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Regulation of TGF- β 1/MAPK-mediated PAI-1 gene expression by the actin cytoskeleton in human mesangial cells

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Abstract

The importance of transforming growth factor- β 1 (TGF- β 1) in plasminogen activator inhibitor-1 (PAI-1) gene expression has been established, but the precise intracellular mechanisms are not fully understood. Our hypothesis is that the actin cytoskeleton is involved in TGF- β 1/MAPK-mediated PAI-1 expression in human mesangial cells. Examination of the distributions of actin filaments (F-actin), α -actinin, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) by immunofluorescence and immunoprecipitation revealed that ERK and JNK associate with α -actinin along F-actin and that TGF- β 1 stimulation promote the dissociation of ERK and JNK with F-actin. Disassembly of the actin cytoskeleton inhibited phosphorylation of ERK and JNK and modulated PAI-1 expression and promoter activity under both basal and TGF- β 1-stimulated conditions. Stabilizing actin prevented dephosphorylation of ERK and JNK. ERK and JNK inhibitors and overexpressed dominant negative mutants antagonized the ability of TGF- β 1 to increase PAI-1 expression and promoter activity. Disassembly of F-actin also inhibited AP-1 DNA binding activity as determined by electrophoretic mobility shift assay using AP-1 consensus oligonucleotides derived from human PAI-1 promoter. F-actin stabilization prevented loss of AP-1 DNA binding activity. Therefore, changes in actin cytoskeleton modulate the ability of TGF- β 1 to stimulate PAI-1 expression through a mechanism dependent on the activation of MAPK/AP-1 pathways.

Keywords

Transforming growth factor- β 1; Plasminogen activator inhibitor-1; MAPK; Actin cytoskeleton; α -actinin; AP-1

Introduction

Glomerulosclerosis is the final common pathway leading to loss of renal function in a variety of primary and secondary glomerular diseases such as diabetic nephropathy, lupus nephritis and chronic glomerulonephritis. Accumulation of mesangial extracellular matrix (ECM) and/or collapse of glomerular basement membranes characterize the glomerulosclerotic process [1]. Although glomerulosclerosis involves multiple mechanisms, inhibition of ECM

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degradation appears to play an important role. In vivo, the plasminogen activation system plays a central role in controlling matrix degradation [2]. Plasminogen activator inhibitor-1 (PAI-1), the main physiological inhibitor of plasminogen activation, is thought to regulate glomerular mesangial matrix turnover by preventing plasmin generation and plasmin-mediated MMP activation. Experimental and clinical studies support an important role for PAI-1 in the pathogenesis of glomerulosclerosis. PAI-1 is not expressed in normal kidney, but is highly expressed in experimental models such as anti-Thy-1 nephritis, lupus nephritis, and crescentic glomerulonephritis [3–5]. In addition, PAI-1 is strongly induced in human diseases such as diabetic nephropathy, thrombotic microangiopathy, acute renal allograft rejection, and focal and segmental glomerulosclerosis [6–9]. PAI-1 deficiency retards diabetic nephritis [10]. Treatment with a mutant, noninhibitory PAI-1 is reported to decrease ECM accumulation in experimental glomerulonephritis [11].

Recognition that transforming growth factor- β 1 (TGF- β 1) is a major mediator of glomerulosclerosis and that TGF- β 1 is a potent inducer of PAI-1 expression led to increased awareness of the possible importance of PAI-1 in progressive renal diseases [12]. Overexpression of TGF- β 1 in progressive glomerulosclerosis is associated with increased PAI-1 expression [13]. In vitro, TGF- β 1 enhances PAI-1 production in glomerular mesangial cells [14]. Thus, the induction of PAI-1 by TGF- β 1 may lead to inhibition of protease-dependent proteolytic activity and accumulated deposits of ECM, resulting in glomerulosclerosis. Despite these observations, the precise intracellular mechanisms that lead to increased PAI-1 expression in human mesangial cells (HMCs) are not fully understood. It has been established that mitogen activated protein kinases (MAPKs), such as ERK, JNK and p38 MAP kinase pathways can be rapidly activated by TGF- β 1 in HMCs [15], but their biological consequences are poorly characterized.

Recent investigations raise the possibility that the actin cytoskeleton also plays a role in ECM accumulation and sclerosis. Hubchak et al [16] found that disruption F-actin with cytochalasin D decreased TGF- β 1-stimulated collagen production. More recently, the use of Rho-kinase (ROCK) inhibitors as potential therapeutic agents to prevent sclerosis in various diseases has received much attention. Y-27632, a specific ROCK inhibitor, markedly decreased collagen accumulation and the progression of fibrosis in experimental models of pulmonary, liver and kidney [17–19]. We have shown previously that preventing actin polymerization either with inhibitors of ROCK or by agents that interact directly with actin inhibits mesangial cell hypertrophy and expression of α smooth muscle actin (α -SMA), characteristics associated with the mesangial cell myofibroblast phenotype [20]. Thus, apart from affecting cell shape and migration, the reorganization of actin cytoskeleton may play important roles in other cellular processes such as gene expression.

In this investigation, we found that PAI-1 expression in basal and TGF- β 1-stimulated HMCs is modulated by changes in actin cytoskeletal structure through ERK- and JNK-dependent signaling pathways and that ERK and JNK distribute along actin stress fibers and are associated with the actin-binding protein, α -actinin. Reorganization of the actin cytoskeleton also affect binding AP-1, a final nuclear mediator of ERK and JNK activation, to a consensus reactive element derived from human PAI-1 promoter. These results demonstrate a possible central role for the actin cytoskeleton in modulating the effects of profibrotic stimuli on extracellular matrix degradation.

