Polarity of Response to Transforming Growth Factor-β1 in Proximal Tubular Epithelial Cells Is Regulated by β-Catenin*

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Mei Zhang, Chien-Hung Lee, Dong Dong Luo, Aleksandra Krupa, Donald Fraser1, and Aled Phillips2

From the Institute of Nephrology, School of Medicine, Heath Park, Cardiff University, Cardiff CF14 4XN, Wales, United Kingdom

Transforming growth factor-β1 (TGF-β1)-mediated loss of proximal tubular epithelial cell-cell interaction is regulated in a polarized fashion. The aim of this study was to further explore the polarity of the TGF-β1 response and to determine the significance of R-Smad-β-catenin association previously demonstrated to accompany adherens junction disassembly. Smad3 signaling response to TGF-β1 was assessed by activity of the Smad3-responsive reporter gene construct (SBE)5-Lux and by immunoblotting for phospho-Smad proteins. Similar results were obtained with both methods. Apical application of TGF-β1 led to increased Smad3 signaling compared with basolateral stimulation. Association of Smad proteins with β-catenin was greater following basolateral TGF-β1 stimulation, as was the expression of cytoplasmic Triton-soluble β-catenin. Inhibition of β-catenin expression by small interfering RNA augmented Smad3 signaling. Lithium chloride, a GSK-3 inhibitor, increased expression of β-catenin and attenuated TGF-β1-dependent Smad3 signaling. Lithium chloride did not influence degradation of Smad3 but resulted in decreased nuclear translocation. Smad2 activation as assessed by Western blot analysis and activity of the Smad2-responsive reporter constructs ARE/My1 was also greater following apical as compared with basolateral TGF-β1 stimulation, suggesting that this is a generally applicable mechanism for the regulation of TGF-β1-dependent R-Smads. Caco-2 cells are a colonic carcinoma cell line, with known resistance to the anti-proliferative effects of TGF-β1 and increased expression of β-catenin. We used this cell line to address the general applicability of our observations. Inhibition of β-catenin in this cell line by small interfering RNA resulted in increased TGF-β1-dependent Smad3 phosphorylation and restoration of TGF-β1 anti-proliferative effects.

It is accepted that the rate of progression of renal disease is closely correlated with the degree of interstitial fibrosis. Much of our work has focused on the possible mechanisms of the pathogenesis of interstitial fibrosis and in particular the role of PTC. It has been shown that the epithelial-mesenchymal transformation in the genesis of renal interstitial fibrosis (1, 2). Transforming growth factor-β (TGF-β) is an important mediator of the pathogenesis of numerous fibrotic diseases and is known to be an important stimulus driving epithelial-mesenchymal transformation (3). One important step in this process is the loss of cell-cell contact (4). Epithelial cells have discrete specialized regions of cell-cell adhesion comprising the tight junction, which forms the main barrier to paracellular traffic and adherens junctions. We have previously demonstrated that alterations in PTC morphology upon TGF-β1 stimulation are associated with disassembly of tight and adherens junction complexes (5, 6). Evidence now suggests that the adherens junction is an important target for the regulation of intracellular signaling pathways (reviewed in Ref. 7). In epithelial cells E-cadherin, a single pass transmembrane molecule, mediates Ca2+-dependent homotypic interactions between adjacent cells. The cytoplasmic tail of E-cadherin binds either β- or γ-catenin, forming a link with the actin cytoskeleton (reviewed in Ref. 8). Cells have two pools of β-catenin, a membrane-associated stable protein involved in cell-cell interaction and a soluble unstable cytoplasmic protein. Our studies suggest that loss of cell-cell contact and disassembly of adherens junctions is a polarized phenomenon, which is more pronounced following addition of TGF-β1 to the basolateral aspect of the cells (6). Furthermore, TGF-β1 binding of TGF-β1 type II receptors, which co-localize with adherens junction complexes in PTC, results in dissociation of β-catenin from the adherens junction complex and an increase in the availability of cytoplasmic β-catenin, which binds to the TGF-β1 signaling molecules, the R-Smads. These data are consistent with previous studies suggesting an interaction between the TGF-β1 and Wnt/β-catenin pathways (9, 10).

