Blockage of Tubular Epithelial to Myofibroblast Transition by Hepatocyte Growth Factor Prevents Renal Interstitial Fibrosis

JUNWEI YANG and YOUHUA LIU
Division of Cellular and Molecular Pathology, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Abstract. Activation of α-smooth muscle actin–positive myofibroblast cells is a key event in the progression of chronic renal diseases that leads to end-stage renal failure. Although the origin of these myofibroblasts in the kidney remains uncertain, emerging evidence suggests that renal myofibroblasts may derive from tubular epithelial cells by a process of epithelial to mesenchymal transition. It was demonstrated that hepatocyte growth factor (HGF) exhibited a remarkable ability to block this phenotypic transition both in vitro and in vivo. HGF abrogated the α-smooth muscle actin expression and E-cadherin depression triggered by transforming growth factor-β1 in tubular epithelial cells in a dose-dependent manner. HGF also blocked morphologic transformation of tubular epithelial cells and inhibited the expression and extracellular deposition of fibronectin. In a mouse model of renal fibrosis disease induced by unilateral ureteral obstruction, transforming growth factor-β1 receptor expression was specifically increased in renal tubules, and myofibroblastically phenotypic transition of the tubules was evident in vivo. Remarkably, injections of exogenous HGF blocked myofibroblast activation and drastically prevented renal interstitial fibrosis in the obstructed kidneys. These results suggest that tubular epithelial to myofibroblast conversion may play an important role in the pathogenesis of renal fibrosis and that blocking this phenotypic transition could provide a novel therapeutic strategy for the treatment of fibrotic diseases.

Despite the diverse initial causes, chronic renal disease that progresses to end-stage renal failure is a remarkably monotonous process that is characterized by the relentless accumulation and deposition of extracellular matrix (ECM) leading to widespread tissue fibrosis (1–3). Activation of α-smooth muscle actin (αSMA)-positive myofibroblast cells is believed to be a central event that plays a key role in the progression of chronic renal fibrosis. Indeed, myofibroblast activation has been identified as a predictor of renal disease progression both in experimental animal models and in patients (4,5). Clearly, a possible key to an effective therapy for progressive renal fibrosis is to find a strategy that prevents the activation and accumulation of renal myofibroblasts in the diseased kidneys.

Although the fundamental role of myofibroblasts in progressive renal fibrosis is well established, the origin of these αSMA-positive cells in the kidney under pathologic conditions remains poorly defined. They are often presumed to derive from local activation of renal interstitial fibroblasts and/or perivascular smooth muscle cells (6). Previous studies from our laboratory showed that blockage of hepatocyte growth factor (HGF) signaling in vivo by a neutralizing antibody markedly induces de novo expression of αSMA in renal tubular epithelium in the remnant kidney model of chronic renal disease (7). Some αSMA-positive renal tubular epithelial cells are found to become elongated and sometimes separated from neighboring cells. This finding is consistent with the hypothesis that, under pathologic conditions, tubular epithelial cells may transdifferentiate into myofibroblasts, as marked by the expression of αSMA (8,9). Furthermore, these observations suggest that endogenous HGF signaling is essential for preserving and maintaining the tubular epithelial cell phenotype by blocking this epithelial to myofibroblastic conversion in vivo.

Here we provide in vitro and in vivo evidence to support that renal myofibroblasts may derive from tubular epithelial cells by an epithelial to myofibroblast transition process under chronically pathologic conditions and that HGF exhibits a remarkable ability to block this epithelial to myofibroblast transition both in vitro and in vivo.

Materials and Methods

Cell Culture and Treatment

Human proximal tubular epithelial HKC cells (clone 8) were obtained from Dr. L. Racusen (The Johns Hopkins University, Baltimore, MD) and maintained in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY), as described previously (10,11). The HKC cells were seeded on six-well culture plates to approximate confluence in complete medium containing 5% fetal bovine serum for 16 h, and then changed to serum-free medium after washing twice with medium. Recombinant human transforming growth factor-β1 (TGF-β1; R & D Systems, Minneapolis, MN) was added to the culture at a final concentration of 2 ng/ml except when indicated otherwise. For dose-dependent studies, TGF-β1 was used at the concentrations of 0.05, 0.1, 0.5, 1, 2, 4, and 10 ng/ml, respectively.
Recombinant human HGF (provided by Genentech, South San Francisco, CA) was also added at the same time at the concentration of 20 ng/ml except when indicated otherwise. The cells typically were incubated for 72 h after addition of cytokines, except when indicated otherwise, before harvesting and subjecting to Western blot or immunofluorescence staining, respectively. For some experiments, the cells were incubated with vehicle (phosphate-buffered saline [PBS]) or 0.2 nM of various cytokines, including epidermal growth factor, insulin-like growth factor-I, platelet-derived growth factor, interleukin 6, monocyte chemotactic protein-1 (R & D Systems), and 100 nM angiotensin II (Sigma Chemical, St. Louis, MO).