Materials and methods

Materials

Active human recombinant TGF- β 1 was purchased from R& D Systems (Minneapolis, MN). PD98059, SP600125, Latrunculin B (Lat B), Y27632, and jasplakinolide (Jas) were purchased

from Calbiochem (San Diego, CA). Cytochalasin B (Cyto B) was purchased from Sigma (St. Louis, MO). Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Antibodies were purchased from the following vendors: phospho-ERK (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), pan-ERK and pan-JNK, phospho-Smad2 (Ser465/467), and Smad2/3 antibodies, Cell Signaling Technology (Beverly, MA); c-Fos, c-Jun, and α -actinin, Santa Cruz Biotechnology (Santa Cruz, CA); Alexa Fluor 594 labeled secondary antibodies, Invitrogen (Carlsbad, CA). Dominant negative plasmids of ERK2 and JNK1 were kindly provided by Dr. Roger J. Davis [21].

Cell culture and treatment

HMCs were isolated and cultured in RPMI 1640 with 16.7% heat-inactivated fetal bovine serum (FBS) as previously described [22]. HMCs at passages 5 through 8 were grown to 70–80% confluence and were serum starved for 24 hours prior to treatment as necessary. Cells analyzed for PAI-1 mRNA were exposed to vehicle, 0.1 μ M Lat B, 1 μ M Cyto B, 10 μ M Y-27632, 30 μ M PD98059 or 10 μ M SP600125 for 2 hours before stimulated with 10 ng/ml TGF- β 1 for 8 hours. Cells analyzed for ERK and JNK activation were stimulated with 10 ng/ml TGF- β 1 for 15 minutes, following 2 hours pretreatment with 0.1 μ M Lat B and/or 0.05 μ M Jas.

PAI-1 promoter cloning and plasmid construction

To construct the human PAI-1 promoter reporter vector, a fragment of the human PAI-1 promoter containing the sequence from –973 to +133 was amplified by polymerase chain reaction (PCR) using HMCs genomic DNA as a template. The forward primer was 5'-CGATCGGTACCTAAAAGCACACCTGCAAAC-3' and the reverse primer was 5'-CGATCAGATCTCAGAGGTGCCTTGCGATTG-3'. To construct a luciferase reporter gene driven by the PAI-1 promoter, the 1106 bp *Kpn I/Bgl II* PCR product was subcloned into pGL3 basic luciferase vector (Promega, Madison, WI) to generate pPAI-1-Luc. The identity of the reporter gene was confirmed by restriction mapping and sequencing (Midland Molecular Biology Group, Midland, TX).

F-actin staining and immunofluorescence

Serum-starved HMCs in 12-well plates were subjected to the indicated stimulations. After that, cells were washed once with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After three further washes and permeabilization with 1% Triton X-100 in PBS for 5 minutes, cells were washed and blocked with in PBS containing 1% BSA and 5% normal goat serum for 60 minutes. Subsequently, Primary antibodies specific for ERK, JNK and α -actinin were applied for 1 hours at room temperature, and staining was detected with Alexa Fluor 594 conjugated secondary antibody for 1 hours. F-actin was detected using Oregon Green 488 phalloidin (Molecular Probes, Eugene, OR). After further washing, slides were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). Fluorescence images were captured using confocal laser scanning microscopy (Zeiss 510, Germany).

Transient transfection and dual-luciferase reporter assay

Cells were split in 6-well plates at 1.6×10^5 /well the day before transfection. pPAI-1-Luc vector was transiently transfected using Eugene 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. After 3 hours, cells were pretreated with 30 μ M PD98059, 10 μ M SP600125, 1 μ M Cyto B, 0.1 μ M Lat B or 10 μ M Y-27632 for 2 hours before stimulated with 10 ng/ml TGF- β 1 for an additional 20 hours. Cells were then lysed and luciferase activity was read using TD20/20 luminometer (Turner Diagnostics, Sunnyvale, CA). Cells cotransfected with either a dominant negative plasmid of ERK2 or JNK1 were cultured for 24 hours. After that, the cells were serum-deprived for 24

hours, stimulated by 10 ng/ml TGF- β 1 for another 24 hours, and then lysed for luciferase assay. In all transfection experiments, phRL-TK, *Renilla* luciferase expression vector (Promega, Madison, WI), was co-transfected as an internal control for normalization of transfection efficiency.

RNA isolation and real-time RT-PCR analysis

Total cellular RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), quantified, and integrity was tested by gel electrophoresis. 1 μ g of total RNA from each sample was reverse transcribed to cDNA. The gene of interest and the housekeeping gene were reverse-transcribed simultaneously using their specific anti-sense primers in the same reaction. After diluting RT products 1:10 in H₂O, 2 μ l of diluted cDNA samples were amplified using the LightCycler (Roche, Indianapolis, IN). Each gene of interest and the reference gene were analyzed in separate glass capillaries. Following cycling parameters were used for the amplification: denaturation at 95°C for 15 seconds, annealing at 60°C for 5 seconds, and extension at 72°C for 18 seconds. Primers for PAI-1: forward primer 5'-TGCTGGTGAATGCCCTCTACT-3', reverse primer 5'-CGGTCATTCCCAGGTTCTCTA-3'. Primers for ubiquitin: forward primer 5'-ATTTGGGTCGCGGTTCTTG-3', reverse primer 5'-TGCCTTGACATTCTCGATGGT-3'. 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 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, melting curve analyses were performed and representative PCR products were run on agarose gels and visualized by ethidium staining.

