

Cytoskeletal Rearrangement and Signal Transduction in TGF- β 1–Stimulated Mesangial Cell Collagen Accumulation

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Abstract. TGF- β 1 has been implicated in glomerular extracellular matrix accumulation, although the precise cellular mechanism(s) by which this occurs is not fully understood. The authors have previously shown that the Smad signaling pathway is present and functional in human glomerular mesangial cells and plays a role in activating type I collagen gene expression. It also was determined that TGF- β 1 activates ERK mitogen-activated protein kinase in mesangial cells to enhance Smad activation and collagen expression. Here, it was shown that TGF- β 1 rapidly induces cytoskeletal rearrangement in human mesangial cells, stimulating smooth muscle α -actin detection in stress fibers and promoting focal adhesion complex assembly and redistribution. Disrupting the actin cytoskeleton with cytochalasin D (Cyto D) selectively decreased basal and TGF- β 1–induced cell-layer collagen I and IV accumulation. The balance of matrix metalloproteinases (MMP) and

inhibitors was altered by Cyto D or TGF- β 1 alone, increasing MMP activity, increasing MMP-1 expression, and decreasing tissue inhibitor of matrix metalloproteinase-2 expression. Cyto D also decreased basal and TGF- β 1–stimulated α 1(I) collagen mRNA but did not inhibit TGF- β –stimulated α 1(IV) mRNA expression. A similar decrease in α 1(I) mRNA expression caused by the actin polymerization inhibitor latrunculin B was partially blocked by the addition of jasplakinolide, which promotes actin assembly. The Rho-family GTPase inhibitor *C. difficile* toxin B or the Rho-associated kinase inhibitor Y-27632 also blocked TGF- β 1–stimulated α 1(I) mRNA expression. Cytoskeletal disruption reduced Smad2 phosphorylation but had little effect on mRNA stability, TGF- β receptor number, or receptor affinity. Thus, TGF- β 1–mediated collagen I accumulation is associated with cytoskeletal rearrangement and Rho-GTPase signaling.

The central role of TGF- β in the pathogenesis of glomerulosclerosis is supported by studies of human kidney diseases and animal models (1,2). TGF- β overexpression causes glomerulosclerosis in transgenic mice (3), whereas extracellular matrix (ECM) accumulation in the acute anti-Thy-1 nephritis model is diminished by the administration of anti-TGF- β serum (4); decorin, a natural TGF- β inhibitor (5); or oligodeoxynucleotides antisense to TGF- β (6). *In vitro*, TGF- β enhances glomerular mesangial cell production of collagen and fibronectin (7–9), suppresses the expression of ECM-degrading proteases, and increases the synthesis of ECM protease inhibitors (10,11). These studies support the hypothesis that TGF- β mediates glomerular disease by altering mesangial matrix turnover. Despite these observations, little information is available regarding the intracellular signals that lead to increased accumulation

of ECM in mesangial cells. In contrast to most growth factors, which transduce signals through receptor tyrosine kinases, members of the TGF- β family use serine/threonine kinase receptors (12,13). A novel group of proteins, the Smads, is activated specifically in response to TGF- β superfamily members (14,15). After TGF- β 1 binding, the pathway-restricted Smads, Smad2 and Smad3, are phosphorylated and associate with the common partner Smad, Smad4. The resulting heteromultimer translocates to the nucleus, where it regulates expression of TGF- β target genes by direct binding to promoter sequences and/or by association with other transcription factors (16–18) or with coactivators such as p300/CBP (18). Previously, our laboratory determined that the Smad pathway is activated by TGF- β 1 in human glomerular mesangial cells to mediate type I collagen gene activation through a complex including Sp1, Smad3, and Smad4 (19,20). TGF- β 1 also activates mitogen-activated protein (MAP) kinases, with ERK MAP kinase enhancing TGF- β 1–stimulated type I collagen synthesis (21). These results suggest that other signaling pathways may contribute to TGF- β 1–stimulated ECM accumulation. Another parameter that may affect the mesangial cell matrix is cell shape or cytoskeletal tension (22). The Rho family of GTPases, which includes RhoA, Rac, and Cdc42, has been implicated in the induction of actin stress fibers, focal adhesion assembly, and the formation of filopodia and lamel-

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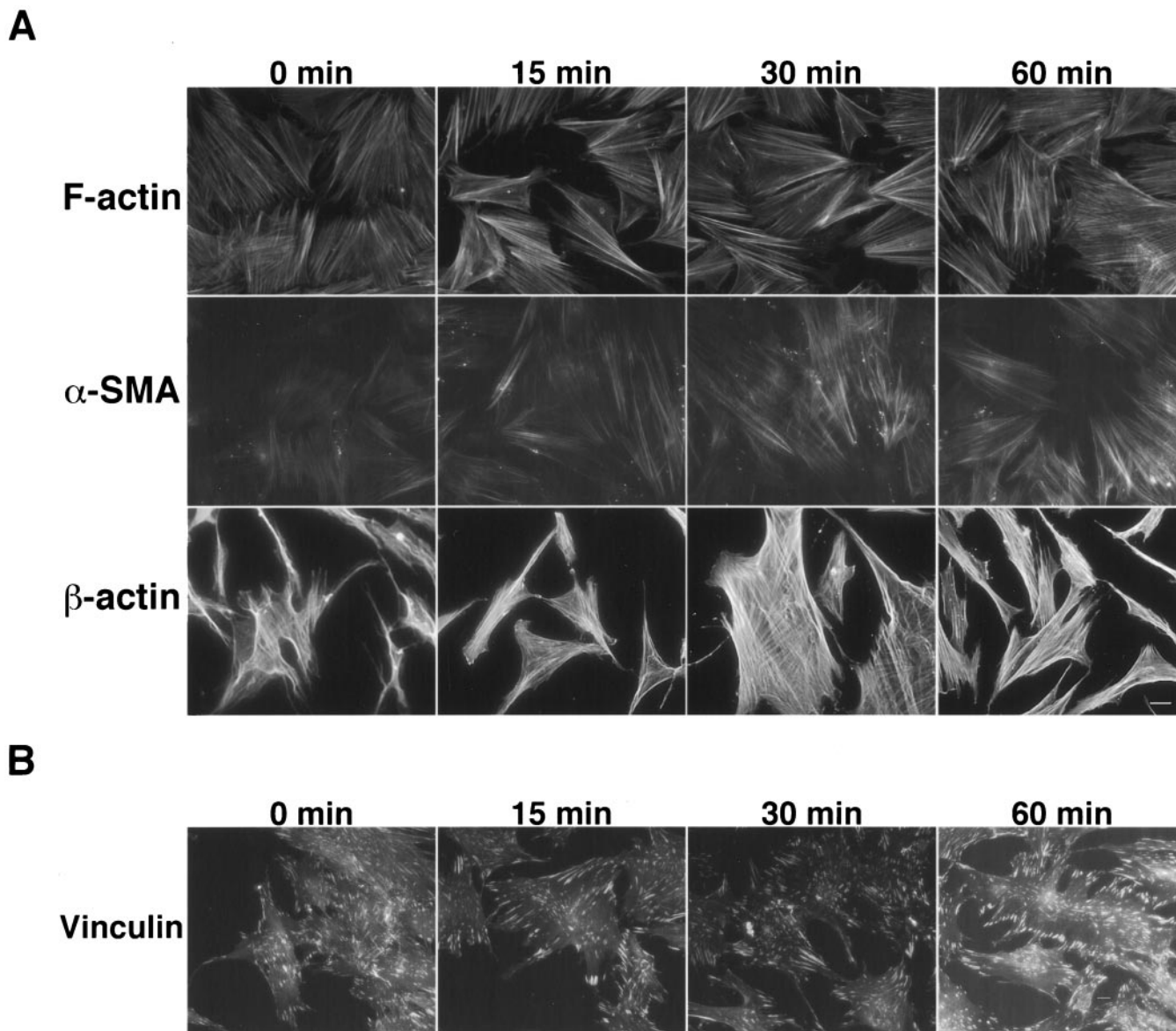