**Animals and HGF Treatment**

Male CD-1 mice that weighed 18 g to 20 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Eighteen mice were randomly assigned to three groups with 6 mice each. Unilateral ureteral obstruction (UUO) was performed using an established procedure (12,13). Briefly, under general anesthesia, complete ureteral obstruction was performed by double-ligating the left ureter using 4-0 silk after a midline abdominal incision. Sham-operated mice had their ureters exposed and manipulated but not ligated. Starting on the day of surgery, mice were administrated recombinant human HGF through tail-vein injections at a dose of 200 μg/kg body wt every 12 h for 6 d. Control mice received an injection of the same volume of vehicle (0.9% saline solution). Mice were killed 7 d after surgery, and the kidneys were removed. One part of the kidneys was fixed in 10% phosphate-buffered formalin for histologic and immunohistochemical studies after paraffin embedding. Another part was immediately frozen in OCT compound for cryosection. The remaining kidneys were snap-frozen in liquid nitrogen and stored at −80°C for protein extractions.

**Western Blot Analysis**

HKC cells and cytokine-treated cells were lysed with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue). Kidney tissues were homogenized by a polytron homogenizer (Brinkmann Instruments, Westbury, NY) in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml antipain, and 2 μg/ml leupeptin in PBS) on ice. The supernatants were collected after centrifugation at 13,000 × g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Sigma), and whole-tissue lysates were mixed with an equal amount 2× SDS loading buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 100 mM DTT, and 0.2% bromophenol blue), as described previously (14). Samples were heated at 100°C for approximately 5 to 10 min before loading and were separated on precasted 10% or 5% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins were electrotransferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) in transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, and 20% methanol at 4°C for 1 h. Non-specific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes were then incubated for 16 h at 4°C with various primary antibodies in blocking buffer containing 5% milk at the dilutions specified by the manufacturers.

The mouse monoclonal anti-αSMA antibody (clone 1A4) was purchased from Sigma. The monoclonal antibodies for E-cadherin (clone 36) and fibronectin (clone 10) were obtained from Transduction Laboratories (Lexington, KY). The goat polyclonal anti–type I collagen antibody was obtained from Southern Biotechnology Associates (Birmingham, AL). The anti–TGF-β1 type I receptor (sc-398) and anti-actin (sc-1616) antibodies were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). After extensive washing in TBS buffer, the membranes were then incubated with horseradish peroxidase–conjugated secondary antibody (Bio-Rad) for 1 h at room temperature in 1% nonfat milk dissolved in TBS. Membranes were then washed with TBS buffer, and the signals were visualized using the enhanced chemiluminescence system (ECL, Amersham).

**Indirect Immunofluorescence Staining**

Indirect immunofluorescence staining was performed using an established procedure (15). Briefly, control or cytokine-treated HKC cells cultured on coverslips were washed with cold PBS twice and fixed with cold methanol:acetone (1:1) for 10 min on ice. After extensive washing with PBS containing 0.5% bovine serum albumin three times, the cells were blocked with 20% normal donkey serum in PBS buffer for 30 min at room temperature and then incubated with the specific primary antibodies described above, except for the mouse monoclonal antivimentin (clone V9) and rat monoclonal anti–E-cadherin (clone DECMA-1), which were obtained from Sigma. To visualize the primary antibodies, cells were stained with FITC-conjugated secondary antibodies (Sigma). After washing, cells were double-stained with 4,6-diamidino-2-phenylindole, HCl to visualize the nuclei. For visualizing F-actin, cells were stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin. Stained cells were mounted with antifade mounting medium (Vector Laboratories, Burlingame, CA) and viewed with a Nikon Eclipse E600 Epi-fluorescence microscope (Melville, NY).

