TGF-β1-mediated alterations of renal proximal tubular epithelial cell phenotype

Ya-Chung Tian,1 Donald Fraser,1 Liliana Attisano,2 and Aled O. Phillips1

1Institute of Nephrology, University of Wales College of Medicine, Cardiff, Wales CF14 4XN; and 2Department of Anatomy and Cell Biology, University of Toronto, Toronto, Canada M5S 1A8

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Transforming growth factor (TGF)-β, which is the prototypic member of the TGF-β1 superfamily, exerts a broad range of biological activities. There is overwhelming evidence implicating TGF-β in the pathogenesis of progressive renal fibrosis associated with numerous diseases. In addition to its role in the modulation of extracellular matrix turnover, studies using normal rat kidney tubular epithelial cells suggest that TGF-β1 may be a key mediator regulating differentiation of tubular epithelial cells into α-smooth muscle actin (SMA)-positive cells (11). Furthermore, recent studies using intermediate filament markers and reorganization of the cytoskeleton as indicators of a “fibroblastic” phenotype suggest that TGF-β1 induces phenotypic alterations in renal PTC. The mechanism by which TGF-β1 regulates cell phenotype is, however, not clear.

TGF-βs elicit their signaling effects by binding mainly to three cell-surface receptors: type I (RI), type II (RII), and type III (RIII). RI and RII are serine/threonine kinases that form heteromeric complexes and are necessary for TGF-β signaling. Ligand binding induces assembly of a heteromeric complex, which results in activation of the signaling intermediates Smads and initiation of transcriptional activation of target genes. Although the Smad proteins are key participants in TGF-β1 signaling, other signaling pathways are known to be activated by TGF-β1 (10, 14). We recently characterized TGF-β1-mediated disassembly of PTC cell-cell junctional complexes and demonstrated a link between the TGF-β type II receptor/Smad pathway and alterations of β-catenin/E-cadherin phosphorylation (44). These results together with recent studies suggesting cooperative effects in terms of cell signaling mediated by the TGF-β1 and Wnt pathways (17) suggest that the Wnt pathway may be a further potential signaling pathway mediating downstream events following TGF-β1 receptor binding. Although the Smad proteins are key participants in TGF-β1 signaling, other signaling pathways have also been shown to be activated by TGF-β1. For example, recent studies in a fibrosarcoma cell line demonstrated induction of fibronectin transcription and synthesis to be dependent on the Smad signaling pathway.

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on TGF-β1-mediated activation of c-Jun NH2-terminal kinase (JNK), a member of the MAP kinase family (14). The aim of the current study was to examine the mechanism by which PTC phenotype is altered in response to TGF-β1. We characterized the relative contributions of alteration in cell-cell contact and the role of cell-matrix interaction in regulation of cell phenotype. In addition, we examined the involvement of numerous potential signaling pathways in mediating these effects. These data suggest that acquisition of a “fibroblastic” phenotype in response to TGF-β1 required coordinated alterations in the cytoskeleton and disruption of cell-cell and cell-matrix interactions. Furthermore, this is not dependent on Smad or Wnt signaling but rather is dependent on activation of the RhoA-ROCK pathway.

EXPERIMENTAL PROCEDURES

Materials. Antibodies for Western blot analysis, immunoprecipitation, and immunocytochemistry and the final working dilution were as follows: (for Western blot analysis) mouse monoclonal anti-E-cadherin antibody (dilution 1:1,500) from Transduction Laboratories (Lexington, KY), rabbit monoclonal anti-β-catenin antibody (dilution 1:1,500) from Transduction Laboratories, mouse monoclonal anti-paxillin antibody (dilution 1:500) from Biogenex (San Ramon, CA), rabbit monoclonal anti-FLAG antibody (dilution 1:500) from Sigma-Aldrich (Saint Louis, MO), and all of FITC-conjugated secondary antibodies from Sigma; (other reagents) recombinant protein expressed in Escherichia coli (RBD; recombinant protein expressed in Escherichia coli) from Upstate Biotechnology (Buckinghamshire, UK), mouse monoclonal anti-phosphotyrosine antibody for immunoprecipitation (dilution 1:100) from Sigma, and all of HRP-conjugated secondary antibodies from Sigma; (for immunocytochemistry) rabbit monoclonal anti-β-catenin antibody (dilution 1:100) from Transduction Laboratories, rabbit monoclonal anti-o-cadherin antibody (dilution 1:100) from Zymed Laboratories (San Francisco, CA), mouse monoclonal anti-vinculin antibody (dilution 1:100) from Sigma, mouse monoclonal anti-paxillin antibody (dilution 1:100), mouse monoclonal anti-β3-integrin antibody (dilution 1:50) from Upstate Biotechnology, anti-FLAG monoclonal antibody (dilution 1:100) from Sigma, mouse TRITC-conjugated phalloidin antibody (dilution 1:50) from Sigma, and all of FITC-conjugated secondary antibodies from Sigma; (other reagents) recombinant TGF-β1 was purchased from R&D Systems (Oxford, UK), cytochalasin D from Sigma, tyrosine phosphatase inhibitor sodium orthovanadate from Calbiochem (San Diego, CA), and the inhibitors of RhoA target protein Rho-associated coiled-coil kinase (ROCK) R(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide (Y-27632) from Calbiochem.