Figure 1. TGF- β 1 treatment modifies the actin cytoskeleton and alters focal adhesions. Serum-starved cells were treated for the indicated periods of time with 1 ng/ml TGF- β 1 before fixation and staining with rhodamine phalloidin, 1:40 anti- α -SMA, 1:1500 anti- β -actin, or 1:400 antivinculin. (A) Total F-actin (top) increases at 15 to 30 min and continues to increase up to 60 min. This reflects increased smooth muscle- α -actin incorporation into stress fibers (center), with little change seen in β -actin (bottom). (B) Focal adhesions, detected by a monoclonal antibody to vinculin, increase in number and size and tend to localize to the periphery. Bar = 20 μ m.

lipodia (23). Kreisberg *et al.* (24) reported that RhoA activation is involved in actin stress fiber assembly in mesangial cells and that cAMP-induced actin stress fiber disassembly is mediated by RhoA inactivation. Downstream effects of these events clearly control cell shape and movement, but it has become apparent that the same pathways also can play a role in gene expression (25,26). Thus, alterations in cell morphology may have functional significance for matrix accumulation. In the present study, we found that administration of TGF- β 1 caused rapid changes in the focal adhesions and stress fibers of mesangial cells. Disruption of the actin cytoskeleton by agents with different mechanisms of action selectively decreased collagen I mRNA expression. TGF- β -stimulated Smad2 phosphoryla-

tion was reduced in the presence of cytoskeletal disruptors, although α 1(I) mRNA stability was unaffected and only minor changes were seen in TGF- β receptor number and affinity. In addition, inhibitors of Rho-GTPase signaling blocked induction of collagen α 1(I) mRNA expression by TGF- β 1. These findings suggest that the cytoskeleton plays a significant role in TGF- β 1-stimulated matrix accumulation.

Materials and Methods

Mesangial Cell Culture

Human mesangial cells were isolated from glomeruli of normal renal cortex as described previously (27). Cells were cultured in DMEM/F12 medium supplemented with 20% heat-inactivated FBS,

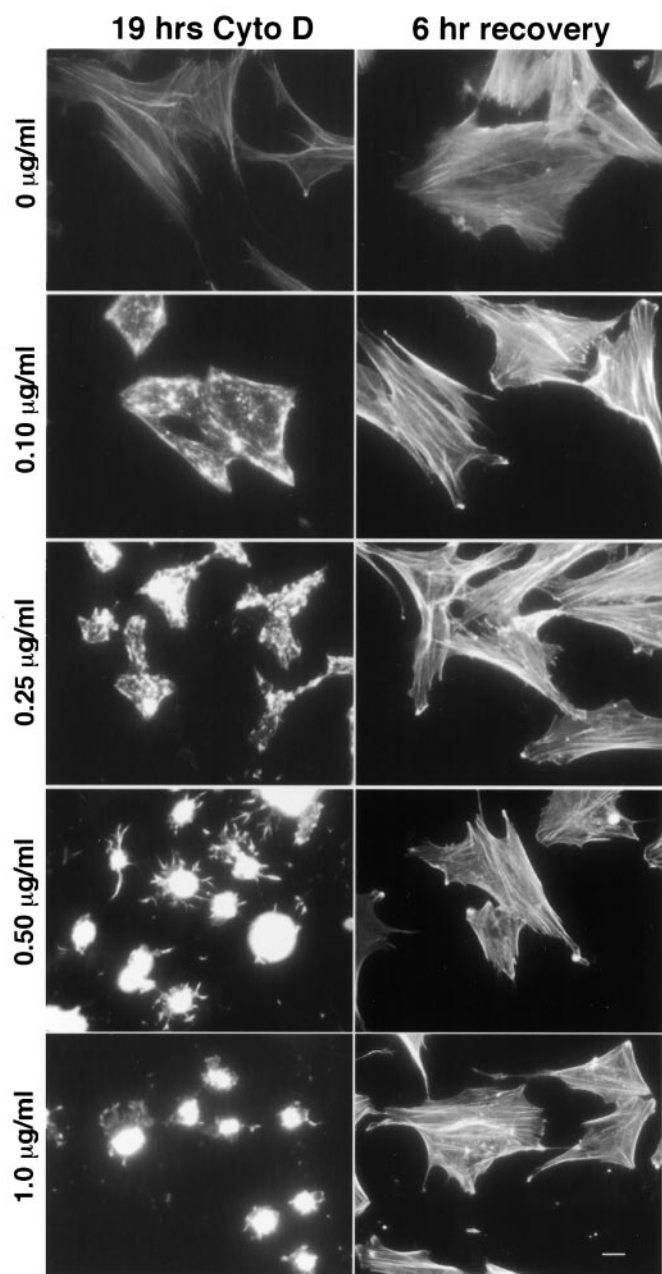


Figure 2. Cytochalasin D (Cyto D) dramatically alters the actin cytoskeleton in mesangial cells. Cells were treated for 19 h under serum-free conditions with vehicle or Cyto D from a 1 mg/ml ethanol stock solution, and F-actin was visualized with rhodamine phalloidin staining. Vehicle-treated cells exhibit abundant stress fibers; the fibers fragment with increasing doses of Cyto D (0.1 to 0.5 $\mu\text{g/ml}$), then are obliterated at a higher dose (1.0 $\mu\text{g/ml}$), causing cells to appear “arborized” (left). Cultures treated with Cyto D for 19 h and then washed free of drug were allowed to recover for 6 h in complete mesangial medium before visualization of F-actin (right). Bar = 20 μm .

glutamine, penicillin/streptomycin, sodium pyruvate, HEPES buffer, and 8 $\mu\text{g/ml}$ insulin (Life Technologies, Gaithersburg, MD) and confirmed as mesangial as described (27). Isolates were mycoplasma-free according to the method of Chen (28) and were used between passages 5 and 8.

Antibodies and Other Reagents

Active, human recombinant TGF- β 1 (R & D Systems, Minneapolis, MN) was reconstituted as a 4 $\mu\text{g/ml}$ stock solution in 4 mM HCl with 1 mg/ml BSA. Cytochalasin D (Cyto D) and actinomycin D were obtained from Sigma Chemicals (St. Louis, MO). Latrunculin B, *C. difficile* toxin B, and Y-27632 were purchased from Calbiochem (San Diego, CA). Jasplakinolide was obtained from Molecular Probes (Eugene, OR). Receptor-binding grade [^{125}I] TGF- β 1 (800 to 2500 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ) and from Perkin-Elmer Life Sciences (Boston, MA). Antibodies were obtained from the following sources: human collagen I and collagen IV, Biodesign International (Saco, ME); smooth muscle α -actin (α -SMA), Dako Corporation (Carpinteria, CA); β -actin and vinculin, Sigma; Smad4, Santa Cruz Biotechnology (Santa Cruz, CA); phospho-Smad2 (pSmad2), Upstate USA (Charlottesville, VA); human tissue inhibitor of matrix metalloproteinase-1 (TIMP-1; MAB1342) and matrix metalloproteinase-1 (MMP-1; AB806, which recognizes both pro and active forms of MMP-1), Chemicon (Temecula, CA). The Chemicon antibody to TIMP-2 (MAB3310) was a gift from Dr. M. S. Stack (Northwestern University Medical School; Chicago, IL). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Promega (Madison, WI) or Santa Cruz Biotechnology. Oregon Green 514 (OG514)-conjugated secondary antibodies and rhodamine phalloidin were from Molecular Probes.

Cell Treatments

Cells seeded on eight-well, gelatin-coated Lab-Tek slides were serum-starved for 24 h before treatment with 1 ng/ml TGF- β 1 or were washed free of serum and exposed to Cyto D or latrunculin B for 19 h. Cells seeded on plastic dishes were grown to 80% confluence; washed free of serum; and exposed to vehicle, Cyto D, latrunculin B, jasplakinolide, or actinomycin D for 1 h before stimulation with 1 ng/ml TGF- β 1. Other cultures were serum-depleted for 24 h with 1% FBS-mesangial medium and exposed to 10 pM Toxin B or 10 μM Y-27632 for 1 h before stimulation with 1 ng/ml TGF- β 1 for 6 h. Cells analyzed for Smad2 activation were grown to 80% confluence before serum starvation and treatment with vehicle or Cyto D or followed by stimulation with 1 ng/ml TGF- β 1. Control cells were exposed to vehicle at a concentration of no more than 0.1%, corresponding to the highest dose used. Conditioned media were collected, centrifuged, aliquoted, and stored at -80°C until required, and cell layers were harvested.