The aim of this study was to further define the polarity of PTC response to TGF-β1. In addition we have examined the hypothesis that interaction between R-Smad β-catenin regulates TGF-β1 signaling and that this association in PTCs is responsible for the polarity of TGF-β1 response.

MATERIALS AND METHODS

Cell Culture—HK-2 cells (human renal proximal tubular epithelial cells immortalized by transduction with human papillomavirus 16 E6/E7 genes (11)) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Invitrogen) supplemented with 10% fetal calf serum (Biological Industries Ltd., Cumbernauld, UK), 2 μM l-glutamine (Invitrogen), 20 mM saline; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; q-PCR, quantitative PCR.
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Hepes buffer (Invitrogen), 5 μg/ml insulin, 5 μg/ml transferring (Sigma), 40 ng/ml hydrocortisone (Sigma), and 5 ng/ml sodium selenite (Sigma). Cells were grown at 37 °C in 5% CO₂ and 95% air. Fresh growth medium was added to cells every 3–4 days until confluent. With the exception of the cells used for transfection, cells were growth-arrested in serum-free medium for 48 h before use in experiments. Determination of polarity of cell response was examined by growing cells on porous tissue culture inserts (0.4 μm pore size) (BD Biosciences). All experiments were performed in serum-free conditions. In all aspects of cell biology that we have studied previously, HK-2 cells respond in an identical fashion to primary cultures of human proximal tubular cells (12–15). Therefore, they are a good model from which general conclusions can be drawn in terms of proximal tubular cell biology. Caco-2 cells (LGC Promochem, Teddington, UK) were cultured under identical conditions to HK-2 cells other than supplementation of medium with 25% fetal calf serum.

Transfection; Reporter Gene Analysis—Transient transfection and reporter gene analysis using HK-2 cells were performed as described previously (16). Briefly, 80% confluent HK-2 cells were changed to serum-free medium for 4 h before addition of plasmids and the mixed lipofection agent FuGENE 6 (Roche Diagnostics) at a ratio of 1 μg of plasmid, 3 μl of transfection agent. After an overnight incubation, the medium was replaced with serum-free medium containing additives as necessary for the experiment. 0.1 μg of the plasmid pRL-CMV Renilla was used to control for transfection efficiency, together with Smad3 or Smad2 reporter plasmids as follows: 0.9 μg of the Smad3-responsive promoter (SBE)₄-Lux (a gift from Aristo-tidis Moustakas, Ludwig Institute for Cancer Research, Uppsala, Sweden (17)) or 0.45 μg of the Smad2/4-specific promoter ARE-Luc together with 0.45 μg of its co-plasmid MF1 (gifts of Lalage Wakefield, NCI, National Institutes of Health, Bethesda (18)). Following lysis of the cells in Reporter Lysis Buffer (Promega Ltd., Southampton, UK), firefly and Renilla luciferase content was quantified using the Dual-Glo assay (Promega).

β-Catenin Gene Silencing siRNA—Transfection of HK-2 cells with siRNAs was optimized using the GAPDH Silencer II kit (Ambion Europe Ltd., Huntingdon, UK) according to the manufacturer’s instructions. Briefly, 9 × 10⁴ cells per 12-well plate were transfected in suspension with 30 nM siRNA and 5 μl of siPORT Amine (Ambion Europe Ltd.) in a final volume of 1000 μl. After 48 h, cells were lysed, and RNA was extracted using Tri-reagent, before detection of gene expression using q-PCR as described below. This protocol was found to give optimal knockdown (reliably 80% or greater reduction in GAPDH mRNA; data not shown). Following optimization, the same protocol was followed for β-catenin siRNA transfection (siRNA ID115717 Ambion Europe Ltd.). Mock-transfected and scrambled siRNA transfected controls were included in all experiments.