For co-localization of αSMA and the proximal tubular marker in kidney sections using immunofluorescence staining, frozen sections at 5 μm thickness were cut using a cryostat, mounted onto poly-l-lysine–coated slides, and fixed in 4% paraformaldehyde in PBS for 30 min. The slides were stained for αSMA using the Vector M.O.M. immunodetection kit by the protocol specified by the manufacturer (Vector Laboratories). The slides were then stained with fluorescein-conjugated lectin from *Tetragonolobus purpureus* (Sigma). Stained slides were viewed and photographed as described above.

**Morphological and Immunohistochemical Studies**

Kidney sections from paraffin-embedded tissues were prepared at 4-μm thickness using a routine procedure (7). Sections were stained with hematoxylin/eosin for routine histology. Another set of sections was stained with Masson-Trichrome method for identifying interstitial collagen by blue color (7,13). Immunohistochemical localization was performed using the Vector M.O.M. immunodetection kit. The primary antibodies used were antifibronectin (Transduction Laboratories), antivimentin and anti–E-cadherin (Sigma), polyclonal anti–TGF-β1 (sc-146), anti–TGF-β type I receptor, and mouse monoclonal anti–proliferating cell nuclear antigen (*sc-36*, Santa Cruz Biochemical). As a negative control, the primary antibody was replaced with either nonimmune mouse or rabbit IgG, corresponding to species of the primary antibodies. Tubular E-cadherin and vimentin expression was semiquantitatively determined by counting positive tubules in at least 10 randomly chosen nonoverlapping high-power (×400) fields for each mouse. Injury score, collagen deposition, and fibronectin expression were scored on a scale from 0 to 3, as previously reported (7,16): 0, absent; 1, mild; 2, moderate; and 3, severe. The overall mean scores were calculated based on individual values, which were determined on at least 10 fields per mouse, six mice per group.
**Determination of Tissue TGF-β1 Levels by Enzyme-Linked Immunosorbent Assay**

For measurement of tissue TGF-β1 level, kidneys were homogenized in the extraction buffer containing 20 mM Tris-HCl (pH 7.5), 2 M NaCl, 0.1% Tween-80, 1 mM ethylenediaminetetraacetate, and 1 mM phenylmethylsulfonyl fluoride, and the supernatant was recovered after centrifugation at 19,000 \(g\) for 20 min at 4°C. Kidney tissue TGF-β1 level was determined by using a commercial Quantikine TGF-β1 enzyme-linked immunosorbent assay kit in accordance with the protocol specified by the manufacturer (R & D Systems). The concentration of active TGF-β1 and total TGF-β1 (acid-activated) in kidneys was expressed as picograms per milligram of total protein.

**Statistical Analyses**

Animals were randomly assigned to control and treatment groups. All data examined were expressed as mean ± SEM. For Western blot analysis, quantitation was performed by scanning and determination of the intensity of the hybridization signals. Statistical analysis of the data were performed by Student-Newman-Kuels test using SigmaStat software (Jandel Scientific, San Rafael, CA). Values of \(P < 0.05\) were considered to be statistically significant.

**Results**

**HGF Blocks the De Novo Expression and Assembly of αSMA In Vitro**

To seek in vitro evidence that tubular epithelial cells can undergo conversion into myofibroblasts, we used a human renal proximal tubular epithelial (HKC) cell line as a model system to screen a panel of cytokines and hormones for their ability to induce the de novo expression of αSMA, the phenotypic marker of myofibroblasts. Among the agents tested, TGF-β1, a well-characterized profibrogenic cytokine, markedly induced de novo αSMA expression in HKC cells (Figure 1). Hepatocyte growth factor (HGF) abrogates the expression and assembly of α-smooth muscle actin (αSMA) in human tubular epithelial HKC cells. HKC cells were incubated for 3 d without (control) or with the same molar concentration (0.2 nM) of various cytokines and 100 nM angiotensin II (a and b), except when otherwise indicated (c and d through g). The cell lysate was immunoblotted with specific antibody against αSMA. The same samples were reprobed with β-actin to ensure equal loading. (a) Transforming growth factor-β1 (TGF-β1) induced the de novo expression of αSMA in tubular epithelial HKC cells. (b) HGF abolished the αSMA expression induced by TGF-β1 in HKC cells. (c) Dose-dependent inhibition of the TGF-β1–induced αSMA expression by HGF in HKC cells. (d through g) Representative photographs of the αSMA visualized by indirect immunofluorescence staining in HKC cells after various treatments; (d) control; (e) 2 ng/ml TGF-β1; (f) 20 ng/ml HGF; (g) TGF-β1 plus HGF. The αSMA-positive microfilaments were evident in TGF-β1–treated cells (e). Simultaneous incubation with HGF blocked TGF-β1–induced αSMA expression and assembly in tubular epithelial HKC cells (g). Scale bar, 10 μm.
cytokine capable of blocking TGF-β1 treatment was suppressed to approximately 10% of that in the control culture. It is interesting that simultaneous incubation of HGF restored the E-cadherin expression depressed by TGF-β1 in HKC cells (Figure 2). These results indicate that HGF not only prevents the de novo expression of the myofibroblast marker αSMA in HKC cells but also prohibits the loss of the epithelial marker E-cadherin. The loss of E-cadherin expression in tubular epithelial cells after TGF-β1 treatment was also independently confirmed by indirect immunofluorescence staining (Figure 2, b through e). In agreement with the Western blot data, treatment of TGF-β1 resulted in total loss of E-cadherin staining in the plasma membrane of HKC cells and co-incubation with TGF-β1 and HGF largely restored the E-cadherin protein staining.