Immunocytochemistry/F-Actin Staining

Cells were fixed with 3.7% formaldehyde in PBS for 20 min and permeabilized with ice-cold 0.5% Triton X-100 in PBS for 7 min or fixed for 10 min with ice-cold methanol/acetone. Primary antibodies (α -SMA, β -actin, or vinculin) were applied for 1 h at room temperature, and specific staining was detected with 30-min incubation of OG514-conjugated secondary antibodies. F-actin was detected in formaldehyde-Triton X-100-fixed slides using rhodamine phalloidin according to the manufacturer’s directions. Slides were mounted with AquaPolymount (Polysciences, Warrington, PA). Fluorescence images were captured using a Photomatrix cooled charged-coupled device digital camera from a Zeiss Axioskop upright microscope.

RNA Isolation and Northern Analysis

Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) and then electrophoresed through a 1.2% agarose-1.1% formaldehyde gel, transferred overnight onto a nylon membrane (MagnaGraph; MSI, Westborough, MA), and immobilized by UV cross-linking with a Stratalinker

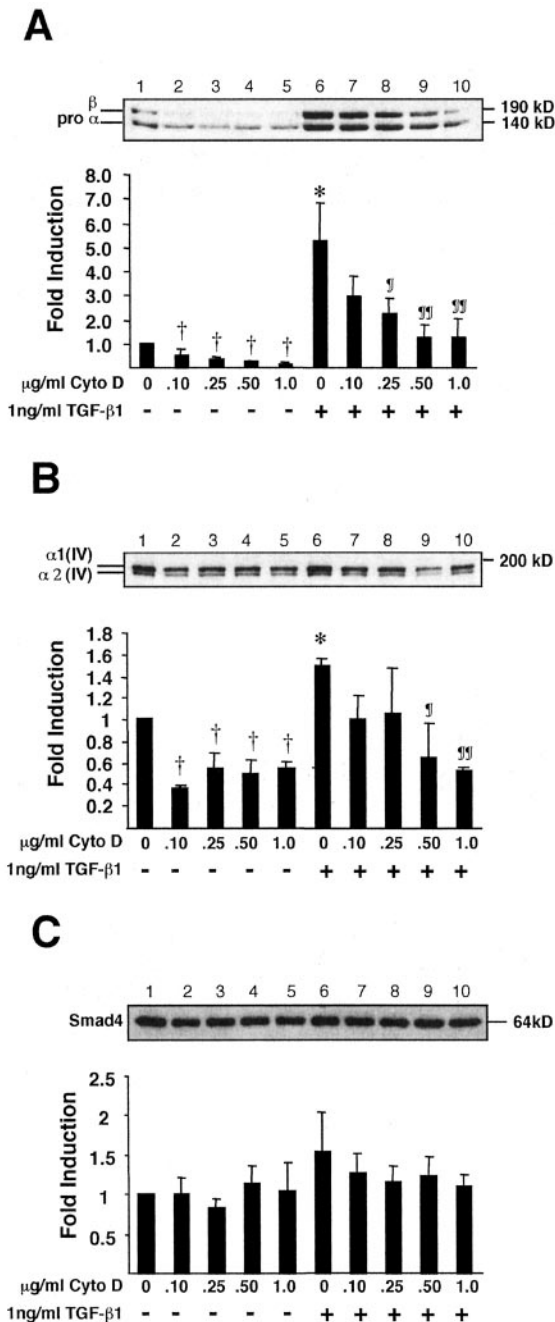


Figure 3. Cyto D diminishes basal and TGF-β1–stimulated collagen I and IV cell layer accumulation. Cells were pretreated for 1 h with Cyto D in serum-free medium before 18-h stimulation with 1 ng/ml TGF-β1. Cell lysates were electrophoresed under reducing conditions for collagen I (20 µg) and under nonreducing conditions for collagen IV (10 µg) before transfer to Immobilon-P membrane and Western analysis. Blots were developed with 1:5000 anti-human collagen I (A) or 1:5000 anti-human collagen IV (B) antibodies. (C) Membranes were then reprobed with anti-Smad4. For each protein, a representative blot is shown at the top and the results of densitometric analysis are shown at the bottom. Values in the graph are expressed as mean fold increase over control ± SEM from four (A and C) or three (B) separate experiments. **P* < 0.04 compared with unstimulated control cells (*t* test); †*P* < 0.015 compared with unstimulated control cells (Fisher PLSD); ¶*P* < 0.04 compared with cells treated with TGF-β1 alone (Fisher PLSD); ¶¶*P* < 0.025 compared with cells treated with TGF-β1 alone (Fisher PLSD).

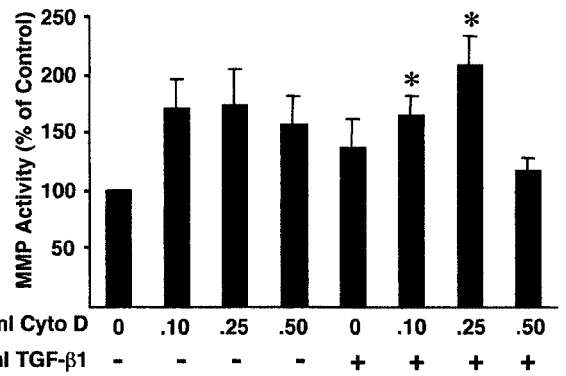


Figure 4. Cyto D enhances TGF-β1–stimulated matrix metalloproteinase (MMP) activity released into conditioned medium. Media were collected from serum-free cultures pretreated for 1 h with Cyto D and then stimulated for 18 h with 1 ng/ml TGF-β1. Basal MMP activity, as measured by hydrolysis of the fluorescence quenching substrate Mca-pro-leu-gly-leu-Dpa-ala-arg-NH₂, is increased by Cyto D treatment alone. TGF-β1 alone had no significant effect on MMP activity, but co-addition of Cyto D up to 0.25 µg/ml caused significant increases. Values in the graph are expressed as mean % of control ± SEM from three separate experiments. **P* < 0.02 compared with unstimulated control cells (*t* test).

2400 (Stratagene, La Jolla, CA). Membranes were probed with ³²P-labeled cDNA probes as described (7). The same blots were successively rehybridized with other probes after stripping membranes with 50% formamide–4X-SSPE–0.25% SDS for 20 min at 70°C.

cDNA Probes

cDNA for human α1(I) (clone Hf677) and α1(IV) collagen chains (1) were obtained from Dr. Y. Yamada (National Institutes of Health, Bethesda, MD). These cDNAs do not cross-hybridize with other known human collagen chain mRNA (Y. Yamada, personal communication). The signals obtained by hybridization with a bovine cDNA for 28S rRNA obtained from Dr. H. Sage (University of Washington, Seattle, WA) were used to correct for loading.