Quantitative RT-PCR—Following cDNA synthesis as described previously (12), TGF-β1 mRNA was quantified by real time RT-PCR. Primers and probe for TGF-β1 quantification were designed using Primer Express Software and purchased from Applied Biosystems (Warrington, UK). The amplification product is intron spanning. Sequences used are as follows: forward primer, 5′-CTTTCTCTGTTCCTACGAC-3′, reverse primer, 5′-CTTTCAAGGGAGTCTTG-3′; probe, 5′-FAM-ACACAAACTTGCTTCAGCCAGGAGAACATGCTGCTG-3′. PCR was performed on an ABI PRISM 7000 (Applied Biosystems) using the standard protocol for the machines in a total volume of 25 μl containing 300 nM forward and reverse primers, 100 nM probe, 50% v/v Taqman reaction buffer (Applied Biosystems). β-Catenin, 18 S rRNA and human large ribosomal protein RPLPO expression (huPO) expression were quantified using pre-developed Taqman assay kits (Applied Biosystems) according to the manufacturer’s instructions. Fold changes in expression were calculated using the formula 2⁻(ΔΔCt sample1 − ΔΔCt sample2), where ΔΔCt is the difference between the amplification threshold for the gene of interest (TGF-β1 or β-catenin) and the housekeeping genes (rRNA and huPO, respectively). Statistical analysis was performed using the unpaired Student’s t test.

Immunoblotting/Western Analysis—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. To obtain Triton-soluble fractions, the 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/m aprotinin) containing 1% Triton X-100 (TX buffer) for 30 min on ice. Samples were centrifuged at 12,500 rpm for 30 min, and the supernatant was transferred to a separate tube and kept at −70 °C until use. To optimize immunoblot analysis, the volume of lysis buffer was minimized by lysis of cells following detachment. Antibodies for immunoblot analysis were as follows: rabbit polyclonal anti-phospho-Smad3/1, rabbit polyclonal anti-phos-ho-Smad2, and rabbit polyclonal anti-Smad3anti Smad3, New England Biolabs (Hitchin, UK); goat polyclonal anti-Smad2 and rabbit polyclonal anti-β-catenin, Autogen Bio- clear (Calne, UK).

To isolate nuclear proteins, the cytoplasmic proteins were first extracted by the addition of 10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 50 mM dithiothreitol, 10 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 50 mM NaF and incubation on ice for 10 min. Subsequently following centrifugation, nuclear protein from the resultant pellet was extracted by washing cells 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/m aprotinin) containing 1% Triton X-100 (TX buffer) for 30 min on ice. Samples were centrifuged at 12,500 rpm for 30 min, and the supernatant was transferred to a separate tube and kept at −70 °C until use. To optimize immunoblot analysis, the volume of lysis buffer was minimized by lysis of cells following detachment. Antibodies for immunoblot analysis were as follows: rabbit polyclonal anti-phospho-Smad3/1, rabbit polyclonal anti-phospho-Smad2, and rabbit polyclonal anti-Smad3anti Smad3, New England Biolabs (Hitchin, UK); goat polyclonal anti-Smad2 and rabbit polyclonal anti-β-catenin, Autogen Bio- clear (Calne, UK).

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lulose membrane (Amersham Biosciences). The membrane was blocked with Tris-buffered saline containing 5% nonfat powdered milk for 1 h and then incubated with the appropriate primary antibody (see materials above) in Tris-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 (Tris-buffered saline/Tween) for 1 h at room temperature. The blots were subsequently washed in Tris-buffered saline/Tween and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Sigma) in Tris-buffered saline/Tween. Proteins were visualized using enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer’s instructions.

Immunoprecipitation—Immunoprecipitation was performed by standard methodologies. Briefly, cell protein samples were pre-cleared 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, 10 min), and the supernatant was collected. Primary antibody (mouse monoclonal anti-β-catenin antibody; Abcam Ltd., Cambridge, UK) (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) supernatant) for 2 h at 4 °C. The beads were removed by centrifugation (13,000 × g for 25 min), and the supernatant was washed twice with 1 ml of ice-cold PBS containing 1 mM thymidine at 4 °C for 1 h. The culture medium was removed, and the cells were washed twice with 1 ml of ice-cold PBS containing 1 mM thymidine. The beads were removed by centrifugation (13,000 × g for 25 min), and the supernatant was added. Subsequently samples were subject to immunoblot/Western analysis as described above. Specificity of immunoprecipitation was confirmed by negative control reactions performed with either no primary antibody or rabbit IgG control.