**HGF Blocks Phenotypic Transformation of Tubular Epithelial to Myofibroblasts**

Concomitant with the alterations in gene expression described above (Figures 1 and 2), we found that HGF blocked the morphologic transformation of tubular epithelial cells into the myofibroblastic appearance induced by TGF-β1. HKC cells displayed typical cobblestone morphology of epithelial cells when grown in culture. TGF-β1 induced profound morphologic changes, with cells becoming elongated in shape, disassociating from neighboring cells, and losing their cobblestone monolayer pattern. Simultaneous incubation of HGF with TGF-β1 largely restored the epithelial morphology of the HKC cells (Figure 3). Because the actin cytoskeleton plays an important role in defining cell shape and morphology (22,23), we examined the F-actin reorganization during TGF-β1–induced tubular epithelial to myofibroblast conversion. TGF-β1 induced marked F-actin reorganization, forming abundant, long stress fibers in HKC cells (Figure 3), providing a structural basis for the defined morphology of the transformed cells.

**HGF Restores the Depression Of E-Cadherin Expression Induced by TGF-β1 in Tubular Epithelial Cells**

E-cadherin is a tubular epithelial cell-cell adhesion receptor that is essential for the formation and maintenance of the homeostasis and architecture of renal epithelia (17–21). We found that TGF-β1 dramatically repressed E-cadherin expression in HKC cells (Figure 2). Western blot analyses revealed that the level of E-cadherin protein expression after TGF-β1

1a). Dose-response studies revealed that TGF-β1 induced αSMA expression at a concentration as low as 0.1 ng/ml. Remarkably, HGF dramatically abrogated TGF-β1–induced αSMA expression in HKC cells in a dose-dependent manner (Figure 1, b and c). At a concentration of 20 ng/ml, HGF completely blocked αSMA expression induced by 2 ng/ml TGF-β1. As the molecular weight of HGF (95 kD) is much larger than that of TGF-β1 (12.5 kD), the molar concentrations of HGF (approximately 0.21 nM) and TGF-β1 (approximately 0.16 nM) were comparable. Other cytokines, such as epidermal growth factor and insulin-like growth factor-I, did not significantly suppress TGF-β1–induced αSMA expression at the same molar concentration as HGF (0.2 nM) (Figure 1b). These results suggest that HGF is the most potent, if not unique, cytokine capable of blocking TGF-β1–induced αSMA expression in tubular epithelial cells in vitro. Using an indirect immunofluorescence staining, we also demonstrated that TGF-β1 induced αSMA expression and assembly in HKC cells, as shown by the presence of cytoplasmic αSMA-positive microfilaments (Figure 1, d through g). HGF markedly blocked this induction of αSMA in tubular epithelial cells. Of note, TGF-β1 was unable to induce αSMA expression and phenotypic conversion in mouse inner medullary collecting duct (mIMCD-3) cells that are developmentally derived from ureteric bud epithelium (data not shown).