Preparation of Cell Lysates and Western Blot Analysis

Cells were washed twice with ice-cold PBS before lysis in RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM PMSF, 2 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Protein concentrations in cleared cell lysates were determined using a Bradford assay kit (BioRad, Hercules, CA). For detection of collagens, protein extracts were subjected to 6% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% milk in Tris-buffered saline (TBS)–Tween 20 (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20 [vol/vol]) for 1 h at room temperature and then incubated with primary antibodies in blocking buffer for 16 h at 4°C. After incubation with HRP-conjugated secondary antibodies, immunoreactive bands were detected by ECL (Santa Cruz Biotechnology) according to the manufacturer’s directions. For detection of C-terminal phospho-Smad2 and total Smad2, protein extracts were separated by 8% SDS-PAGE. Membranes were probed with anti-pSmad2 and secondary antibody before ECL detection as described above. Membranes were then stripped with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8) at 60°C for 20 min and washed with TBS-Tween 20

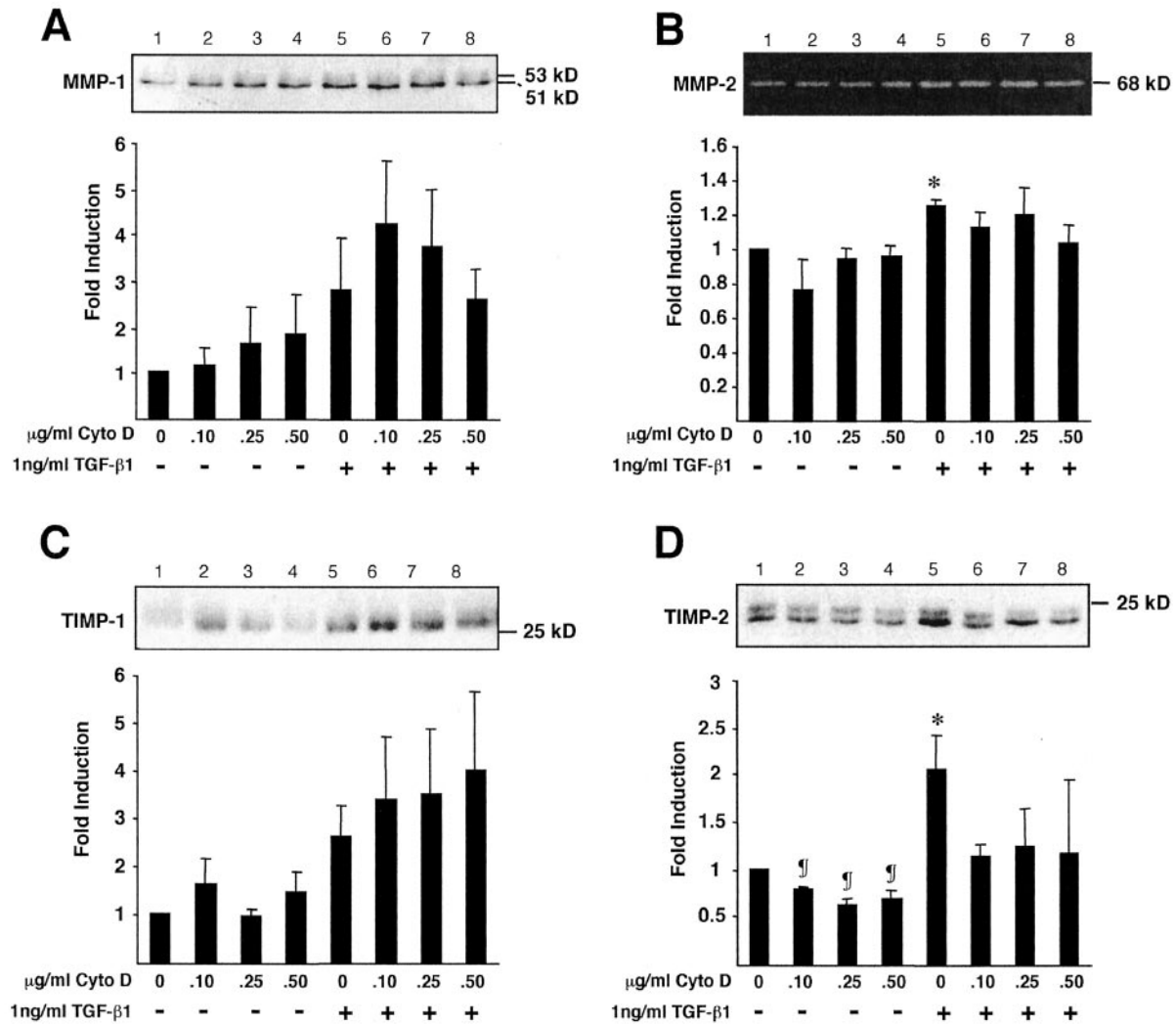


Figure 5. Cyto D affects the concentration of MMP and tissue inhibitor of matrix metalloproteinases (TIMP) released into conditioned medium. Conditioned media (36 $\mu\text{l}/\text{lane}$) from cells treated as described in Figure 4 were electrophoresed under reducing conditions before transfer to Immobilon-P membrane and Western analysis. Blots were developed with 1 $\mu\text{g}/\text{ml}$ anti-MMP-1 (A), 1:200 anti-TIMP-1 (C), or 1 $\mu\text{g}/\text{ml}$ TIMP-2 (D). MMP-2 was analyzed by gelatin zymography (B). Cyto D dose-dependently increases basal levels of MMP-1 protein (A) and augments TGF- β 1-induced increases, mirroring the profile seen in Figure 4 for MMP activity. Neither TGF- β 1 nor Cyto D, alone or in combination, affect the activation of MMP-2, although TGF- β 1 alone significantly increased the amount of 68-kD protein (B). Cyto D had no effect on basal or stimulated TIMP-1 protein (C) but significantly decreased basal levels of TIMP-2 and abrogated TGF- β 1-induced increases (D). Representative blots/gels are shown at the top and the results of densitometric analysis are shown at the bottom. Values in the graph are expressed as mean fold increase over control \pm SEM from three separate experiments. * $P < 0.05$ compared with control cells (t test); $\text{¶}P < 0.05$ compared with unstimulated control cells (Fisher PLSD).

before being reprobed for total Smad2 protein. For detection of MMP-1 and TIMP, equal volumes of conditioned media (36 μl) were prepared with Laemmli buffer and separated on 10% (MMP-1) or 12% (TIMP) SDS-PAGE and transferred to Immobilon-P membranes. Membranes were blocked in 3% BSA-TBS-Tween 20 and then probed with antibodies against MMP-1, TIMP-1, and TIMP-2 for 1 h at room temperature or overnight at 4°C before developing with HRP-conjugated secondary antibodies and ECL reagent.

MMP Activity

Total net MMP activity in conditioned media was determined using the fluorescence quenching substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ dissolved in DMSO as described (29). Briefly, duplicate ali-

quots of conditioned media (100 μl) were mixed with 100 μl of 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, and DMSO (2% vol/vol), containing 5 μM substrate in 96-well Greiner black plates and incubated at 37°C in the dark for 20 to 24 h. After incubation, substrate hydrolysis was measured using a Molecular Devices Spectra Max Gemini spectrofluorimeter (Sunnydale, CA) with excitation and emission wavelengths set at 326 and 396, respectively.

Gelatin Zymography

Equal volumes of conditioned media (36 μl) were prepared with Laemmli buffer under nonreducing conditions and separated on 10% SDS-PAGE gels containing 1 mg/ml gelatin. Gels were developed according to standard methods (27).