Immunoblot assay

After treatment, the cells were washed twice with ice-cold PBS before lysis in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (2 mM EDTA, 1 mM PMSF, 10 μ M leupeptin, 1 μ M pepstatin A, 1 μ g/ml aprotinin) and then centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant, determined for protein content by BCA protein assay (Pierce, Rockford, IL), was mixed with an equal volume of 2 $\times$ SDS sample buffer and boiled for 3 minutes before loading. Cell lysates were resolved on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Bedford, MA) with an electrophoretic transfer unit (Bio-Rad, Hercules, CA). After transfer, membranes were blocked in 5% non-fat dry milk, 0.1% Tween 20 in TBS (TBST) for 1 hour at room temperature and then incubated with the indicated diluted primary antibody in 5% BSA/TBST overnight at 4°C. Membranes were washed three times with TBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibody in TBST for 1 hour at room temperature. After three further washes, immunoreactive bands were detected by ECL reagents (Amersham Bioscience, Piscataway, NJ) and exposed to X-ray film. Immunoreactive bands were scanned with an UMAX PowerLook II Scanner in transparency mode, and densitometric analysis was performed using Kodak 1D Image Analysis Software for Windows.

Immunoprecipitation

HMCs were split in 150 mm dish and cultured for 48 hours until 70–80% confluence was reached. After serum-starved for 24 hours, the cells were scraped in a lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β -glycerophosphate, 2.5 mM Sodium pyrophosphate, and 1mM Na₃VO₄) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO). After 15 minutes on ice, cell lysates were centrifuged (14,000g, 10 minutes, 4 °C), and the resulting clarified supernatants were collected. Equal amounts of proteins were incubated with anti- α -actinin antibody (5 μ g) at 4°C for 2

hours. Then, protein G plus-agarose beads were added, and the incubation was continued at 4°C for 1 hour. The beads were pelleted by centrifugation and washed three times with PBS. After the final wash, aspirate and discard supernatant and resuspend pellet in 40 µl 2× sample buffer. After boiling for 2–3 minutes, proteins were separated by SDS-PAGE and detected by immunoblot with specific primary antibodies as shown above.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (PIERCE Biotechnology, Rockford, IL) according to the instructions. Briefly, subconfluent HMCs were stimulated with 10 ng/ml TGF-β1 for 30, 60 minutes, following 2 hours pretreatment with 0.1 µM Lat B and/or 0.05 µM Jas. The cells were then washed, collected in cold phosphate-buffered saline, and resuspended in ice-cold hypotonic homogenization buffer, CER I containing protease inhibitors. After 10 minutes swelling on ice, 5.5% (v/v) detergent, CER II, was added to the cells followed by vigorous vortexing for 10 seconds. Nuclei were pelleted at 16,000g for 5 minutes and resuspended in nuclear extraction buffer, NER. Following rotation at 4 °C for 40 minutes, the nuclear lysates were centrifuged for 10 minutes, and the supernatant was collected for EMSA.

The double-strand oligonucleotide corresponding to the AP-1 binding site in the human PAI-1 promoter was end-labeled with DIG-11-dUTP and terminal transferase (Roche Diagnostics Corporation). 30 fmol of DIG-labeled human PAI-1 AP-1 probes were incubated with 5 µg of nuclear protein extract in binding buffer (0.2 M potassium cacodylate, 25mM Tris-HCl, 0.25 mg/ml BSA, pH 6.6, 2 µg of poly (dI-dC), 0.1 µg of poly L-lysine) for 20 minutes at room temperature. In competition experiments, the extract was preincubated for 30 minutes with a 200-fold molar excess of cold competitor. For supershift analysis, nuclear extracts were preincubated for 30 minutes at room temperature with 4 µg of antibody followed by the addition of DIG-labeled probe. The binding reactions were separated by nondenaturing 6% polyacrylamide gel electrophoresis at 4°C. After electrophoresis, the gel was transferred onto nylon membrane with electro-blotting followed by UV cross-linking for 50 seconds. After washing and blocking, the nylon membrane was incubated with anti-Digoxigenin-AP for 30 minutes at room temperature. The bands were detected by CSPD reagent and exposed to X-ray film. Oligonucleotides used in EMSA were as follows: human PAI-1 AP-1 wild-type sequence 5'-AGGTTG TTGACACAAGAGAGC-3'; human PAI-1 AP-1 mutant sequence 5'-AGGTTG TGGACATGAGAGAGC-3'; Consensus SBE sequence 5'-AGTATGTCTAGACTGA-3'.

Statistical analysis

Data are presented as mean ± s.e.m and represent the averages of at least three independent experiments. Differences between the mean values were analyzed by Student's *t*-test. A *P* value of <0.05 was considered significant.

Results

Effects of F-actin disruption on PAI-1 gene expression

Recent investigations suggested that the reorganization of actin cytoskeleton might be closely associated with ECM accumulation. We therefore hypothesize that the integrity of F-actin may play a role in TGF-β1-mediated PAI-1 gene transcription. As shown in Fig. 1A, TGF-β1 increased PAI-1 mRNA level by 2.3-fold after 8hours treatment. Lat B, a marine toxin that disrupt actin polymerization by binding one to one with monomeric G-actin, suppressed basal PAI-1 mRNA by 54% and TGF-β1-induced PAI-1 mRNA level by 50%. Cyto B and Y-27632 treatment gave comparable results. Cyto B, a fungal toxin, binds to the barbed end of actin

filaments, inhibiting actin polymerization, while Y-27632 is a selective inhibitor of Rho-associated protein kinase.