Cell culture. HK2 cells [human renal PTC immortalized by transduction with human papilloma virus 16 E6/E7 genes (38)] were cultured in DMEM/Ham’s F-12 Life Technologies, Paisley, UK supplemented with 10% FCS (Biological Industries, Cumbernauld, UK), insulin, transferrin, and sodium selenite (Sigma). Cells were grown at 37°C in 5% CO2-95% air. Fresh growth medium was added to cells every 3–4 days until confluent. Cells were grown to confluence and serum deprived for 48 h before experimental manipulation. All experiments were performed under serum-free conditions. Selective experiments with primary human PTC (HPTC) were performed to confirm the observations obtained in HK2 cells. HPTC were isolated from normal tissue obtained from nephrectomy specimens, characterized as previously described (31) and grown under the same culture conditions as HK2 cells.

The effects of TGF-β on cell phenotype were determined by addition of recombinant cytokine (0–50 ng/ml) to confluent growth-arrested monolayers of cells. All experiments were performed under serum-free conditions. Cell phenotype was monitored by light microscopy. In all experiments, supernatant samples were collected and stored at −70°C. Subsequently, cell protein was prepared as described below. For all experiments, a known number of cells were seeded into each flask, and all results were normalized for this cell number. Similarly for Western blot analysis, equal amount of total protein was loaded as assessed by the Bradford protein assay (Bio-Rad Laboratories, Hertfordshire, UK).

Immunocytochemistry. Cells were grown in eight-well multichamber slides (Invitrogen, Paisley, UK) under serum-free media for 48 h and then stimulated with recombinant TGF-β1 (50 ng/ml) for up to 4 days. At each time point, cells were rinsed three times in PBS for 5 min each, before fixation in 3% paraformaldehyde for 15 min at room temperature and subsequent permeabilization with 0.2% Triton X-100 for 5 min at room temperature. After a blocking step (1% BSA/PBS for 1 h), cells were incubated with the primary antibodies for 1 h at room temperature followed by the incubation of FITC-conjugated secondary antibodies. After being washed with PBS, cells were mounted with fluorSave reagent (Calbiochem, Nottingham, UK) and analyzed by confocal microscope (Leica TCS 4D).

Double immunofluorescence was performed to examine stress fiber organization resultant from Smad overexpression. For fixation and a blocking step as described above, cells were incubated with TRITC-conjugated phalloidin and FITC-conjugated anti-FLAG monoclonal antibody for 1 h at room temperature.

Alteration in E-cadherin expression. Total cell lysates were obtained by washing cells once with cold PBS; cells were subsequently detached by scraping into 5 ml of cold PBS. After centrifugation at 2,500 rpm for 10 min, cell pellets were mixed with SDS sample buffer (reducing sample buffer) and stored at 20°C until use. Immunoblot analysis of lysate samples was performed by standard methodologies. Tyrosine phosphorylation of E-cadherin was examined by E-cadherin immunoblot analysis, following phosphotyrosine immunoprecipitation performed by standard methodologies. Briefly, cell protein samples were precleared with 50 µl of packed protein A cross-linked 4% beaded agarose (Sigma) at 4°C for 1 h. The beads were removed by centrifugation (13,000 rpm for 10 min) and the supernatant was collected. Primary antibody (2 µg/ml) was added to the cleared supernatant and incubated at 4°C with constant mixing for 12 h. The immune complex was captured by the addition of packed agarose-protein A beads (50 µl/500 µl supernatant) for 2 h at 4°C. Separation of the beads was achieved by centrifugation (13,000 g for 25 min), and the supernatant was removed. Specificity of immunoprecipitation was confirmed by negative control reactions performed with either no primary antibody or rabbit IgG control.