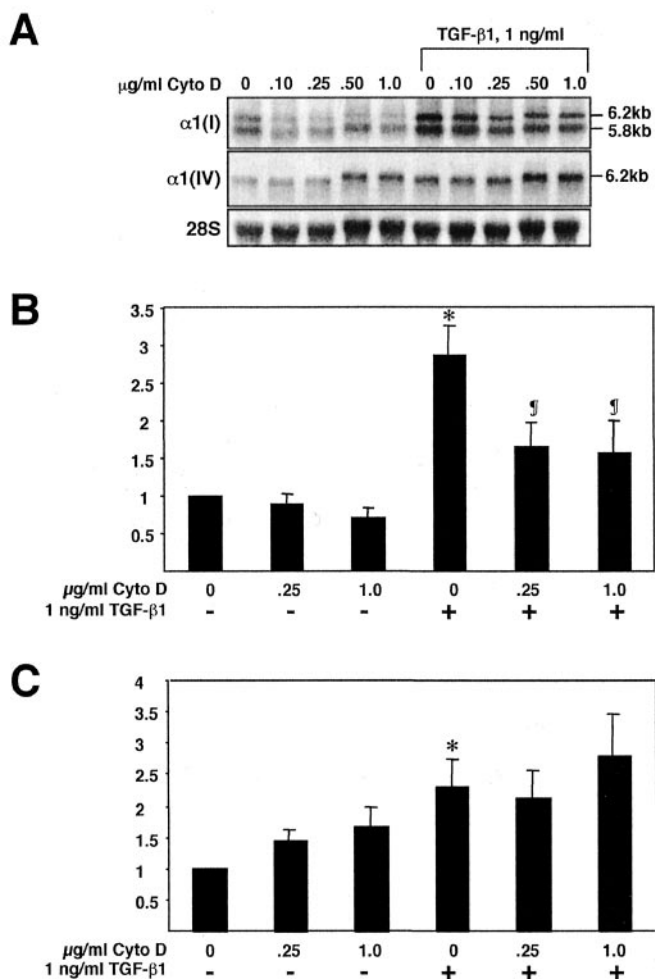


Figure 6. Effect of Cyto D on $\alpha 1(I)$ and $\alpha 1(IV)$ expression. Cells were pretreated for 1 h with Cyto D in serum-free medium before 18-h stimulation with 1 ng/ml TGF- $\beta 1$. Total cellular RNA was isolated and subjected to denaturing gel electrophoresis (8 μ g/lane) before transfer to a nylon membrane. Blots were sequentially probed for mRNA expression of the $\alpha 1$ -chain of types I and IV collagen. (A) Cyto D slightly decreased basal and significantly decreased TGF- $\beta 1$ -stimulated steady-state expression of $\alpha 1(I)$ message. Both basal and TGF- $\beta 1$ -stimulated $\alpha 1(IV)$ message levels were slightly increased in the presence of Cyto D. Blots are representative of at least three separate experiments. (B) Densitometric representation of selected Cyto D doses for $\alpha 1(I)$ mRNA corrected for loading with 28S mRNA represented as fold induction over control \pm SEM from six separate experiments. * $P < 0.001$ compared with unstimulated control cells (t test); § $P < 0.05$ compared with cells treated with TGF- $\beta 1$ alone (Fisher PLSD). (C) Densitometric representation of selected Cyto D doses for $\alpha 1(IV)$ mRNA corrected for loading with 28S mRNA represented as fold induction over control from five separate experiments. * $P < 0.03$ compared with unstimulated control cells (t test).

TGF- β Binding Assay

Cells grown to 90% confluence in 24-well plates were analyzed for TGF- β binding as described (30). Briefly, cells were washed free of media and incubated for 2 min at 4°C in 20 mM glycine–135 mM NaCl, pH 3.0) to remove endogenous TGF- β . After two rinses with binding buffer (0.2% BSA in 128 mM NaCl, 5 mM KCl, 5 mM

MgSO₄, 1.2 mM CaCl₂, 50 mM HEPES [pH 7.4]), ¹²⁵I-labeled TGF- $\beta 1$ was added to triplicate wells to achieve concentrations of 1 to 40 pM or added to tubes for counting. Nonspecific binding was determined in wells that contained 100-fold or greater excess unlabeled TGF- $\beta 1$. After a 2-h incubation at 22°C with gentle rotation, the supernatant was removed for determination of free radioactivity and cells were quickly washed four times with ice-cold binding buffer and solubilized and with 1% Triton X-100, containing 10% glycerol and 20 mM HEPES (pH 7.4), and counted. Specific TGF- β binding was determined from bound *versus* free radioactivity after subtracting the amount of nonspecifically bound ligand. Binding data were analyzed with Prism3 software from GraphPad Software (www.graphpad.com).

Image Analysis

Developed films or zymograms were scanned with an Arcus II Scanner (AGFA), and densitometric analysis was performed using NIH Image 1.61 program for Macintosh.

Statistical Analyses

Differences between experimental groups were analyzed by ANOVA (Fisher PLSD test) for comparison of three or more groups or by t test (unpaired, two-tailed) for comparison of two groups. Values of $P < 0.05$ were considered significant. All of the analyses were performed using StatView 4.02 software program for Macintosh.

Results

Effect of TGF- $\beta 1$ on Collagen Accumulation and the Cytoskeleton

As reported by our laboratory and others, human mesangial cells treated with 1 ng/ml TGF- $\beta 1$ for 18 h under serum-free conditions showed increased deposition of type I and type IV collagens. Because TGF- $\beta 1$ begins to increase collagen mRNA expression as early as 1 h after stimulation (7), we investigated whether cytoskeletal architecture was affected within this time frame. Stress fibers became more prominent within 15 min of TGF- $\beta 1$ treatment (Figure 1A, top), increasing in intensity and thickness for up to 60 min. Concomitantly, there was increased detection of α -SMA into stress fibers (Figure 1A, middle), whereas little change was noted in the amount or distribution of β -actin (Figure 1A, bottom). Vinculin staining for focal adhesions showed increases in their size and changes in shape by 15 min of TGF- $\beta 1$ treatment, with increasing localization of these structures to the cell periphery by 60 min (Figure 1B). Thus, TGF- $\beta 1$ treatment markedly alters the actin cytoskeleton in mesangial cells by stimulating an increase in the number, thickness, and composition of stress fibers and by changing the size, shape, and localization of focal adhesions.

Effect of Actin Cytoskeleton Disruption on TGF- $\beta 1$ -Mediated Collagen Accumulation

Because the actin cytoskeleton is affected by TGF- $\beta 1$ less than 1 h after stimulation, we speculated that it may play a role in transducing signals responsible for the downstream accumulation of cell layer collagens I and IV. Cyto D, a fungal alkaloid, binds to the plus ends of actin filaments, disrupting the normal polymerization process. When added to mesangial cells at low concentrations (0.1 to 1.0 μ g/ml), it dose-depen-