To further confirm the effects of F-actin depolymerization on PAI-1 expression, we then evaluated the involvement of Lat B, Cyto B or Y-27632 in PAI-1 gene transcription using a PAI-1 promoter construct, pPAI-1-Luc, containing the sequence from -973 to +133 of the human PAI-1 gene fused with a luciferase reporter gene. 3 hours after transfection, HMCs were pretreated with Lat B followed by 24 hours stimulation with TGF- β 1. Fig. 1B showed that PAI-1 promoter activity was induced 4.3-fold by TGF- β 1. Lat B inhibited basal and TGF- β 1-induced promoter activities 37% and 33%, respectively. Similarly, the inhibitory effects of Cyto B and Y-27632 on PAI-1 gene transcription are comparable to those of Lat B although Cyto B appeared to have a stronger inhibitory effect. Therefore, agents that inhibit actin disassembly directly or through signal cascades also cause inhibit both basal and TGF- β 1-stimulated PAI-1 transcription in HMCs.

Association of ERK and JNK with the actin cytoskeleton

Association of ERK with F-actin been previously reported in other cell types [23,24]. As shown in Fig. 2A using confocal microscopy, ERK was distributed diffusely in HMCs, with a strong signal in perinuclear regions, as well as along F-actin, which was prominent in cell protrusions (arrow). Although JNK was detected in cytosol, stronger colocalization with F-actin was also apparent. TGF- β 1 stimulation promoted the dissociation of ERK and JNK from F-actin. Dissociation occurred even though we also found that TGF- β 1 can induce actin polymerization (Fig. 2B). ERK has also been reported to bind the actin cross-linking protein, α -actinin [24]. Fig. 2C shows that antibodies to α -actinin co-immunoprecipitated ERK and JNK in HMCs as well. Fig. 2D demonstrates the distribution of α -actinin along actin filaments detected by immunofluorescence.

Effects F-actin reorganization on ERK and JNK activation

If ERK and JNK are associated with α -actinin in actin stress fibers, then it is plausible that disruption of the stress fibers could affect MAPK signaling. Therefore, we assessed the effects of actin filaments disruption on ERK and JNK activation. As shown in Fig. 3A, TGF- β 1 stimulation increased ERK phosphorylation by 2.9-fold compared to unstimulated cells at 15 minutes. Preincubation with Lat B at 0.1 μ M inhibited basal and TGF- β 1-stimulated ERK activation by 54% and 45%. Jasplakinolide (Jas) is a pharmacologic agent that stabilizes the actin cytoskeleton [25]. We found that 0.05 μ M Jas rescued ERK activation in the presence of Lat B in both basal and TGF- β 1-stimulated conditions. In addition, both Lat B and Jas had no effects on total ERK levels. We also found that Lat B and/or Jas had similar effects on JNK activation in HMCs (Fig. 3B). These results suggest that disruption of F-actin by Lat B decreased TGF- β 1-induced PAI-1 expression in HMCs by interfering with ERK and JNK signaling.

Effects F-actin disruption on TGF- β 1/Smad activation

TGF- β 1 exerts most of its major effect through the Smad pathways [26]. Previous investigations also found that Smads mediated TGF- β 1-induced PAI-1 gene expression [27]. We then examined whether actin reorganization affects Smad activation in HMCs, as it does MAPKs phosphorylation. As shown in Fig. 4, TGF- β 1 activated Smad2/3 as early as 15 minutes. F-actin disruption by 0.1 μ M Lat B, 1 μ M Cyto B or 10 μ M Y-27632 had no effects on Smad phosphorylation, even with 10-fold higher concentrations of F-actin inhibitors.

Effects of inhibiting ERK or JNK MAPK signaling pathways on PAI-1 gene expression

As reported here by our laboratory and by others [15], TGF- β 1 can activate both the ERK and JNK MAP kinase pathways in HMCs. Although TGF- β 1 is known to increase expression of PAI-1 in HMCs, whether TGF- β 1 requires the ERK or the JNK pathways for induction of PAI-1 expression has not been examined yet in HMCs. Thus, we first used biochemical inhibitors of ERK and JNK to assess their effects on PAI-1 expression. Although inhibitors may cause non-specific effects, PD98059 has been reported to be a specific inhibitor of ERK with minimal inhibition of other kinases at doses higher than that used in our experiments [28]. SP600125 was reported to cause >20-fold selective inhibition of JNK with an IC₅₀ of 10 μ M [29], the same dose as used in this study. PD98059 or SP600125 were incubated with cells for 2 hours prior to stimulation with TGF- β 1 for an addition 24 hours. As shown in Fig. 5A, the basal and TGF- β 1-induced PAI-1 promoter activities were abrogated by 54% and 55% at 10 μ M PD98059. SP600125 had no significant effect on basal PAI-1 promoter activity, while it decreased TGF- β 1-induced PAI-1 promoter activity by 51% at 10 μ M.

Because the nonspecific effects may exist between these inhibitors, we further determined the abilities of dominant negative plasmids of ERK2 and JNK1 to effect PAI-1 promoter activity. As shown in Fig. 5B, specifically inhibiting ERK or JNK by this means significantly blocked TGF- β 1-induced PAI-1 promoter activity. These data further support the hypothesis that both the ERK and the JNK signaling pathways contribute to TGF- β 1-induced PAI-1 expression.