Detection of focal adhesion proteins. Association of focal adhesion components and adherens junction proteins with the actin cytoskeleton was indirectly assessed by examining their Triton solubility as previously described (2). With the use of this method, the Triton-soluble component represents membrane and cytosolic fractions and not proteins associated

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with the cytoskeleton. After an equal number of cells were seeded, confluent monolayers were washed once with cold PBS, scraped, and rinsed into 5 ml of cold PBS. After centrifugation at 2,500 rpm for 10 min, cell pellets were extracted in buffer (150 mM NaCl, 50 mM Tris·Cl, 0.01% NaN₃, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin) containing 1% Triton X-100 (TX buffer) for 30 min on ice. Samples were centrifuged at 12,500 rpm for 30 min on ice. The supernatant (Triton-soluble component containing membrane and cytosolic fraction) was transferred to a separate tube and kept at −70°C until use. Expression and state of “activation” of the focal adhesion component paxillin and the adherens junction components β-catenin and E-cadherin were determined following photophototroside immunoprecipitation by Western blot analysis performed by standard methodologies as described below.

Detection of alterations in type IV collagen generation. Analysis of type IV collagen in the culture supernatant was performed by Western blot analysis by standard methodologies. Briefly, samples were prepared in SDS sample buffer and boiled for 5 min at 95°C. For Western blot analysis, equal amounts of cell protein, or equal volumes of culture supernatant, were loaded onto 10% SDS-PAGE gradient gels and electrophoresis was carried out under reducing conditions according to the procedure of Laemmli (18). After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was blocked with Tris-buffered saline (TBS) containing 5% nonfat powdered milk for 1 h and then incubated with the primary antibody in TBS containing 1% bovine serum albumin and 0.1% Tween 20 (TBS-Tween) for 1 h at room temperature. The blots were subsequently washed in TBS-Tween and then incubated with an appropriate HRP-conjugated secondary antibody in TBS-Tween. Proteins were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer’s instructions.

Transfection of reporter constructs and expression vectors. Xtn and Topflash (obtained from B. Vogelstein, John Hopkins University, Baltimore, MD) (17), TGF-β1 promoter (ph TG1–1,362 + 11; gift from Dr. S. J. Kim, National Cancer Institute, National Institutes of Health, Bethesda, MD) (16), luciferase reporter constructs, and the Smad2, 3, 4, and LEF-1 expression vectors (17) have been described previously.

For luciferase assays, HK2 cells were transiently transfected using the mixed lipofection reagent Fu-gene 6 (Roche) at a ratio of 3 μl Fu-gene 6 to 1 μg DNA in serum-free medium. Transfections contained 1 μg of Xtn-w-lux, 1 μg of Topflash, or 3 μg of the TGF-β1 reporter construct. Transfection efficiency was monitored by cotransfection with a β-galactosidase reporter plasmid. Luciferase activity was normalized to β-galactosidase activity. After cell lysis, luciferase content was quantified by glow-type luminescence assay (Promega) with a standard curve of recombinant luciferase.

Expression vectors contained 2 μg of either Smad or LEF-1. Smad or LEF-1 overexpression following transfection was confirmed by immunoblot analysis of total cell protein using anti-FLAG antibodies. After transfection with the expression vectors, alterations in cell phenotype were monitored by light microscopy and immunohistochemistry and changes in cell function by collagen IV immunoblot analysis as described above.

Assessment of RhoA activation. With the use of RBD, which specifically binds to GTP-Rho, not GDP-Rho to precipitate GTP-Rho, followed by immunoblotting with specific RhoA antibody, we are able to detect the activity of activated RhoA, GTP-RhoA. This method has been well established to measure activated RhoA activity. Briefly, following addition of TGF-β1 (10 ng/ml) for 2 h or 2 days, the cell monolayer was rinsed twice with ice-cold TBS. Cell lysate was obtained by adding Mg²⁺ buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, and 10% glycerol) onto the cell monolayer and scraping with a cell scraper, followed by centrifugation at 14,000 g for 5 min at 4°C. The supernatant was transferred to a microfuge tube and further incubated with 30 μg of rhokin TGBD-agarose slurry for 45 min at 4°C. Agarose beads were then pelleted by brief centrifugation, washed with Mg²⁺ buffer three times, and subjected to Western blot analysis as described above. Activated GTP-RhoA was detected by immunoblotting with specific RhoA antibody.

Results

Morphological alterations. The culture of HK2 cells in six-well plates or eight-chamber glass slides produced a confluent monolayer with cobblestone morphology (Fig. 1A). Serum deprivation to achieve cell cycle synchronization did not alter cell morphology. In contrast, addition of recombinant TGF-β1 (10 ng/ml) to serum-deprived confluent monolayers of HK2 cells resulted in a marked alteration in cell morphology (Fig. 1B). Cells developed marked hypertrophy, becoming elongated and spindle shaped and losing the cobblestone pattern. These effects were apparent within 2 days of addition of TGF-β1. Alteration of cell morphology following addition of recombinant TGF-β1 was also seen following culture of primary cultures of HPTC in the presence of recombinant TGF-β1 (Fig. 1C). Again, these effects become apparent within 2 days of addition of TGF-β1, with cells becoming elongated, spindle shaped, and fibroblastic in appearance.