Immunohistochemistry—Cells were grown in 8-well multichamber slides (In Vitrogen). Following experimental manipulation, cells were rinsed three times in PBS for 5 min each, prior to fixation in ice-cold acetone/methanol (1:1 v/v) for 15 min at −20 °C. Following a blocking step (1% bovine serum albumin/PBS for 1 h), cells were incubated with the primary antibodies (1:50 dilution of rabbit anti-Smad3; Abcam Plc Cambridge, UK), for 1 h at room temperature followed by the incubation of fluorescein isothiocyanate-conjugated secondary antibody (1:50 dilution anti-rabbit immunoglobulin; Sigma). After washing with PBS, cells were mounted with fluorSave™ reagent (Calbiochem) containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc, Peterborough, UK).

Assessment of Caco-2 Cell Proliferation by Thymidine Incorporation—For assessment of cell proliferation, serum-free medium containing 1 µCi/ml of [3H]thymidine (2.0 Ci/mmol; Amersham Biosciences) was added to serum-deprived cells for 24 h. The culture medium was removed, and the cells were washed twice with 1 ml of ice-cold PBS containing 1 mM thymidine, prior to fixation in 500 µl of ice-cold 5% trichloroacetic acid containing 1 mM thymidine at 4 °C for 1 h. Subsequently, trichloroacetic acid was removed, and 0.5 ml of 0.1 M NaOH was added at room temperature overnight. Lysates were then neutralized with 1 M HCl prior to addition to scintillation vials and quantitation of incorporated radioactivity using a β-scintillation counter.

Statistical Analysis—Unless otherwise specified statistical analysis was performed using the unpaired Student’s t test, with a value of p < 0.05 considered to represent a significant difference. The data are presented as means ± S.D. of n experiments as indicated in figure legends. For each individual experiment the mean of duplicate determinations was calculated.

RESULTS

Apical Stimulation Leads to Enhanced TGF-β1 Smad3 Signaling—Polarity of TGF-β1 response PTC was assessed by its addition either to the apical or basolateral aspect of cells grown in tissue culture inserts. Smad3 activation was assessed by Western blot analysis of Smad3 phosphorylation. HK-2 cells were grown to confluence in tissue culture inserts, serum-deprived for 48 h, and subsequently stimulated with 1 ng/ml TGF-β1 for up to 24 h. At each time point following cell lysis, both phosphorylated Smad3 and total Smad3 were analyzed.
Polarity of TGF-β1 Response

![Image](49x482 to 408x733)

**FIGURE 2. β-Catenin regulates polarity of Smad3 response.** HK-2 cells were grown to confluence on porous tissue culture inserts, serum-deprived for 48 h, and stimulated with 1 ng/ml TGF-β1 added to either their apical (apic) or basolateral (baso) aspect. Following 3 or 6 h of stimulation, β-catenin (β-caten) was immunoprecipitated (IP) as described under “Materials and Methods.” A, Smad3 and β-catenin in the precipitate examined by Western analysis. In control experiments, cell lysates were prepared from unstimulated cells grown on porous tissue culture inserts. One representative experiment of at least four individual replicate experiments is shown. Cytoplasmic β-catenin was also examined by polarized stimulation of HK-2 with TGF-β1 for up to 24 h prior to addition of 1% Triton (Triton-soluble extract) and Western blot analysis for β-catenin. IB, Immunoblot. B, Western blot analysis for GAPDH was used as the loading control. β-Catenin gene silencing using siRNA was used to determine the role of β-catenin in TGF-β1-mediated activation of Smad3. Cells were transfected in suspension with 30 nM siRNA and 5 μl of siPORT Amine in a final volume of 1000 μl C, after 48 h, cells were lysed and RNA extracted using Tri-reagent, and β-catenin mRNA suppression was confirmed by q-PCR. Results represent mean ± S.D., n = 4. Parallel experiments were performed to confirm inhibition of β-catenin protein expression. Cells were transfected with β-catenin siRNA or scrambled oligonucleotide control. Cell lysates were prepared 48 and 72 h following transfection for analysis of β-catenin. D, Immunoblot. E, Western blot analysis for GAPDH was used as the loading control. Stimulating cells transfected with β-catenin siRNA with TGF-β1 subsequently determined the impact of suppression of β-catenin expression. Following introduction of β-catenin siRNA, HK-2 cells subsequently transfected with the Smad3-responsive promoter (SBE)4-Lux and pRL-CMV Renilla. 24 h following the second transfection, cells were stimulated with 1 ng/ml TGF-β1 for 6 h prior to lysis of the cells in Reporter Lysis Buffer. In control experiments, cell lysates were prepared from unstimulated cells. E, firefly and Renilla luciferase content was quantified using the Dual-Glo assay. F, in parallel experiments Smad3 activation was also examined by Western blot analysis of Smad3 phosphorylation. HK2 cells transfected with either β-catenin siRNA or scrambled (Scr) control oligonucleotide were stimulated with 1 ng/ml TGF-β1 for 24 h prior to cell lysis and analysis of phosphorylated and total Smad3.