Effects of F-actin rearrangement on TGF- β 1-induced AP-1 DNA binding activity in human PAI-1 promoter

The human PAI-1 promoter contains a region (−711 to −717) which has DNA sequences homologous to the activator protein-1 (AP-1) binding site [30]. We evaluated whether reorganization of F-actin affects TGF- β 1-induced AP-1 DNA binding activity to synthetic nucleotides corresponding to this human PAI-1 promoter sequence. As shown in Fig. 5A, TGF- β 1 stimulated AP-1 DNA binding activity in a time-dependent manner with maximum at 30 minutes. The binding interaction was specific, since it was blocked by competition with excess unlabeled AP-1 wide-type probe but not by point-mutated AP-1 or unrelated SBE probes (Fig. 6A, compare lane 5 and 6–7). Using specific antibodies, we identified c-fos and c-jun as components of the induced DNA-protein complexes (Fig. 6B, lane 4, 5). Disruption of actin cytoskeleton by Lat B inhibited TGF- β 1-induced AP-1 DNA binding activity (Fig. 6C, compare lane 3 and 4), while Jas can antagonize the effect of Lat B, partly restoring TGF- β 1-induced AP-1 DNA binding activity (Fig. 6C, compare lane 4 and 5).

Discussion

The actin cytoskeleton plays important roles in many cell functions including cell shape, migration, intracellular transport, and contraction. The accumulating evidence of recent investigations indicates that the actin cytoskeletal structure plays a coordinating role in regulating myofibroblast differentiation and accumulation of ECM deposits and fibrosis. Our laboratory recently demonstrated that the state of actin polymerization controls the expression of α -SMA and hypertrophy in mesangial cells, two major features of the profibrotic, myofibroblast phenotype [20]. Rearrangement of the actin cytoskeleton has been reported to affect α 1 (I) collagen expression and the activities of MMPs [16]. The small GTPase Rho is involved in actin polymerization, with Rho-kinases as its downstream effectors [31,32]. Rho-kinase inhibition was found to markedly decrease TGF- β 1-induced α -SMA expression, collagen accumulation, and the extent of fibrosis [17,18,33]. These observations suggest that the reorganization of actin cytoskeleton may play a role in ECM accumulation.

In the present study, we evaluated how changes in actin cytoskeletal organization affect PAI-1 expression in HMCs and found that disrupting the F-actin with actin depolymerization agents, Lat B and Cyto B or with Rho-kinase inhibitor, Y-27632 significantly inhibited basal and TGF- β 1-stimulated PAI-1 mRNA levels and PAI-1 promoter activity. Further analysis demonstrated that disassembly of F-actin by Lat B decreased basal and TGF- β 1-induced ERK and JNK phosphorylation, and this inhibition was antagonized by Jas, an agent promoting F-actin polymerization. We also found that Lat B inhibited TGF- β 1-induced AP-1 DNA binding activity in human PAI-1 promoter region, while Jas can diminish the effects of LatB. Because PAI-1 is a potential target in renal fibrogenesis, the disassembly of actin cytoskeleton may prevent mesangial matrix accumulation in glomerulus by suppressing PAI-1 expression. These findings also suggested that the dynamic integrity of actin cytoskeleton might play significant roles in regulating AP-1 activation by MAPK cascades.

How does the actin cytoskeleton work in the activation of MAPK/AP-1 signal transduction? We found that both ERK and JNK can co-localize to stress fibers in HMCs, which was partly supported by Leinweber's findings that recombinant ERK co-sedimented with purified actin filaments and induced a fluorescence change in pyrene-labeled F-actin [23]. We also found that α -actinin can form complexes with ERK and JNK in mesangial cells. α -Actinin is composed of two identical anti-parallel peptides, including two actin domains (also known as CH domains) at amino-terminus [34]. It has been noted that the CH domain has a new potential function; that is, interacting with signaling molecules [34]. Purified α -actinin has been shown to interact with recombinant ERK through its CH domain [24]. Our data also suggested that JNK is bound to stress fibers and that changes in actin cytoskeleton may affect JNK signal transduction, although we find no previous reports that α -actinin or F-actin can bind JNK directly. However, MEKK1, which can bind to JNK and regulate its activity, has been reported to be associated with α -actinin and actin stress fibers [35]. We also found that the interaction of ERK or JNK was disrupted by TGF- β 1 stimulation. This change may reflect release and translocation of the activated MAPKs for participation in AP-1 activation. The interaction of MAPKs with actin cytoskeleton raises the possibility that the actin cytoskeleton may function as a scaffold that tether MAPKs and their upstream activators into specific modules, thereby facilitating the activation of MAPKs and their downstream effectors, such as AP-1 (Fig. 7). The disassembly of actin filaments may therefore abrogate MAPKs activation by TGF- β 1. Thus, the actin cytoskeleton with inherent dynamic stability appears to be crucial to MAPK/AP-1 signal transduction.