Organization of the actin cytoskeleton was examined by fluorescein-conjugated phalloidin staining of F-actin. HK2 cells under control, unstimulated conditions contained both peripheral bands of actin near cell membranes and randomly oriented cytoplasmic fibers (Fig. 1D) as described previously in epithelial cells (22). Addition of recombinant TGF-β1 to either HPTC or HK2 cells resulted in a reorganization of the actin filament architecture with coalescence of actin fibers into stress fibers with direct extension of these fiber formations from cell to cell (Fig. 1, E and F). As with alteration in cell morphology, these effects were apparent 2 days after the addition of recombinant TGF-β1. The importance of the cytoskeleton in alteration of the cell phenotype was demonstrated by using cytochalasin D, as disruption of the actin cytoskeleton with cytochalasin D prevented phenotypic alterations following addition of TGF-β1 (Fig. 2).

In addition to an alteration in cell-cell contact, TGF-β1-mediated alteration in cell phenotype was also associated with alterations in cell-matrix interactions, as TGF-β1 induced the expression of paxillin and vinculin and their incorporation into dense focal adhesion.
plaques (Fig. 3). There was also an increase in β3-integrin associated with these dense adhesion plaques (Fig. 3).

Tyrosine phosphorylation of E-cadherin is known to critically regulate adherens junction protein complex assembly, and we demonstrated that loss of cell-cell contact in response to TGF-β1 is associated with E-cadherin phosphorylation and adherens junction disassembly manifested by relocation of β-catenin away from the cell periphery. We used the tyrosine phosphatase inhibitor sodium orthovanadate to determine whether E-cadherin phosphorylation and adherens junction disassembly regulate cell phenotype. Addition of sodium orthovanadate resulted in transient loss of cell-cell contact (Fig. 4A). Loss of cell-cell contact was also accompanied by a transient increase in tyrosine phosphorylation of E-cadherin without a change in total E-cadherin expression (Fig. 4C) and relocaliza-

Fig. 1. Effect of transforming growth factor (TGF)-β on cell morphology and cytoskeletal organization. A: appearance of control HK2 grown on a glass surface without any treatment following serum deprivation for 7 days. Cells grew as a typical “cobblestone” monolayer. B: TGF-β1 (10 ng/ml) under serum-free conditions resulted in marked changes in HK2 cell morphology at day 2. Cells lost their regular cuboidal appearance, becoming elongated and spindle shaped. C: appearance of control HK2 grown on a glass surface without any treatment following serum deprivation for 7 days. D: stimulation of primary cultures of human proximal tubular cells (HPTC) with TGF-β1 also resulted in marked alteration in cell morphology. Confluent monolayers of HPTC were stimulated under serum-free conditions. Two days after the addition of TGF-β1, cells became elongated and spindle shaped and lost cell-cell adhesion. A-D: ×100 magnification. Filamentous actin was visualized by staining with fluorescein-conjugated phalloidin. E: monolayer of HK2 cells under control conditions. F: TGF-β1 (10 ng/ml) treatment of confluent monolayer of HK2 under serum-free conditions for 6 days. G: monolayer of HPTC cells under control conditions. H: TGF-β1 (10 ng/ml) treatment of confluent monolayer of HPTC under serum-free conditions for 6 days. A and C: ×400 magnification; B: ×250 magnification; E–H: ×400.

Fig. 2. Cytochalasin inhibits phenotypic change and F-actin reorganization. Growth-arrested HK2 cells were stimulated with recombinant TGF-β1 (10 ng/ml) under serum-free conditions for 48 h in the presence (C) or absence of cytochalasin D (2 μg/ml) (B). A: in control experiments, cells were exposed to serum-free medium alone. Cell morphology and filamentous actin were visualized by staining with fluorescein-conjugated phalloidin and visualization was by confocal microscopy.
tion of the adherens junction component β-catenin from the cell periphery (Fig. 4B). The time course of loss of cell-cell contact was, however, very different from that induced by TGF-β1. Alteration in cell phenotype was apparent within 2 h; however, 12 h following addition of sodium orthovanadate, a confluent monolayer reformed in which cells retained their characteristic epithelial phenotypic appearance, E-cadherin phosphorylation decreased, and adherens junctional complexes reformed (Fig. 4). During the phase of transient E-cadherin relocalization and loss of cell-cell contact following addition of sodium orthovanadate, there were no changes in the organization of the F-actin cytoskeleton. Addition of sodium orthovanadate resulted in opposite effects to TGF-β1 on focal adhesion assembly as expression of vinculin and paxillin was reduced and focal adhesions became sparse and smaller (Fig. 5).