which TGF-β1 was added to the apical aspect as compared with the basolateral aspect of the cells.

Suppression of Smad3 Activation Is Accompanied by Increased Smad3 Association with β-Catenin—Previously we have demonstrated that loss of PTC cell-cell contact results in association of the adherens junction protein component β-catenin with R-Smads. By using our polarized tissue culture system, addition of TGF-β1 to the basolateral aspect of PTC resulted in increased association of β-catenin with Smad3 (Fig. 2A). Basolateral stimulation of PTC was also associated with an increase in Triton-soluble, cytoplasmic β-catenin as compared with apical stimulation (Fig. 2B).

Inhibition of β-Catenin Expression by siRNA Enhances Smad3 Activation—We used β-catenin by gene silencing using siRNA to inhibit β-catenin expression and to determine the role of β-catenin in TGF-β1-mediated activation of Smad3. 48 h after transfection, β-catenin mRNA was significantly reduced in cells transfected with β-catenin siRNA compared with the scrambled siRNA (Fig. 2C). In addition β-catenin protein expression was significantly reduced 48 and 72 h following transfection of cells with β-catenin siRNA compared with the scrambled siRNA (Fig. 2D).

The impact of suppression of β-catenin expression was determined by stimulating cells transfected with β-catenin siRNA with TGF-β1. Using the Smad3 reporter construct, inhibition of β-catenin resulted in roughly a 3-fold increase in luciferase activity compared with nontransfected control and a 4-fold increase when compared with the scrambled siRNA (Fig. 2E). Phosphorylation of Smad3 as determined by Western analysis confirmed increased activation of Smad3 following β-catenin siRNA transfection compared with the scrambled siRNA control (Fig. 2F).

Inhibition of β-Catenin Degradation Suppresses Smad3 Activation—The signals resulting in increased cytoplasmic β-catenin include inhibition of the serine-threonine kinase glycogen synthase kinase-3 (GSK-3), which phosphorylates β-catenin on specific serine and threonine residues, resulting in subsequent degradation of β-catenin via the ubiquitin-protosme pathway (20). Lithium chloride is a specific inhibitor of GSK-3 that results in accumulation of cytoplasmic β-catenin (21). Our previous studies have demonstrated a decrease in total β-catenin following TGF-β1 stimulation. In contrast, addition of lithium resulted in increased expression of β-catenin both when added alone and also in the presence of TGF-β1 (Fig. 3A). Pretreatment of PTC with lithium for 6 h resulted in decreased Smad3 phosphorylation following addition of TGF-β1 compared with control cells to which no lithium had been added (Fig. 3B). In addition, lithium pretreatment significantly attenuated TGF-β1-stimulated Smad3-responsive luciferase reporter activity (Fig. 3C).