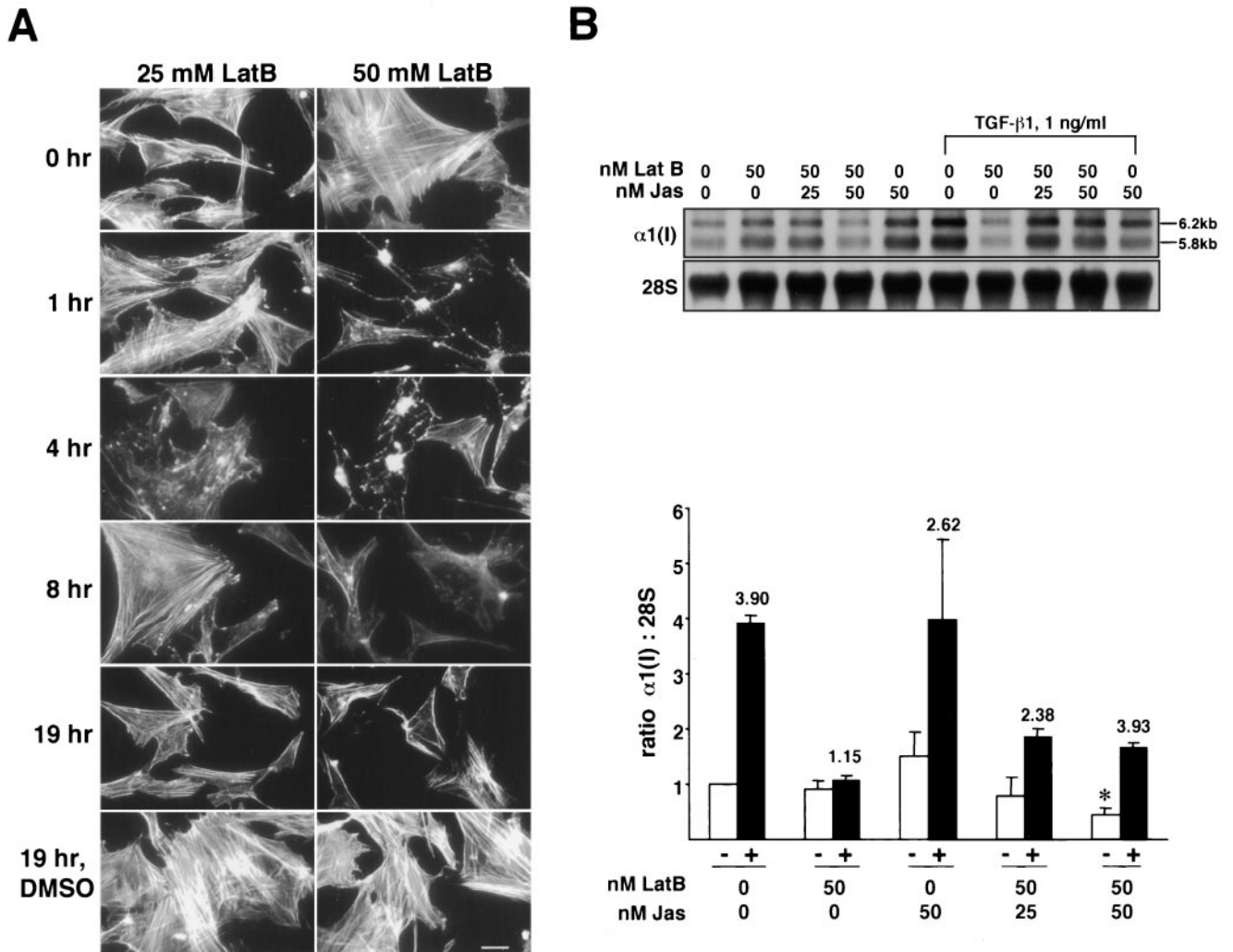


Figure 7. Latrunculin B (Lat B) disrupts mesangial cell stress fibers and blocks TGF- β 1-stimulated collagen expression. (A) Cells were treated for 19 h under serum-free conditions with DMSO or Lat B, and F-actin was visualized with rhodamine phalloidin staining. Vehicle-treated cells exhibit well-formed stress fibers, which fragment with increasing doses of Lat B (25 and 50 nM) at 1 h and are substantially intact at 19 h. Bar = 20 μ m. (B) Cells were pretreated for 1 h with vehicle, Lat B, and/or jasplakinolide (Jas) in serum-free medium before 18-h stimulation with 1 ng/ml TGF- β 1. Total cellular RNA was isolated and subjected to denaturing gel electrophoresis (4 μ g/lane) before transfer to a nylon membrane. Blots were probed for mRNA expression of the α 1-chain of type I. cDNA for 28S rRNA was used as a control for loading. A representative blot is shown (top), and the results of densitometric analysis are shown below. Values in the graph are expressed as mean fold increase over control \pm SEM for three separate experiments. * P < 0.015 compared with vehicle-treated cells (t test).

dently fragmented stress fibers, causing cells to retract and acquire an “arborized” appearance at higher concentrations (Figure 2). These effects were seen by 1 h, remained constant for up to 19 h, and were reversible upon removal of the drug (Figure 2). TGF- β 1 significantly increased (fivefold) the amount of collagen I detected in cell lysates (Figure 3A). Cyto D significantly reduced basal accumulation of cell layer collagen I to a similar extent at all doses and dose-dependently reduced TGF- β 1-stimulated levels to near basal amounts at 1.0 μ g/ml. TGF- β 1 treatment significantly increased cell layer collagen IV levels (Figure 3B). Again, Cyto D significantly decreased basal levels of cell layer collagen IV at all doses and dose-dependently reduced TGF- β 1-stimulated levels to half of

the basal amount at 1.0 μ g/ml. Smad4 protein levels were essentially unaffected by treatment with Cyto D, indicating that the effects of actin cytoskeleton disruption on TGF- β 1-stimulated collagen accumulation do not represent a generalized effect on cellular protein expression (Figure 3C). Therefore, actin cytoskeleton disruption decreases both basal and TGF- β 1-stimulated collagen accumulation in human mesangial cells.

Effect of Actin Cytoskeleton Disruption on ECM Protease Activity and Expression

Cyto D could decrease basal and TGF- β 1-stimulated cell layer collagen I and IV in mesangial cells by altering the

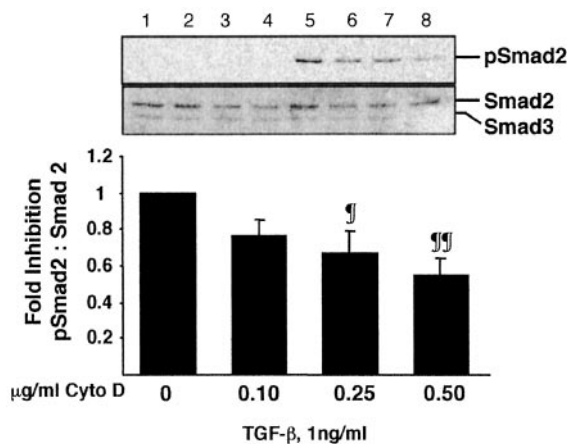


Figure 8. Cyto D diminishes TGF- β 1-induced C-terminal Smad2 phosphorylation. Serum-starved cells were pretreated for 1 h with Cyto D before 30-min stimulation with 1 ng/ml TGF- β 1. Cell lysates (25 μ g) were electrophoresed under reducing conditions before transfer to Immobilon-P membrane and Western analysis for C-terminal phospho-Smad2 using 1 μ g/ml anti-phospho Smad2. Blots were then stripped and reprobed with 0.2 μ g/ml anti-Smad2 antibody (N19) for protein expression. Cyto D dose-dependently reduced TGF- β 1-stimulated phosphorylation of Smad2. A representative blot is shown at the top, and the results of densitometric analysis are shown at the bottom. Values in the graph are expressed as mean fold inhibition over cells treated with TGF- β 1 alone \pm SEM from five separate experiments. ¶ P < 0.020 compared with control; ¶¶ P < 0.002 compared with control (Fisher PLSD).

balance between MMP and their inhibitors (TIMP). To address this possibility, we estimated total MMP activity in conditioned media harvested from cultures, using the nonspecific MMP substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂. Cyto D treatment slightly increased MMP activity over control levels at all doses (Figure 4), consistent with previous reports of increased MMP-2 activity in the conditioned media of Cyto D-treated rat mesangial cells (31,32). In accordance with our previous finding (7), TGF- β 1 alone had no significant effect on MMP activity. In contrast, addition of TGF- β 1 to cells pretreated with 0.1 to 0.25 μ g/ml Cyto D significantly increased MMP activity over basal levels, although activity returned to near basal levels in cells stimulated with TGF- β 1 and 0.5 μ g/ml Cyto D (Figure 4).

We then examined levels of specific MMP and their inhibitors in these conditioned media. Cyto D or TGF- β 1 alone slightly increased levels of pro-MMP-1 over vehicle-treated control (Figure 5A). Cyto D and TGF- β 1 together increased levels by 4.22 and 3.76-fold at 0.10 and 0.25 μ g/ml Cyto D, respectively. However, at the highest dose of Cyto D, pro-MMP-1 expression decreased to the level seen with TGF- β 1 stimulation alone. Gelatin zymography revealed one major band of MMP-2 activity at approximately 68 kD (Figure 5B), corresponding to a truncated but not fully active form seen under nonreducing conditions (7,27). This band could be further activated to a 57-kD form upon p-aminophenylmercuric acetate treatment following a standard protocol (33) (data not

shown). TGF- β 1 significantly increased the amount of MMP-2, which differs from our previous findings (7) (Figure 5C). In contrast to previous reports of rat mesangial cell responses (31,32), Cyto D did not significantly alter the levels of different MMP-2 cleavage forms regardless of whether TGF- β 1 was present. MMP-9 was barely detectable and did not change substantially with treatment (data not shown).

TGF- β 1 alone increased TIMP-1 expression, with Cyto D slightly increasing both basal and induced levels (Figure 5C). TGF- β 1 significantly increased TIMP-2 expression by twofold (Figure 5D). Cyto D treatment significantly decreased basal TIMP-2 protein expression and reduced TGF- β 1-stimulated levels of at all doses (Figure 5D). Except for the results with TIMP-1, these changes are consistent with the increased net MMP activity depicted in Figure 4. However, because we did not evaluate all MMP, others could also contribute to increased net activity with cytoskeletal disruption.