Role of the Smad signaling pathway. Activation of TGF-β1 type I receptor kinase is known to propagate its signal through the Smad family of proteins. Although structurally very similar, different Smad proteins have specific and different roles in TGF-β1 signaling. After addition of TGF-β1 to HK2 cells, activation of the Smad signaling pathway was confirmed by demonstration of Smad2, 3, and 4 translocation to the nucleus (data not shown). To determine whether signaling through specific Smad proteins differentially regulates cell phenotype and function, cells were transfected with Smad2 and Smad3 expression vectors either alone or in combination with Smad4. After transfection, overexpression of each of the proteins was confirmed by FLAG immunoblot analysis of cell lysates (data not shown).

Despite confirmation of Smad production by the expression vectors, overexpression of Smad2 or Smad3 either alone or in combination with Smad4 for up to 2 days did not influence cell phenotype, as assessed by confocal microscopy. Furthermore, there was no change in organization of the F-actin cytoskeleton in the transiently transfected cells overexpressing Smad2 or Smad3 either alone (Fig. 6) or in combination with
Smad4, although Smad overexpression could be confirmed by immunocytochemistry (Fig. 6).

Given the lack of effect of Smad transfection of cell phenotype to demonstrate the efficacy of transfected Smad proteins, we examined the effect of Smad overexpression on ECM remodeling and TGF-β autoinduction. Although transient transfection with Smad expression vectors did not influence cell morphology and cytoskeletal organization, overexpression of Smad proteins stimulated type IV collagen generation as assessed by immunoblot analysis of the cell culture supernatant taken from transfected cells (Fig. 7A). Similarly, Smad transient transfection was associated with increased transcriptional activation of the TGF-β promoter reporter construct (Fig. 7B). Transfection with either Smad2 or Smad3 had a similar effect on TGF-β promoter transcriptional activity. In contrast to the stimulation of TGF-β promoter activity, there was no associated increase in TGF-β protein synthesis as assessed by commercial ELISA (12) (data not shown). Maximal TGF-β promoter activity was seen with cotransfection of either Smad2 or 3 together with Smad4, and this was similar to promoter activity following addition of recombinant TGF-β1. These results therefore confirm the “functionality” of the Smad expression vectors.

Involvement of the Wnt signaling pathway. We showed previously that TGF-β1-mediated disassembly of adherens junctional complexes induced β-catenin association with the TGF-β1 signaling molecules Smad3 and Smad4 and its relocation to the cell nucleus. β-Catenin is not only involved in the formation of adherens junctional complexes, but it is also involved in Wnt signaling, thus raising the possibility that TGF-β1 may mediate some of its effect on PTC phenotype through this signaling pathway. To test this hypothesis, HK2 cells were transfected with two reporter constructs: first, a 3,220-bp fragment of the Xwnt promoter upstream of a luciferase construct and, second, Topflash, a Wnt-responsive reporter that contains three multimerized LEF-1-binding sites, but in contrast to the Xwnt promoter, no Smad binding elements. Neither of the reporter constructs showed significant transcriptional activity following addition of TGF-β1 (Fig. 8). We were unable to demonstrate expression of LEF-1 mRNA or LEF protein in untransfected HK2 cells, and abundant constitutive expression of LEF-1 mRNA in fibroblasts was used as the positive control (data not shown). Transfection of LEF-1 had no effect on PTC phenotype but activated Xwnt promoter upstream of a luciferase construct and, second, Topflash, a Wnt-responsive reporter that contains three multimerized LEF-1-binding sites, but in contrast to the Xwnt promoter, no Smad binding elements.

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RhoA signaling and phenotypic change. Addition of TGF-β1 led to a dose-dependent activation of RhoA. Increased expression of activated RhoA was demonstrated following GST-Rho immunoprecipitation followed by RhoA immunoblot analysis (Fig. 9). Inhibition of RhoA downstream target ROCK by addition of