**Figure 2.** HGF restores the depression of E-cadherin expression induced by TGF-β1 in tubular epithelial HKC cells. HKC cells were incubated without (control) or with 2 ng/ml TGF-β1, 20 ng/ml HGF, or both for 3 d. (a) Western blot demonstrates that HGF restored the loss of E-cadherin expression induced by TGF-β1 in HKC cells. The cell lysate was immunoblotted with antibody against E-cadherin. The same samples were reprobed with actin to ensure equal loading. (b through e) Immunofluorescence staining for the distribution of E-cadherin in tubular epithelial cells after incubation with TGF-β1 or/and HGF; (b) control; (c) TGF-β1; (d) HGF; (e) TGF-β1 plus HGF. Scale bar, 10 μm.
HGF also significantly abolished this TGF-β1–induced actin reorganization and stress fiber formation (Figure 3).

We next examined the phenotypic consequence of the myofibroblastically transformed cells induced by TGF-β1. Following TGF-β1 treatment, the transformed cells exhibited robust expression of vimentin, another marker for mesenchymal cells, which was organized in marked intermediate filaments all over the cells; and HGF significantly blocked this induction of vimentin (Figure 3). Furthermore, the transformed cells began to markedly produce the components of interstitial matrix, such as fibronectin and collagen I. Western blot analyses revealed that TGF-β1 induced the expression of fibronectin by HKC cells (Figure 4). Immunofluorescence staining displayed a dramatic increase in the extracellular assembly and deposition of fibronectin after TGF-β1–induced cell transdifferentiation (Figure 4). These results indicate that the transformed cells, like activated myofibroblasts, have acquired the capacity to produce and to assemble properly a large amount of interstitial matrix in the extracellular compartment. Although HGF alone only slightly reduced basal fibronectin expression in tubular epithelial HKC cells, simultaneous incubation of HGF markedly blocked the TGF-β1–induced expression and extracellular assembly and deposition of fibronectin (Figure 4). TGF-β1 also induced collagen I, another major component of interstitial matrix, after epithelial to myofibroblast conversion, and HGF abolished the induction (data not shown). Taken together, these data clearly suggest that HGF blocks tubular epithelial to myofibroblast conversion and prevents subsequent interstitial matrix accumulation in vitro.

**HGF Blocks the Induction of αSMA Expression and Prevents Myofibroblast Activation In Vivo**

To examine whether exogenous HGF prevents myofibroblast activation in vivo, we administered recombinant human HGF by intravenous injection into mice with renal fibrosis disease induced via UUO. This model of fibrosis is characterized by renal myofibroblast activation, tubular atrophy, and interstitial fibrosis with minimal glomerular lesions (24). The obstructed kidneys displayed marked activation of myofibroblasts at 7 d after surgery, characterized by a dramatic increase in αSMA abundance (Figure 5a). Strikingly, intravenous injections of exogenous HGF (200 μg/kg body wt every 12 h for 6 d) largely blocked renal αSMA expression (Figure 5a). Quantitative studies revealed that the level of αSMA protein in the obstructed kidneys after HGF administration was markedly inhibited compared with that given vehicle (Figure 5b), suggesting that HGF prevents renal myofibroblast activation in vivo.

To localize the αSMA-positive cells in the diseased kidney, we used a double immunofluorescence staining for both αSMA and a proximal tubule marker, FITC-labeled lectin from *Tetragonolobus purpureas* (25,26). The αSMA staining typically was limited to small arteries and arterioles in the kidneys of sham-operated mice. However, a large increase in αSMA...
staining was observed in the widened interstitium and proximal tubular epithelium of the obstructed kidneys (Figure 5). Co-localization (yellow) of αSMA (red) and the proximal tubular cell marker (green) indicates that the tubular epithelial cells acquired a phenotypic marker of myofibroblasts in the obstructed kidney (Figure 5), suggesting that there are cells at the transitional stage between the epithelial and myofibroblast phenotypes. Of note, the αSMA-positive proximal tubular epithelial cells typically were disorganized, irregular in shape, and estranged from neighboring cells (Figure 5). After injection of exogenous HGF, renal αSMA staining in the obstructed kidneys declined toward the baseline level (Figure 5), suggesting that, similar to the in vitro situation, HGF drastically blocks this phenotypic conversion in vivo.