Effect of Actin Cytoskeleton Disruption on Expression of mRNA for Collagens

Another way that actin cytoskeleton disruption could diminish collagen I and IV accumulation is by altering the steady-state expression of specific mRNA, thereby decreasing protein synthesis. Cyto D did not significantly change basal α 1(I) collagen mRNA expression but significantly decreased TGF- β 1-stimulated expression by approximately 40% at all doses (Figure 6A). This reduction was not due to decreased message stability, as cells treated with control vehicle, TGF- β 1, and/or Cyto D in the presence or absence of actinomycin D exhibited identical rates of decay for α 1(I) mRNA (data not shown). In contrast, mesangial cells treated with Cyto D exhibited a slight but nonsignificant increase in basal expression of α 1(IV) collagen mRNA (Figure 6B). TGF- β 1 induced a twofold increase in α 1(IV) collagen mRNA; expression was further enhanced by Cyto D treatment. Thus, Cyto D differentially affects α 1(I) and α 1(IV) collagen steady-state mRNA expression. Similarly, our laboratory has observed differential regulation of these two mRNA in mesangial cells pretreated with cycloheximide before exposure to TGF- β 1 (7).

To determine whether the mechanism of actin filament destabilization is important in the reduction of TGF- β 1-stimulated collagen I mRNA expression, we used an alternative method of disrupting cytoskeletal assembly. Latrunculin B (Lat B) prevents polymerization of actin filaments by forming 1:1 complexes with monomeric actin. It disrupted mesangial cell stress fibers by 1 h (Figure 7A), although the degree of disorganization varied among the cells. Unlike Cyto D, washout of the drug was not required for morphologic recovery after 8 h of exposure (Figure 7A). However, like Cyto D, Lat B blocked TGF- β 1-stimulated collagen I mRNA expression (Figure 7B). The cells were further treated with jasplakinolide (Jas), a marine sponge toxin that promotes actin polymerization. Although treatment of the cells with Lat B plus Jas reduced the basal expression of α 1(I) mRNA, Jas restored the TGF- β 1-stimulated collagen response to control levels (3.9-fold). These results indicate that two agents that inhibit actin stress fiber assembly by different mechanisms each decreases TGF- β 1-

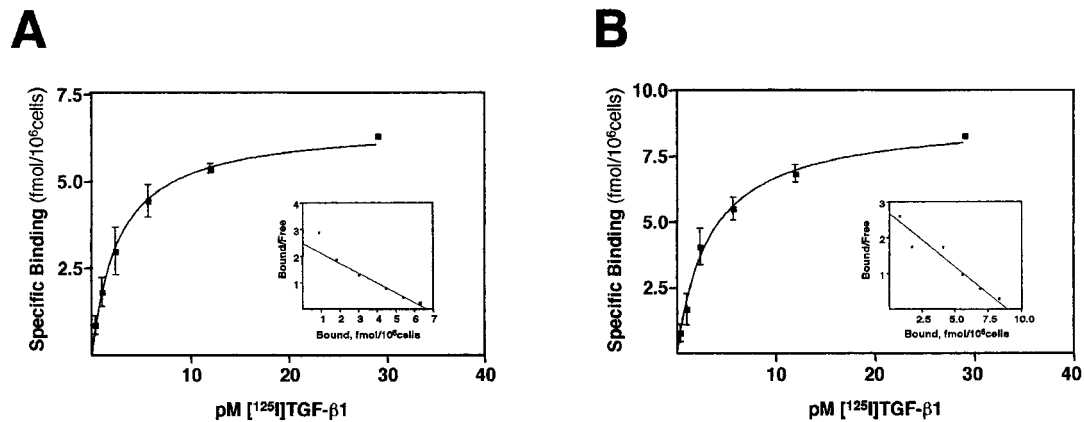


Figure 9. Cyto D has no effect on TGF- β 1 binding or affinity to its receptor. Cells were pretreated for 60 min with vehicle (A) or 0.25 μ g/ml Cyto D (B) before addition of 125 I-TGF- β 1 \pm 100-fold excess cold TGF- β 1. Values represent mean specific binding of 125 I-TGF- β 1 to mesangial cells \pm SEM from four (A) or three (B) separate experiments.

stimulated α 1(I) collagen mRNA expression, and, in one case, the effect is reversed by a compound that promotes actin polymerization.

Taken together, our findings suggest that disruption of stress fibers by Cyto D or Lat B reduces type I collagen accumulation at least in part at the level of mRNA expression (Figures 6 and 7), whereas decreased type IV collagen protein expression is more likely to be regulated posttranslationally (Figures 4 and 5).

Effect of Actin Disruption on TGF- β 1-Induced Smad2 Phosphorylation

Because our laboratory has shown a role for the Smad pathway in activating type I collagen gene expression in human glomerular mesangial cells (19), we evaluated the effect of cytoskeletal disruption on Smad activation. Little to no pSmad2 was detected in the absence of TGF- β 1, but phosphorylation increased 11.8-fold over vehicle-treated cells after TGF- β 1 stimulation (Figure 8). Cyto D treatment dose-dependently inhibited this activation, decreasing phosphorylation by nearly half at 0.5 μ g/ml. These results show that actin cytoskeleton disruption by Cyto D decreases Smad2 activation by TGF- β 1. This action could result in reduced TGF- β 1-specific gene expression.

Effect of Actin Disruption on TGF- β Receptor Binding

One way in which cytoskeleton disruption could reduce TGF- β 1-stimulated Smad phosphorylation and α 1(I) mRNA expression is by decreasing binding of TGF- β to its receptor or downregulating the number of TGF- β receptors. We therefore examined the effect of Cyto D on TGF- β 1 binding kinetics. Vehicle-treated cells were found to have 3988 ± 359 high-affinity receptors/cell (Figure 9A), with an apparent K_d of $2.68 \pm .73$ pM. Cyto D-treated cells were found to have 5366 ± 437 high-affinity receptors/cell (Figure 9B) with an apparent K_d of $3.31 \pm .78$ pM (Figure 9B). These results were not significantly different. Thus, cytoskeleton disruption does not decrease TGF- β 1 receptor expression or binding kinetics.

Effect of Rho Signaling Pathway Inhibitors on TGF- β 1-Induced Collagen Expression

Because actin assembly is regulated by members of the Rho-GTPase family, we investigated whether inhibitors of Rho signaling could affect TGF- β 1-stimulated collagen expression. *C. difficile* toxin B, an inhibitor that inactivates the Rho-GTPases Rho, Rac, and Cdc42, abolished mesangial cell stress fibers (Figure 10A, middle) and blocked induction of α 1(I) mRNA by TGF- β 1 (Figure 10B). Pharmacologic blockade of the RhoA effector Rho-associated kinase, with its inhibitor Y-27632, reduced stress fibers (Figure 10A, right) and prevented TGF- β 1-stimulated α 1(I) expression at 6 h (Figure 10B). These data support the notion that activation of cytoskeletal signaling through Rho-GTPases or an effector of RhoA plays a role in TGF- β -stimulated collagen I expression.

Discussion

TGF- β 1 seems to play a significant role in progressive renal disease. Previous studies have shown that TGF- β significantly affects cytoskeletal architecture in fibroblast (34,35), smooth muscle (36), epithelial (35,37,38), and endothelial (39) cells. TGF- β also induces α -SMA expression (40), a marker of activation and progression in diseased kidneys (41,42). These observations suggest that TGF- β 1 effects on the cytoskeleton could play a role in mesangial cell collagen accumulation. In the present study, TGF- β 1 rapidly induced cytoskeletal rearrangement in mesangial cells, promoting the formation of stress fibers and increasing the amount of α -SMA visualized in stress fibers. Focal adhesions changed in shape, size, and distribution. Disrupting the actin cytoskeleton with Cyto D reduced basal and TGF- β 1-stimulated accumulation of collagen I and IV protein and increased net proteolytic activity. Cyto D also decreased α 1(I) mRNA expression while having no effect on or slightly increasing α 1(IV) mRNA levels. Lat B, which disrupts the actin cytoskeleton by a different mechanism, also blocked induction of α 1(I) mRNA expression by TGF- β 1, and this induction was rescued by Jas, which stabilizes