Fig. 4. Alteration in cell phenotype and E-cadherin phosphorylation following inhibition of tyrosine phosphatase. Confluent monolayers of growth-arrested HK2 cells were incubated with orthovanadate (250 ng/ml) for up to 12 h. A: cell phenotype following addition of sodium orthovanadate was monitored by light microscopy; ×100 magnification. B: alteration in β-catenin distribution was examined by immunocytochemistry and analyzed by confocal microscopy. C: total E-cadherin expression and tyrosine phosphorylation of E-cadherin assessed following phosphotyrosine immunoprecipitation were monitored by E-cadherin immunoblot analysis of cell lysates. Both E-cadherin and phosphotyrosine analysis demonstrated bands of identical molecular weight. One representative experiment is shown.
Y27632 inhibited the TGF-β1-mediated change in cell phenotype (Fig. 10). Y27632 also inhibited TGF-β1-mediated relocation of the adherens junction component β-catenin to the cytoplasm (Fig. 11) as well as its phosphorylation (Fig. 9), redistribution of tight junction protein components (Fig. 11), and TGF-β1-mediated formation of focal adhesion formation (Fig. 11). Although TGF-β1-mediated alteration in cell morphology was prevented by inhibition of ROCK, Y27632 did not prevent TGF-β1 stimulation of type IV collagen synthesis (Fig. 9).

In contrast to inhibition of RhoA downstream targets, inhibitors of phosphatidylinositol 3-kinase (LY284002; Calbiochem), p38 MAP kinase (SB202580; Calbiochem), or MAP kinase kinase/MEK (PD-98059; Calbiochem) did not influence TGF-β1-mediated alteration in cell phenotype nor any of the associated rearrangement of adherens junction, tight junction, or focal adhesion components (data not shown).

**DISCUSSION**

The clinical course of patients with renal insufficiency can be predicted by the extent of fibrosis in the corticointerstitium (20, 21), and a large body of evidence supports a role for TGF-β1 in its pathogenesis. Work carried out in numerous animal models of renal injury demonstrated that strategies aimed at inhibition of TGF-β1 activity may have a beneficial effect. The widely cited work of Border et al. (5–7) in the thy1.1 model of nephritis, using either neutralizing antibodies to TGF-β or decorin as its natural antagonist, first drew attention to the potential therapeutic benefit of such a strategy in progressive renal disease.
Subsequently, work performed in animal models of diabetes also suggested that such an approach may ameliorate features associated with the development of diabetic nephropathy (8, 41).

The predominant cell type in the renal cortex is the PTC, and numerous studies identified the PTC as a major potential source of profibrotic growth factors such as TGF-β1 (24, 31, 33, 37). A further mechanism, by which PTC may influence the renal corticointerstitium, is that they may acquire a fibroblastic phenotype, as assessed by the expression of fibroblast-specific markers in vitro and in vivo (42). De novo expression of α-SMA, a marker of myofibroblast phenotype, by PTC may be associated with disruption of the tubular basement membrane and migration of these cells into the corticointerstitium (27), a process which may be mediated by TGF-β1. Therefore, through a process of “transdifferentiation,” PTC may also contribute to increased numbers of fibroblastic cells that mediate the fibrotic process. The mechanistic basis for the TGF-β1-regulated alteration in PTC cell phenotype is currently not known. Our data confirm that stimulation of renal PTC with recombinant TGF-β1 regulates their phenotypic characteristics. Specifically, addition of TGF-β1 led to cells assuming a fibroblastic spindle-shaped morphology accompanied by a reorganization of the actin cytoskeleton and the formation of dense focal adhesion plaques. By the use of the tyrosine phosphatase inhibitor sodium orthovanadate, we were able to induce transient adherens junctions. Our previous studies demonstrated that stimulation with TGF-β1 led to sustained decreased expression of E-cadherin and loss of cell-cell contact, and furthermore, we demonstrated an association of E-cadherin with the TGF-β RII (44). Previous studies also demonstrated that activation of TGF-β signaling may target TGF-β1 receptors for degradation (15). It is interesting to speculate therefore that the different kinetics and transient nature of E-cadherin phosphorylation, without alteration in total E-cadherin expression following addition of sodium orthovanadate, may result from phosphorylation of a different pool of E-cadherin to that seen following addition of TGF-β1 and that this may result in different functional consequences. These data, however, would suggest that transient loss of cell-cell contact in isolation is insufficient to trigger a cascade of events leading to a stable change in cell phenotype. We therefore postulate that induction of a stable alteration in cell phenotype requires a coordinated change in the regulation of cell-cell contact and cell-matrix contact and reorganization of the actin cytoskeleton.

