209, 210]. Second, a larger study with more animals in each group may help the trends seen in the result achieve a statistical significance. Third, the effect of treatment was inconsistent, making it difficult to grade the injury even by an experienced pathologist. Although the drugs used in these studies were active and effective in blocking stress fiber formation in cell culture, their in vivo effective dose could not be assessed. Optimization of therapeutic dose in this particular model system could potentially give more consistent therapeutic effects.
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Dear Sir/Madam,

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I have created a modified diagram based on the figure 3 of the article to show information pertinent to my thesis. I am hereby requesting a kind permission to use the modified (not the original) diagram for the thesis. Please find attached the original and modified diagram for your review.

Also, I am acknowledging at several places in my thesis that the diagram was modified from an original publication by Binder et al and giving a reference for the same.

Following is the figure legend I am planning to use with the modified figure pending your approval.

"Figure 21: Schematic representation of PAI-1 promoter.-RE, response element; TGFβ, transforming growth factor; VLDL, very low density lipoprotein; CTF, CCAAT box binding transcription factor; NF1, nuclear factor 1; PMA, phorbol 12-myristate 13-acetate; AP, activator protein; TRE, tetradecanoyl phorbol acetate response element; GRE, glucocorticoid response element. Adopted with modification from Binder et al [Binder, 2002]."

I will really appreciate your prompt and favorable consideration of my request. Your approval will help me immensely in completing my thesis in time for submission in December.

Sincerely,

Keyur Patel, MD
(Ph.D. Candidate)

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CC: "Patel, Keyur MD" <KPatel1@LIJ.EDU>
VITA

Name: Keyur Pravinchandra Patel

Title: Graduate Student
Department of Pathology and Anatomy
Eastern Virginia Medical School
700 W Olney Rd
Norfolk, VA 23507

Education: 1999-present Ph.D. Candidate,
Biomedical Sciences Ph.D. Program
(Cell Biology and Molecular Pathogenesis Track)
Eastern Virginia medical school, Norfolk, VA, USA
1993-1999 M.B.B.S. (Bachelor of Medicine, Bachelor of Surgery), Smt. N.H.L. municipal medical college, Ahmedabad, India

Awards: Young Pathologist Fellowship, American Society of Clinical Pathologists, 2004
Best Graduate Student Poster, Cardiovascular Focal Research Group, Annual Research Day, Eastern Virginia Medical School, 2003

Publications:

Oral Presentation:

Poster Presentations:

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