A significant body of research supports that TGF- β 1 plays a pivotal role in the pathogenesis of glomerulosclerosis, although the precise intracellular mechanisms have not been fully elucidated. While Smads are considered the primary intracellular downstream effectors of TGF- β 1 signal pathway [36–38], there is increasing evidence that TGF- β 1-induced MAPKs activation plays an important role in regulating ECM accumulation and degradation [15,39]. In HMCs, TGF- β 1 can activate ERK and JNK MAPK pathways, which then activate their downstream transcription factors such as AP-1. Because there are several consensus AP-1 binding sites in PAI-1 promoter [30], TGF- β 1 may induce PAI-1 expression at least in part through activating MAPK pathways. In our research, we found that inhibiting ERK or JNK either by their biochemical inhibitors or by their dominant negative mutants decreased TGF- β 1-induced human PAI-1 promoter activities and that TGF- β 1 stimulation promoted the binding activity of AP-1 to consensus AP-1 binding site derived from human PAI-1 promoter. These results indicate that MAPK/AP-1 pathways play an important role coordinating the effects of TGF- β 1 on PAI-1 gene expression with the state of organization of the actin cytoskeleton. The fact that that basal PAI-1 expression is also dependent on ERK, JNK and an intact actin cytoskeleton suggests that these factors are likely to play important roles in modulating the effects of additional factors, including growth factors and cell-to-matrix interactions on PAI-1 expression. However, we have also observed that basal PAI-1 expression

is largely inhibited by anti-TGF- β 1 antibody (not shown) indicating that inhibition of endogenously produced TGF- β 1 may be largely responsible for the effects of agents on basal PAI-1 expression.

Since TGF- β 1/Smads participate in PAI-1 gene expression [27], we also examined the possibility of actin reorganization on Smad activation and found that TGF- β 1-mediated Smad phosphorylation is actin polymerization independent, which is coincident with Dong's finding [40]. They further reported that Smad activation is closely associated with microtubule reorganization. The cooperation between MAPKs and Smads pathways in TGF- β 1-stimulated human PAI-1 gene expression deserves further investigation.

In summary, we present new mechanistic evidence of a potential function for the actin cytoskeleton in gene expression. These results suggest that the dynamic integrity of the actin cytoskeleton plays an important role in modulating the effect of TGF- β 1 on PAI-1 gene expression via MAPK signal cascades. The actin cytoskeleton itself may serve as a frontier effector of glomerular response to renal injury, contributing to the distributions of actin-associated signaling molecules of the glomerular mesangial cells as well as to altered cellular mechanical characteristics. Therefore, the actin cytoskeleton may represent a new therapeutic target of glomerulosclerosis.

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References

1. Schnaper HW, Hayashida T, Hubchak SC, Poncelet AC. TGF-beta signal transduction and mesangial cell fibrogenesis. *Am J Physiol Renal Physiol* 2003;284:F243–F252. [PubMed: 12529270]
2. Rerolle JP, Hertig A, Nguyen G, Sraer JD, Rondeau EP. Plasminogen activator inhibitor type 1 is a potential target in renal fibrogenesis. *Kidney Int* 2000;58:1841–1850. [PubMed: 11044203]
3. Feng L, Tang WW, Loskutoff DJ, Wilson CB. Dysfunction of glomerular fibrinolysis in experimental antiglomerular basement membrane antibody glomerulonephritis. *J Am Soc Nephrol* 1993;3:1753–1764. [PubMed: 8329670]
4. Moll S, Menoud PA, Fulpius T, Pastore Y, Takahashi S, Fossati L, Vassalli JD, Sappino AP, Schifferli JA, Izui S. Induction of plasminogen activator inhibitor type 1 in murine lupus-like glomerulonephritis. *Kidney Int* 1995;48:1459–1468. [PubMed: 8544402]
5. Tomooka S, Border WA, Marshall BC, Noble NA. Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. *Kidney Int* 1992;42:1462–1469. [PubMed: 1474781]
6. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA. Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci U S A* 1993;90:1814–1818. [PubMed: 7680480]
7. Xu Y, Hagege J, Mougenot B, Sraer JD, Ronne E, Rondeau E. Different expression of the plasminogen activation system in renal thrombotic microangiopathy and the normal human kidney. *Kidney Int* 1996;50:2011–2019. [PubMed: 8943484]
8. Wang Y, Pratt JR, Hartley B, Evans B, Zhang L, Sacks SH. Expression of tissue type plasminogen activator and type 1 plasminogen activator inhibitor, and persistent fibrin deposition in chronic renal allograft failure. *Kidney Int* 1997;52:371–377. [PubMed: 9263992]
9. Yamamoto T, Noble NA, Cohen AH, Nast CC, Hishida A, Gold LI, Border WA. Expression of transforming growth factor-beta isoforms in human glomerular diseases. *Kidney Int* 1996;49:461–469. [PubMed: 8821830]
10. Nicholas SB, Aguiniga E, Ren Y, Kim J, Wong J, Govindarajan N, Noda M, Wang W, Kawano Y, Collins A, Hsueh WA. Plasminogen activator inhibitor-1 deficiency retards diabetic nephropathy. *Kidney Int* 2005;67:1297–1307. [PubMed: 15780082]