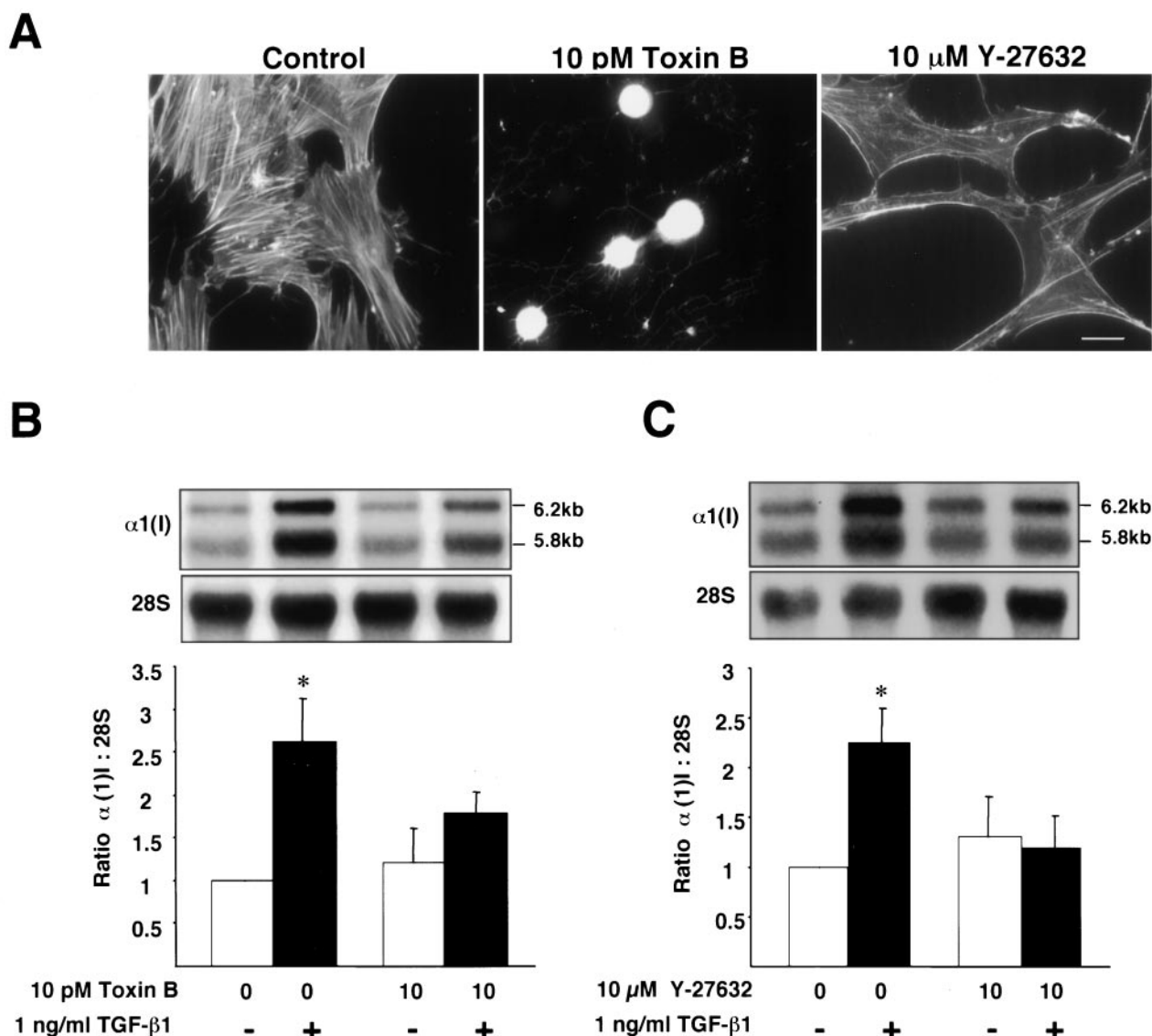


Figure 10. Rho signaling pathway inhibitors affect mesangial cell stress fibers and block induction of TGF-β1 α1(I) mRNA expression. (A) Cells serum-depleted for 24 h with 1% FBS mesangial medium were treated for 7 h with 10 pM *C. difficile* Toxin B or 10 μM Y-27632, and F-actin was visualized with rhodamine phalloidin. Control cells exhibit abundant stress fibers, which Toxin B abrogates. Some loss of stress fibers is seen in 10 μM Y-27632-treated cultures. Bar = 20 μm. (B) Cells were pretreated for 1 h with Toxin B before 6-h stimulation with 1 ng/ml TGF-β1. Total cellular RNA was isolated and subjected to denaturing gel electrophoresis (4 μg/lane) before transfer to a nylon membrane. Blots were probed for mRNA expression of the α1-chain of type I. cDNA for 28S rRNA was used as a control for loading. A representative blot is shown at the top, and the results of densitometric analysis are shown at the bottom. (C) Cells were pretreated for 1 h with Y-27632 before 6-h stimulation with 1 ng/ml TGF-β1. Total RNA isolation, electrophoresis, transfer, and probing were as in B. Values in the graphs are expressed as mean fold increase over control ± SEM from three (B) or four (C) separate experiments. **P* < 0.04 compared with unstimulated control cells (*t* test).

actin polymers. Cyto D also did not affect expression of Smad4, indicating that the cytoskeletal disruption did not cause a generalized decrease in cellular proteins.

Our findings regarding the net balance in collagen turnover partially agree with reports showing that Cyto D treatment of rat mesangial cells decreases TIMP-2 protein and increases activation of latent MMP-2 in conditioned medium, most likely through increased expression of its activator, membrane-type MMP (31,32,43). Although these results support a posttrans-

lational mechanism for decreased collagen accumulation, the results of our mRNA expression studies suggest a role for the cytoskeleton in regulating collagen synthesis as well. Thus, an intact cytoskeleton may be important in TGF-β1 signal transduction leading specifically to collagen I expression.

Cytoskeletal disruption could negatively affect TGF-β-stimulated collagen I mRNA expression through a number of mechanisms. However, our data indicate that Cyto D does not decrease receptor number or specific TGF-β ligand binding. It

is possible, nonetheless, that Cyto D affects an aspect of T β R activation subsequent to ligand binding. We also did not detect an effect of Cyto D on α 1(I) mRNA decay in the presence or absence of TGF- β . In contrast, Smad2 phosphorylation is decreased, indicating that TGF- β 1 signaling is less active after cytoskeletal disruption.

The mechanism by which TGF- β triggers cytoskeletal assembly is not well studied. Edlund *et al.* (44) reported that TGF- β 1-dependent stress fiber assembly in prostate carcinoma cells requires signaling by Cdc42 and RhoA. Bhowmick *et al.* (45) showed that TGF- β rapidly activates RhoA normal mouse mammary cells. We found that inhibition of Rho-GTPases by *C. difficile* toxin B blocks induction not only of stress fiber formation but also of α 1(I) mRNA by TGF- β 1. Furthermore, blocking the action of the RhoA effector, Rho kinase, with the inhibitor Y-27632 also abrogated induction of α 1(I) mRNA, raising the possibility that cytoskeletal rearrangement and TGF- β -stimulated collagen production are functionally related. It is noteworthy that this same Rho-associated kinase inhibitor has been shown to decrease progressive hepatic (46) and renal (47) fibrosis.

Glomerular injury is frequently associated with loss of cell attachment or synthesis of additional and possibly abnormal matrix. Thus, our data are consistent with the hypothesis that actin cytoskeleton alteration is an effector mechanism for glomerular injury. Our results regarding actin cytoskeletal involvement with the Smad pathway parallel the work of Dong *et al.* (48), who reported that Smad proteins bind to microtubules and that Smad2 and Smad3 are released upon TGF- β stimulation, freeing them for receptor-mediated phosphorylation, nuclear translocation, and gene transcription. Because stretch enhances TGF- β 1-stimulated mesangial cell collagen production (22), the data together indicate that cytoskeletal signaling may play a significant role in TGF- β 1-induced type I collagen accumulation at the level of mRNA expression and may also affect type IV collagen accumulation by altering the balance of proteases and their inhibitors. The effects of Cyto D and Lat B on collagen I mRNA expression suggest that the cytoskeleton cooperates with other TGF- β signals in this process. The cross-talk among cytoskeletal, Smad, and other signaling pathways stimulated by TGF- β and its importance ECM accumulation in mesangial cells remain topics for further exploration.

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