Previous studies have demonstrated that Smad signaling may be regulated through modulation of ubiquitin-mediated degradation (22). To determine whether lithium-dependent β-catenin accumulation influenced Smad3 degradation, we examined its in vitro ubiquitin-mediated degradation as described previously (23). The degradation pattern of endogenous Smad3 from cells pretreated with lithium was identical to Smad3 from control cells (Fig. 4A).
Altered Smad Signaling—In the context of TGF-β1 auto-induction we have previously demonstrated that Smad3 signaling is specifically involved in AP-1-dependent activation of the TGF-β1 promoter (24). To examine the functional consequence of enhanced Smad3 signaling following apical TGF-β1 stimulation, in this study we therefore examined the polarity of TGF-β1 transcription by q-PCR. An increase in TGF-β1 mRNA was demonstrated following either apical or basolateral stimulation with TGF-β1 for either 24 or 48 h (Fig. 5). Consistent with increased Smad3 signaling following apical stimulation, induction of TGF-β1 mRNA following apical stimulation with TGF-β1 was significantly greater than following basolateral stimulation at both time points.

Polarity of TGF-β1 Is Not Con- fined to Smad3 Activation—In addition to Smad3, TGF-β1 may alter cell function via activation of an alternative R-Smad, Smad2. The influence of polarity on TGF-β1-dependent Smad2 activation and the role of β-catenin were therefore examined. As with Smad3, activation of a Smad2 luciferase reporter construct was significantly greater following apical stimulation compared with basolateral stimulation (Fig. 6A). In addition Smad2 phosphorylation assessed by Western analysis was also significantly greater following apical stimulation (Fig. 6B).

The role of β-catenin in Smad2 activation was determined by both β-catenin gene silencing using siRNA and also by increas-
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ing β-catenin accumulation using lithium chloride. Inhibition of β-catenin expression was associated with enhanced Smad2 phosphorylation assessed by Western blot analysis (Fig. 6C). In contrast pretreatment of cells with lithium and β-catenin accumulation attenuated Smad2 activation quantified as luciferase activity of the Smad2-responsive reporter construct (Fig. 6D).

General Applicability of Observations—Caco-2 cells, a colonic cancer cell line in which there is increased in β-catenin expression (25) and TGF-β1 unresponsiveness (26), were used to determine the wider implications of our observations and to test the hypothesis that β-catenin-mediated negative regulation of TGF-β1-dependent R-Smads may have wider applicability beyond renal epithelial cells. To address this issue, we examined the effect of suppression of β-catenin using siRNA on TGF-β1-dependent phosphorylation of Smad3. By Western blot analysis at all time points up to 24 h post-transfection, β-catenin was significantly reduced in Caco-2 cells transfected with β-catenin siRNA (Fig. 7A). The impact of suppression of β-catenin expression on TGF-β1 signaling was determined by stimulating Caco-2 cells transfected with β-catenin siRNA with TGF-β1. Phosphorylation of Smad3 as determined by Western blot analysis confirmed increased activation of Smad3 following β-catenin siRNA transfection as compared with the scrambled siRNA control (Fig. 7B). Caco-2 cells are known to be unresponsive to the anti-proliferative effects of TGF-β1. We therefore evaluated the effect of inhibition of β-catenin on TGF-β1-dependent cell proliferation, using thymidine incorporation as a measure of cell proliferation. Suppression of β-catenin by gene silencing resulted in a significant anti-proliferative response following addition of TGF-β1 compared with that seen following addition of TGF-β1 to cells transfected with scrambled β-catenin siRNA (Fig. 7C).

DISCUSSION

TGF-β1 is a multifunctional cytokine, which is involved in the maintenance of normal homeostasis. It is important in development and tissue differentiation and normal wound healing through its effects on cell proliferation, migration, differentiation, and apoptosis (27). TGF-β1 is also important in the pathological process of fibrosis and subsequent organ failure, leading to the concept of fibrotic disease as, “the dark side of tissue repair” (28, 29). Its importance in renal fibrosis is illustrated by a recent report in which ultrasound microbubble-mediated gene transfer of inducible Smad7 blocks TGF-β1 signaling and fibrosis in rat remnant kidney (30).