11. Huang Y, Haraguchi M, Lawrence DA, Border WA, Yu L, Noble NA. A mutant, noninhibitory plasminogen activator inhibitor type 1 decreases matrix accumulation in experimental glomerulonephritis. *J Clin Invest* 2003;112:379–388. [PubMed: 12897205]
12. Lund LR, Riccio A, Andreasen PA, Nielsen LS, Kristensen P, Laiho M, Saksela O, Blasi F, Dano K. Transforming growth factor-beta is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. *EMBO J* 1987;6:1281–1286. [PubMed: 3111844]
13. Krag S, Osterby R, Chai Q, Nielsen CB, Hermans C, Wogensen L. TGF-beta1-induced glomerular disorder is associated with impaired concentrating ability mimicking primary glomerular disease with renal failure in man. *Lab Invest* 2000;80:1855–1868. [PubMed: 11140698]
14. Baricos WH, Cortez SL, Deboisblanc M, Xin S. Transforming growth factor-beta is a potent inhibitor of extracellular matrix degradation by cultured human mesangial cells. *J Am Soc Nephrol* 1999;10:790–795. [PubMed: 10203363]
15. Hayashida T, Poncelet AC, Hubchak SC, Schnaper HW. TGF-beta1 activates MAP kinase in human mesangial cells: a possible role in collagen expression. *Kidney Int* 1999;56:1710–1720. [PubMed: 10571779]
16. Hubchak SC, Runyan CE, Kreisberg JI, Schnaper HW. Cytoskeletal rearrangement and signal transduction in TGF-beta1-stimulated mesangial cell collagen accumulation. *J Am Soc Nephrol* 2003;14:1969–1980. [PubMed: 12874450]
17. Murata T, Arai S, Nakamura T, Mori A, Kaido T, Furuyama H, Furumoto K, Nakao T, Isobe N, Imamura M. Inhibitory effect of Y-27632, a ROCK inhibitor, on progression of rat liver fibrosis in association with inactivation of hepatic stellate cells. *J Hepatol* 2001;35:474–481. [PubMed: 11682031]
18. Nagatoya K, Moriyama T, Kawada N, Takeji M, Oseto S, Murozono T, Ando A, Imai E, Hori M. Y-27632 prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. *Kidney Int* 2002;61:1684–1695. [PubMed: 11967018]
19. Shimizu Y, Dobashi K, Iizuka K, Horie T, Suzuki K, Tukagoshi H, Nakazawa T, Nakazato Y, Mori M. Contribution of small GTPase Rho and its target protein rock in a murine model of lung fibrosis. *Am J Respir Crit Care Med* 2001;163:210–217. [PubMed: 11208648]
20. Patel K, Harding P, Haney LB, Glass WF 2nd. Regulation of the mesangial cell myofibroblast phenotype by actin polymerization. *J Cell Physiol* 2003;195:435–445. [PubMed: 12704653]
21. Chen YR, Wang X, Templeton D, Davis RJ, Tan TH. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem* 1996;271:31929–31936. [PubMed: 8943238]
22. Stephenson LA, Haney LB, Hussaini IM, Karns LR, Glass WF 2nd. Regulation of smooth muscle alpha-actin expression and hypertrophy in cultured mesangial cells. *Kidney Int* 1998;54:1175–1187. [PubMed: 9767533]
23. Nakamura H, Nagaoka N, Hirata A, Inoue M, Ozawa H, Yamamoto T. Distribution of actin filaments, non-muscle myosin, M-Ras, and extracellular signal-regulated kinase (ERK) in osteoclasts after calcitonin administration. *Arch Histol Cytol* 2005;68:143–150. [PubMed: 16079460]
24. Leinweber BD, Leavis PC, Grabarek Z, Wang CL, Morgan KG. Extracellular regulated kinase (ERK) interaction with actin and the calponin homology (CH) domain of actin-binding proteins. *Biochem J* 1999;344:117–123. [PubMed: 10548541]
25. Bubb MR, Spector I, Beyer BB, Fosen KM. Effects of jaspaklinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations. *J Biol Chem* 2000;275:5163–5170. [PubMed: 10671562]
26. Poncelet AC, de Caestecker MP, Schnaper HW. The transforming growth factor-beta/SMAD signaling pathway is present and functional in human mesangial cells. *Kidney Int* 1999;56:1354–1365. [PubMed: 10504488]
27. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J* 1998;17:3091–3100. [PubMed: 9606191]
28. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000;351:95–105. [PubMed: 10998351]

29. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A* 2001;98:13681–13686. [PubMed: 11717429]
30. Keeton MR, Curriden SA, van Zonneveld AJ, Loskutoff DJ. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. *J Biol Chem* 1991;266:23048–23052. [PubMed: 1744101]
31. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995;81:53–62. [PubMed: 7536630]
32. Edlund S, Landstrom M, Heldin CH, Aspenstrom P. Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. *Mol Biol Cell* 2002;13:902–914. [PubMed: 11907271]
33. Masszi A, Di Ciano C, Sirokmany G, Arthur WT, Rotstein OD, Wang J, McCulloch CA, Rosivall L, Mucsi I, Kapus A. Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial-mesenchymal transition. *Am J Physiol Renal Physiol* 2003;284:F911–F924. [PubMed: 12505862]
34. Otey CA, Carpen O. Alpha-actinin revisited: a fresh look at an old player. *Cell Motil Cytoskeleton* 2004;58:104–111. [PubMed: 15083532]
35. Christerson LB, Vanderbilt CA, Cobb MH. MEKK1 interacts with alpha-actinin and localizes to stress fibers and focal adhesions. *Cell Motil Cytoskeleton* 1999;43:186–198. [PubMed: 10401575]
36. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;390:465–471. [PubMed: 9393997]
37. Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998;95:737–740. [PubMed: 9865691]
38. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700. [PubMed: 12809600]
39. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584. [PubMed: 14534577]
40. Dong C, Li ZRA Jr, Feng XH, Goldschmidt-Clermont PJ. Microtubule binding to Smads may regulate TGF-beta activity. *Mol Cell* 2000;5:27–34. [PubMed: 10678166]