Both TGF-β1 and TGF-β Type I Receptor Are Specifically Upregulated in Tubular Epithelium In Vivo

Because TGF-β1 initiates tubular epithelial to myofibroblast conversion in vitro, we next examined the expression of TGF-β1 and its type I receptor in the diseased kidneys. The obstructed kidneys displayed a marked increase in TGF-β1 expression at 7 d after surgery (Figure 6). Quantitative determination by a specific enzyme-linked immunosorbent assay exhibited approximately 15-fold and 22-fold increases in active and total TGF-β1 protein, respectively, in the obstructed kidneys at 7 d after UUO (Figure 6b). Importantly, immunohistochemical staining revealed that TGF-β1 expression was largely upregulated in renal tubular epithelium in the diseased kidneys (Figure 6g). Injection of exogenous HGF markedly inhibited TGF-β1 expression in the obstructed kidney (Figure 6).

TGF-β type I receptor was also drastically increased in the obstructed kidney compared with that from sham-operated animals, as demonstrated by Western blot analyses of whole-kidney lysates (Figure 6a). After HGF administration, the level of TGF-β type I receptor in the obstructed kidneys was similar to that of control kidneys from sham-operated mice, indicating that exogenous HGF markedly inhibits TGF-β type I receptor expression in vivo (Figure 6a). Of interest, immunolocalization studies exhibited that the TGF-β type I receptor was specifically increased in renal tubular epithelium of the obstructed kidneys (Figure 6d). As the TGF-β1 receptor determines the cell-type specificity of TGF-β1’s actions, tubular epithelial cells seem to be the natural in vivo targets of this potent profibrogenic cytokine in the diseased kidneys.

HGF Preserves Tubular Epithelial Phenotypes and Ameliorates Renal Fibrosis In Vivo

Because HGF blocks epithelial to myofibroblast conversion in vitro and myofibroblast activation in vivo, we reasoned that blockage of this phenotypic transition by HGF might provide a novel strategy for preventing renal fibrosis. To this end, we investigated the consequence of HGF injections in vivo by examining the expression of various cell-type–specific markers and renal fibrosis in the obstructed kidneys after UUO. As shown in Figure 7, epithelial cell marker E-cadherin was markedly decreased in the tubular epithelium from the obstructed kidneys of UUO mice, whereas de novo expression of the mesenchymal marker vimentin was evident in the renal tubules. These data essentially recapitulate our in vitro observations and suggest that cell phenotypic transdifferentiation may be of importance in the pathogenesis of renal fibrosis. Administration of exogenous HGF largely prevented this phenotypic conversion. Of interest, despite tubular atrophy as a characteristic feature of this model, increased cell proliferation, as demonstrated by proliferating cell nuclear antigen staining, was evident in the obstructed kidneys.

Blocking of cell phenotypic transition by administration of recombinant HGF resulted in marked inhibition of renal tubulointerstitial fibrosis in vivo (Figure 7). The obstructed kidneys...
Figure 5. HGF blocks the induction of αSMA expression in a mouse model of renal fibrosis. (a) Western blot demonstrates a marked induction of αSMA expression in the obstructed kidneys after unilateral ureteral obstruction (UUO) for 7 days and HGF inhibition of renal αSMA expression in vivo. The kidney tissue lysate was immunoblotted with specific antibodies against αSMA and β-actin, respectively. (b) Graphic presentation of the relative abundance of αSMA protein in the kidneys from sham-operated mice, mice that had UUO and received an injection of vehicle, and mice that had UUO and received an injection of HGF. Data are presented as mean ± SEM of the relative abundance of αSMA normalized to β-actin from six animals per group (n = 6). **P < 0.01, sham versus UUO. †P < 0.01, UUO + vehicle versus UUO + HGF. (c through k) Double immunofluorescence staining shows co-localization of αSMA and proximal tubular epithelial cell marker. (c through e) Staining with proximal tubular marker, FITC-conjugated lectin from *Tetragonolobus purpureas* (green). (f through h) Staining with anti-αSMA antibody (red). (i through k) Merging of two images. (c, f, and i) Sham-operated; (d, g, and j) UUO + vehicle; (e, h, and k) UUO + HGF. Co-localization of αSMA and proximal tubular marker can be easily found in the kidney from UUO mice (yellow) (j). Arrows (d, g, and j) indicate proximal tubular cells positive for αSMA. Scale bar, 50 μm.
from the control group that received an injection of vehicle displayed typical tubular atrophy, interstitial expansion, and extensive fibrosis (Figure 7). Masson-Trichrome staining for collagen revealed that there was an increasing amount of collagen accumulation and deposition in the obstructed kidneys after UUO for 7 d. Administration of HGF largely protected the obstructed kidneys from developing these lesions, as the morphology, tubular integrity, collagen deposition, and fibronectin expression were significantly improved (Figures 7 and 8). Similar results were obtained by Western blot analyses of whole-kidney lysates using antibodies against collagen I, fibronectin, and vimentin (Figure 9). These results indicate that specific blockage of epithelial to myofibroblast transition by HGF markedly ameliorates renal interstitial fibrosis in vivo.