Although traditionally PTC is viewed as a cell that is important in maintaining fluid and electrolyte balance, it is also an important cell type involved in the pathogenesis of renal fibrosis through the generation of pro-fibrotic cytokines, and in particular TGF-β1. We have demonstrated previously that disease-associated stimuli such as glucose (31), pro-inflammatory cytokines (32), and direct contact of PTC with macrophages (33) all modulate TGF-β1 synthesis. Generation of de novo bio-active TGF-β1 is however complex with independent regulation at the level of transcription, translation, secretion, and activation of the latent protein complex.
In addition to these well described stages regulating TGF-β1 synthesis, altering sensitivity of signaling may regulate the biological consequence of an increase in TGF-β1 concentration. As TGF-β1 drives PTC epithelial-mesenchymal transformation, understanding regulation of PTC response to TGF-β1 is an important goal in elucidating the mechanisms that contribute to interstitial fibrosis and progressive renal disease. Although our primary focus is renal fibrosis, it is clear that understanding TGF-β1 signaling and its regulation has implications far beyond this specific research area.

TGF-βs elicit their signaling effects by binding mainly to the following 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, which is initiated when the ligand induces assembly of a heteromeric complex of type II and type I receptors. The RI kinase then phosphorylates RI on a conserved glycine-serine-rich domain. This activates the RI kinase, which subsequently recognizes and phosphorylates members of the intracellular receptor-regulated Smads (R-Smads) signal transduction pathway. 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 (reviewed in Ref. 34).

In this study we have confirmed our previous observations that the polarity of the PTC influences the response of the cell to TGF-β1. More specifically, TGF-β1-dependent activation of R-Smad is greater following apical as compared with basolateral stimulation. The functional significance of this was assessed by examining TGF-β1 auto-induction. Consistent with our previous data demonstrating Smad3-mediated, AP-1-dependent transcriptional activation of the TGF-β1 promoter (24), in this study apical stimulation with TGF-β1 results in a significantly greater increase in TGF-β1 mRNA than basolateral stimulation. It is widely accepted that proteinuria is an adverse prognostic indicator in various renal diseases. Furthermore, there is mounting evidence that urinary protein may directly affect PTC function thus contributing to the pathogenesis of renal fibrosis (35–40). An increase in urinary TGF-β1 is associated with proteinuria, and urinary TGF-β1 levels have been proposed to represent a noninvasive tool to evaluate disease activity (41). More recent studies suggest that reduction in urinary TGF-β1 may in itself be of therapeutic benefit, contributing to the renoprotective effects of blockade of the renin-angiotensin system in diabetic nephropathy (41) and accounting for the added benefit of combination therapy with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers (42, 43). This suggests that in addition to the direct effects of apical presentation of albumin, which may activate PTC, increases in urinary TGF-β1 associated with proteinuria may also have direct effects leading to activation of profibrotic effects of TGF-β1.

TGF-β1 receptors internalize into both caveolin- and EEA1-positive vesicles and reside in both lipid raft and nonraft membrane domains (44). Clathrin-dependent internalization into the EEA1-positive endosome promotes TGF-β1 signaling. In contrast the lipid raft-caveolar internalization pathway contains Smad7-bound receptor and is required for receptor turnover. We have demonstrated that altered TGF-β1 function may be associated with trafficking of receptors between these two pools (45). The polarity of the observed effects of TGF-β1, described in this study, suggests an alternative mechanism other than a generalized change in receptor trafficking. Our previous data demonstrated that basolateral stimulation led to disassembly of adherens junctions, which was less apparent following apical stimulation. Furthermore, we identified a pool of TGF-β receptors, which specifically associate with the adherens junction complex. The data in this study suggest that adherens junction disassembly and the subsequent alteration in β-catenin expression is a negative regulator of TGF-β1 signaling. Following basolateral application of TGF-β1, there is enhanced dissociation of β-catenin from the adherens junction complex resulting in increased availability of cytoplasmic β-catenin expression by increased Triton-soluble β-catenin expression (6). The result of this is an increased level of association of the released cytoplasmic β-catenin with R-Smad. Direct involvement of β-catenin in modulating R-Smad signaling is supported by the data demonstrating that inhibition of β-catenin expression led directly to increased Smad2 and Smad3 phosphorylation and signaling following addition of...
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TGF-β. The subcellular localization of β-catenin is under strict control. It is either bound to the intracellular domain of E-cadherin and the actin cytoskeleton at the plasma membrane or is targeted for ubiquitin-mediated degradation through the interaction with the adenomatous polyposis coli, axin, and GSK-3 complex. Ubiquitin-mediated degradation of R-Smad protein has also been demonstrated to regulate cytoplasmic protein levels and influence TGF-β1 signaling. In this study, increased β-catenin expression following addition of lithium, although attenuating Smad activation, did not alter the rate of in vitro ubiquitin-mediated Smad degradation, which suggests that association of β-catenin does not increase Smad degradation. That the degradative pathway is not involved is also consistent with the mode of action of lithium, which prevents GSK-3 activity and β-catenin degradation. Nuclear translocation of R-Smad, however, is attenuated by lithium pretreatment. These data collectively suggest that interaction of R-Smad with β-catenin allows persistence of β-catenin in the cytoplasm and that the physical interaction of the proteins prevents R-Smad movement into the nucleus thus suppressing R-Smad gene target activation.