It has been well established that TGF-βs elicit their signaling effects by binding mainly to three cell-surface receptors: type I (RI), type II (RII), and type III (RIII) (reviewed in Ref. 25). Receptor ligand binding results in formation of an RI/RII complex in which RII phosphorylates RI on a conserved glycine-serine-rich domain. This activates the RI kinase, which subsequently phosphorylates members of the intracellular receptor-regulated Smads (R-Smads). For TGF-β1, these include Smad2 and Smad3. This causes dissociation of the R-Smads from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smad and the Co-Smad Smad4, and induces the nuclear accumulation of this heteromeric Smad complex. Although they are structurally very
Despite stimulation of TGF-β1, latent form by these cells (30, 32) and thus without activation, even stimulation of TGF-β1 synthesis would not be expected to result in its generation in a biological active form. Furthermore, TGF-β1-mediated translocation of Smad4 into the nucleus occurred as early as 2 h following its addition to cells, whereas alteration in cell morphology was only apparent after 2 days, suggesting that a Smad signaling pathway does not affect cell morphological change directly. This is consistent with recently published studies of mammary epithelial cell differentiation in which TGF-β1 alterations in cell morphology were not Smad dependent (3).

We previously demonstrated that TGF-β1 leads to loss of PTC cell-cell contact and disassembly of adherens junctional protein complexes, with release of β-catenin from the complex (44). There is a large body of evidence to support a cadherin-independent role for β-catenin. This involves translocation of β-catenin to the nucleus, which is preceded by its accumulation in the cytoplasm. Alteration in β-catenin phosphorylation status is known to influence binding to E-cadherin and the regulation of cell-cell contact, β-catenin degradation via the ubiquitin-proteasome pathway, and also a mechanism that regulates β-catenin transcription factor recognition and binding (1, 26, 34, 40). Our previous studies demonstrating TGF-β1-induced stimulation of β-catenin tyrosine phosphorylation therefore suggest that this may also facilitate cadherin-independent, β-catenin-dependent signaling. The Wnt pathway is distinct from that of TGF-β and is mediated by β-catenin; however, several recent studies showed that cooperation may exist between TGF-β1 and Wnt/β-catenin pathways. This may occur both by interactions of intracellular proteins of both pathways facilitating translocation into the nucleus (28) and also by cooperative regulation of the target gene within the cell.

Similar, Smad2 and Smad3 have specific and different roles in TGF-β1 signaling as they activate different subsets of target genes following TGF-β1 receptor activation (9, 35). Our data, however, would suggest that PTC cell phenotype is not solely regulated by activation/phosphorylation of either Smad2 or Smad3, as cell morphology was unaffected by transient transfection with Smad expression vectors. This does not rule out the possibility that a competent Smad pathway is required, together with a second signaling pathway, to induce phenotypic change. Such cooperative effects have been described during malignant phenotypic changes (29). We demonstrated the efficacy of the expression both in terms of overexpression of Smad protein and also their functionality as type IV collagen synthesis and TGF-β1 promoter transcriptional activity were stimulated in transiently transfected cells. Despite stimulation of TGF-β1 promoter activity, we were unable to detect alteration in TGF-β1 protein production in Smad-overexpressing cells. This may suggest that either TGF-β1 levels were too low for detection by ELISA or that despite transcriptional activation of the TGF-β1 gene, that there was no de novo protein synthesis, consistent with previous studies that demonstrated independent regulation of TGF-β transcription and translation in PTC (12, 24, 31, 33). In addition, TGF-β1 is produced almost exclusively in its latent form by these cells (30, 32) and thus without activation, even stimulation of TGF-β1 synthesis would not be expected to result in its generation in a biological active form.
nucleus (19). Although TGF-β1 stimulates the interaction between the TGF-β1-signaling intermediates Smad3 and 4 and β-catenin within the cytoplasm in PTC, data in the current manuscript suggest that this does not lead to cooperative activation of Wnt target gene expression as the Xtwn promoter containing both SBE- and LEF-1-binding sites and was unaffected by stimulation by TGF-β1. In addition, there was no alteration in Topflash, which does not contain SBE, thus confirming the lack of stimulation of Xtwn target genes. Previous studies suggested that renal epithelial cell Wnt unresponsiveness may be the result of lack of expression of LEF-1-related proteins in this cell type (13). Furthermore, studies of renal fibrosis of diverse etiology suggested that increased Wnt expression may be confined to areas of fibrosis surrounding the collecting ducts predominantly in the renal medulla (43). Overexpression of LEF-1 in PTC, although stimulating exogenous Wnt target genes (Xtwn and Topflash), did not however influence PTC morphology or function. This suggests that this pathway was not involved in TGF-β1-mediated signaling at least in this cell type. It is interesting to speculate, however, that Smad-β-catenin interactions may be involved in regulation of nucleo-cytoplasmic shuttling of the Smad proteins. Ligand binding to the integrin family of cell adhesion molecules leads to integrin clustering and subsequent recruitment of actin filaments to the cytoplasmic domains of integrins. Focal adhesions form the link between the matrix-attached transmembrane integrin receptors and the actin cytoskeleton and are composed of a complex of cytoskeletal interacting proteins including talin, vinculin, paxillin, and α-actinin. The significance of coupling of integrins to F-actin via different actin-binding proteins is unclear. Recent work, however, demonstrated that TGF-β1-mediated alteration in the composition of the extracellular matrix may induce alteration in cell phenotype. More specifically, it has been shown that corneal myofibroblast differentiation requires extracellular fibronectin assembly and that this transformation may involve an RBD-dependent phosphotyrosine signal transduction pathway. Members of the Rho subfamily of small GTPases are known to control the adhesion and morphology of