Fig 1. Effects of F-actin disruption on PAI-1 gene expression

(A) Effects of F-actin disruption on PAI-1 mRNA levels. Subconfluent HMCs were serum-starved for 24 hours and then pretreated for 2 hours with 0.1 μ M Lat B, 1 μ M CytoB or 10 μ M Y27632 followed by stimulation with 10 ng/ml TGF- β 1 for 8 hours. Cellular RNA was collected to assess the changes of PAI-1 mRNA levels by real-time RT-PCR. (B) Effects of F-actin disruption on PAI-1 promoter activity. HMCs were transfected with PAI-1 promoter construct, pPAI-1-Luc, together with pRL-TK, *Renilla* luciferase expression vector, as an internal control. 3 hours after transfection, cells were incubated with Lat B, CytoB or Y27632 for 2 hours prior to the exposure to TGF- β 1 for 24 hours. Promoter activity was assessed by dual-luciferase reporter assay. Values represent the means $\pm$ s.e.m of four independent

experiments. $^{\#}P < 0.01$ vs. untreated control; $^{\P}P < 0.05$ vs. untreated control; $^{\dagger}P < 0.005$ vs. untreated control; $^{*}P < 0.001$ vs. untreated control; $^{\P\P}P < 0.05$ vs. TGF- β 1 treated control; $^{\dagger\dagger}P < 0.005$ vs. TGF- β 1 treated control; $^{**}P < 0.001$ vs. TGF- β 1 treated control.

Fig 2. Association of ERK and JNK with the actin cytoskeleton

(A) HMCs were fixed and stained by indirect immunofluorescence with primary antibodies to ERK and JNK and secondary antibody conjugated with Alexa Fluor 594. F-actin was stained with Oregon Green 488 phalloidin. Bar = 20 μ m. These images demonstrate that ERK and JNK colocalize with actin filaments. TGF- β 1 promotes the dissociation of ERK or JNK with actin filaments. (B) TGF- β 1 induces actin polymerization as early as 15 minutes. (C) Anti- α -actinin antibody was used to pull down endogenous α -actinin and associated proteins. Co-precipitated ERK and JNK were identified by immunoblot assay. (D) HMCs were stained by indirect immunofluorescence with primary antibody to α -actinin. These images confirm colocalization of α -actinin with actin filaments. Bar = 20 μ m.

Fig 3. Effects F-actin reorganization on ERK and JNK activation

Serum-starved HMCs were pretreated with 0.1 μ M Lat B and/or 0.05 μ M Jas for 2 hours before stimulation with 10 ng/ml TGF- β 1 for 15 minutes. Equal amount of total cell lysates were subjected to SDS-PAGE. Immunoblotting was then performed using the indicated antibodies. **(A)** Effects of the reorganization of F-actin on ERK activation. **(B)** Effects of the reorganization of F-actin on JNK activation. Representative blots are shown at the top. The results of densitometric analysis of three separate experiments are shown at the bottom. * $P < 0.001$, # $P < 0.01$, ¶ $P < 0.05$.

Fig 4. Effects F-actin disruption on TGF-β1/Smad activation

Serum-starved HMCs were pretreated with different concentrations of Lat B, Cyto B or Y 27632 for 2 hours before stimulation with 10 ng/ml TGF-β1 for 15 minutes. Equal amount of total cell lysates were subjected to SDS-PAGE. Immunoblotting was then performed using the indicated antibodies. Representative blots are shown at the top. The results of densitometric analysis of three separate experiments are shown at the bottom. * $P < 0.001$.

Fig 5. Effects of inhibiting ERK or JNK signaling pathways on PAI-1 gene expression

(A) Inhibitors of ERK or JNK pathways on PAI-1 gene expression. HMCs were transfected with pPAI-1-Luc, together with phRL-TK as an internal control. 3 hours after transfection, cells were treated with 10 μ M PD98059 or 10 μ M SP600125 for 2 hours. Promoter activity was read after 24 hours of stimulation with 10 ng/ml TGF- β 1. (B) Dominant negative plasmids of ERK2 or JNK1 on PAI-1 gene expression. HMCs were transfected with pPAI-1-Luc and phRL-TK for 24 hours. Then, cells were quiescent for 24 hours. Promoter activity was read after exposure to 10 ng/ml TGF- β 1 for another 24 hours. Values represent the means $\pm$ s.e.m of three independent $^{\#}P < 0.01$ vs. untreated control; $^{##}P < 0.01$ vs. TGF- β 1 treated control; $^{\P}P < 0.05$ vs. TGF- β 1 treated control.

Fig 6. Effects of F-actin rearrangement on TGF-β1-induced AP-1 DNA binding activity in human PAI-1 promoter

(A) Nuclear extracts were prepared from HMCs that were either untreated or treated with 10 ng/ml TGF-β1. EMSA was performed using DIG-labeled AP-1 probes derived from human PAI-1 promoter according to "Materials and Methods". A 200-fold excess of unlabeled competitor, mutant or unrelated oligonucleotides were added as indicated in the figure. (B) Supershifts were performed with DIG-labeled AP-1 probes and specific antibodies to c-Fos and c-Jun. (C) Nuclear extracts were prepared from HMCs that were pretreated with LatB and or Jas for 2 hours followed by treatment with 10 ng/ml TGF-β1 for 30 minutes. EMSA was

performed using DIG-labeled AP-1 probes. Figures are representative of three separate experiments.

Fig 7. Regulation TGF-β1/MAPK cascades by the actin cytoskeleton

TGF-β1 has been shown to stimulate both ERK and JNK pathways in human mesangial cells. The data in this article suggest that actin cytoskeleton may function as a scaffold that tether MAPKs and their upstream activators into a specific, thus facilitating TGF-β1/MAPK pathways and their downstream gene expression.