The general importance of signaling molecules downstream of TGF-β1 and β-catenin is well established in both cell fate and proliferation in health and disease. Numerous studies have demonstrated cross-talk between TGF-β and Wnt/β-catenin signaling. For example β-catenin regulates wound size and mediates the effect of TGF-β in cutaneous healing (46). Cooperation between β-catenin and TGF-β1 signaling has also been demonstrated in chondrocytes, playing a key role in chondrocyte differentiation, suggesting that integration of these signaling pathways are important in controlling endochondral ossification (47, 48). Smad-3-dependent nuclear translocation of β-catenin is also required for TGF-β1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells (49). These data therefore suggest that the cooperative effects of β-catenin and TGF-β1 might be applicable to all mesenchymal cells. In contrast our data suggest that this is not the case in epithelial cells. In contrast to the data generated in mesenchymal cells, our data suggest that in renal epithelial cells, β-catenin is associated with down-regulation of TGF-β1 signaling. This contrast between epithelial and mesenchymal cells is also supported by previous published studies, for example in the context of cutaneous wound healing although β-catenin plays a crucial role in regulating mesenchymal cell function promoting successful wound healing, and in contrast β-catenin is not transcriptionally active in epithelial cells (46). Furthermore, there is no wound phenotype described in mice expressing conditional β-catenin null alleles in keratinocytes (50). This cell type specificity may also be explained by our previous observations suggesting that the Wnt-responsive Txwtn reporter and the Wnt/LEF-1-responsive reporter are both silent in response to TGF-β1 in our experimental system (5) suggesting the cooperativity only exists in terms of the TGF-β1 signaling pathway in this cell type.

The applicability of the data to epithelial cells beyond the areas of fibrosis and wound healing is suggested by our data generated using the Caco-2 cells. Inactivation of the TGF-β1 pathway occurs often in malignancies of the gastrointestinal tract. Lack of anti-proliferative effect of TGF-β1 is also a feature of the Caco-2 cells, a cell line derived from colonic carcinoma in which the resistance to the anti-proliferative effects of TGF-β (26) is associated with an increase in β-catenin expression (25). We have demonstrated that suppression of β-catenin by gene silencing in this cell line results in increased R-Smad activation and restoration of TGF-β1-dependent suppression of cell proliferation. Therefore, this further supports the hypothesis that β-catenin in epithelial cells, in contrast to mesenchymal cells, suppresses TGF-β1-dependent signaling. Altered β-catenin expression may therefore play a pivotal role in altered epithelial cell responses associated with carcinogenesis.

In summary we have demonstrated that in epithelial cells, association of β-catenin with R-Smads decreases their nuclear translocation and TGF-β1-dependent signaling. In terms of PTC this suggests a further mechanism by which apical presentation of protein and TGF-β1 may contribute to progressive renal fibrosis. In addition the data support the notion that there may be a general applicability of the data to epithelial cell biology both in health and disease in which β-catenin expression may be important in regulating the response of the cell to TGF-β1.

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