![Image of activated Rho A](#)

**Fig. 9.** Relationship between RhoA activation TGF-β1-mediated changes in type IV collagen and β-catenin expression. A: activation of RhoA was assessed by immunoprecipitation of GTP-RhoA 2 h after the addition of increasing doses of recombinant TGF-β1 (0–10 ng/ml) under serum-free conditions. Growth-arrested HK2 cells were also stimulated with recombinant TGF-β1 (10 ng/ml) under serum-free conditions for 48 h in the presence or absence of Y27632 (10 μM) as indicated. Alteration in collagen in the cell culture supernatant and β-catenin in the Triton-soluble cell lysates were assessed by Western blot analysis. B: results of 2 separate experiments.

![Image of Type IV collagen](#)

![Image of Phosphotyrosine β-catenin](#)

**Fig. 10.** Inhibition of ROCK abrogates TGF-β1-mediated alteration in cell morphology. Growth-arrested HK2 cells were stimulated with recombinant TGF-β1 (10 ng/ml) under serum-free conditions for 48 h in the presence (C) or absence (B) of Y27632 (10 μM) as indicated. A: in control experiments, cells were incubated with Y27632 (10 μM) alone under serum-free conditions. Alterations in cell morphology were monitored by phase contrast light microscopy (×100 magnification).
mammalian cells. These function as binary switches that cycle between an active GTP-bound form and an inactive GDP-bound form. Previous studies suggest that Rho increases focal adhesion and actin stress fibers (36). RhoA activation has also been implicated in F-actin remodeling and relocalization of E-cadherin in adherens junctions following TGF-β1-induced mammary epithelial-mesenchymal transdifferentiation (3). It is clear, however, that RhoA function is dependent on the cell context, as depending on the cell system studied, RhoA can either positively or negatively affect adherens junctions, an effect that may be dependent on different effector proteins that bind to Rho-GTP. For example, of the RhoA effector kinases, ROCK and Dia have opposing effects on adherens junctions downstream of Rho. More specifically, using colonic epithelia, it has been demonstrated that activation of the effector kinase ROCK disrupts adherens junctions downstream of RhoA, whereas signaling through the Rho effector Dia 1 maintains the integrity of adherens junction complexes (39). Our data suggest that TGF-β1-mediated alteration in PTC morphology is also dependent on a similar mechanism as alteration in cell morphology was clearly related to activation of RhoA. Furthermore, inhibition of the RhoA downstream effector ROCK abrogated TGF-β1-mediated alteration in PTC morphology and all the associated changes in cell-cell contact, focal adhesion formation, and F-actin reorganization. In conclusion, we demonstrated that TGF-β1-mediated alteration in PTC phenotype occurs independently of TGF-β1-Smad nuclear transcription-dependent events, which regulate collagen synthesis.

Fig. 11. Inhibition of ROCK prevents TGF-β1-mediated rearrangement of both tight and adherens junctions and formation of focal adhesion. Growth-arrested HK2 cells were stimulated with recombinant TGF-β1 (10 ng/ml) under serum-free conditions for 48 h in the presence (C, F, and I) or absence (B, E, and H) of Y27632 (10 μM) as indicated. A, D, and G: under control conditions, cells were incubated with serum-free medium alone. After fixation in 3% paraformaldehyde, occludin (A, B, and C), β-catenin (D, E, and F), and paxillin (G, H, and I) expressions were examined by immunohistochemistry and stained cells were analyzed by confocal microscope (Leica TCS 4D).
In addition, it seems likely that the Wnt pathway is not involved in this process, but rather that TGF-β-mediated phenotypic alterations are regulated by the RhoA-ROCK-dependent signaling pathway. Integrins mediate cell adhesion and signal transduction at focal adhesions, and recent studies demonstrate that the extracellular domains of β3-integrin subunits play a role in the regulation of Rho (23). It is interesting therefore to speculate that TGF-β mediates co-operative signalling by transforming growth factor-β and Wnt pathways.

REFERENCES


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