**Discussion**

Clinical studies suggest that in patients with chronic renal disease, the decline in renal function often correlates more closely with the extent of tubular and tubulointerstitial lesions than with that of glomerular injury (27–29). Despite this, the potential roles of tubular epithelial cells in the progression of chronic renal disease are often underestimated, if not ignored. This is largely due to lack of crucial evidence that connects tubular epithelial cells with fibrotic matrix accumulation and deposition. In this study, we provide evidence that under pathologic conditions, tubular epithelial cells may transdifferentiate into myofibroblasts by a conversion process known as epithelio-mesenchymal transition.

Figure 6. Both TGF-β type I receptor and TGF-β1 are specifically upregulated in renal epithelia in vivo. (a) Western blot shows a marked increase in TGF-β type I receptor in the kidney after UUO for 7 d and inhibition of renal TGF-β type I receptor expression with HGF. Numbers (1 through 9) indicate individual animals. (b) Quantitative determination of TGF-β1 protein abundance by enzyme-linked immunosorbent assay shows a dramatic increase in both active (■) and total (□) TGF-β1 protein in the obstructed kidneys after UUO for 7 d. Administration of HGF inhibited renal TGF-β1 expression. Data are presented as mean ± SEM from six animals per group (n = 6). *P < 0.01, sham versus UUO. †P < 0.05, **P < 0.01, UUO versus UUO + HGF. (c through e) Immunohistochemical staining shows that increased expression of TGF-β type I receptor was found specifically in renal tubules after UUO for 7 d. (f through h) Expression of TGF-β1 was also observed largely in renal tubules after UUO. (c and f) Sham-operated animal. (d and g) UUO + vehicle. (e and h) UUO + HGF. Asterisks (*) denote positive tubules. Scale bar, 40 μm.
Figure 7. HGF blocks tubular epithelial to myofibroblast transition and ameliorates renal fibrosis in vivo. Representative light micrographs of kidney sections are taken from various groups of mice after UUO for 7 d. Left column (a, d, g, j, m, and p), normal, sham-operated mice; middle column (b, e, h, k, n, and q), mice that had UUO and received injections of vehicle; right column (c, f, i, l, o, and r), mice that had UUO and received injections of HGF. (a through c) E-cadherin; (d through f) vimentin; (g through i) proliferating cell nuclear antigen; (j through l) hematoxylin and eosin staining; (m through o) Masson-Trichrome staining; (p through r) fibronectin. Arrows (a, e, and q) indicate positive tubular cells. Scale bar, 20 μm.
After the appropriate stimulus, the tubular epithelial cells acquire the myofibroblast marker αSMA, display myofibroblastic morphology, lose epithelial E-cadherin, and produce interstitial matrix components such as fibronectin. Profibrogenic cytokine TGF-β1 is a strong initiator of this conversion, and HGF dramatically blocks this process both in vitro and in vivo. These observations suggest that tubular epithelial cells actively play a role in the pathogenesis of chronic renal fibrosis, rather than being innocent bystanders, as previously thought. Our data also establish that tubular epithelial to myofibroblast transition perhaps is a new, novel target for therapeutic strategies designed to treat chronic renal fibrotic diseases.

The identification of TGF-β1 as a key player in renal epithelial to myofibroblast conversion allows us to reexamine its role and mechanisms in progressive renal fibrosis. It is widely accepted that TGF-β1 is a key modulator of organ fibrosis after tissue injury (31,32). However, previous studies emphasized the role of TGF-β1 in stimulating myofibroblasts to produce fibrogenic proteins. Little is known about the origin of renal myofibroblasts or the effects of TGF-β1 on tubular epithelial cells in renal fibrogenesis. Paradoxically, it is the tubular epithelium where the TGF-β type I receptor, which determines which cells respond to TGF-β1, is specifically and almost exclusively upregulated in diseased kidneys (Figure 6) (33), suggesting that tubular epithelial cells are the in vivo targets of TGF-β1 under pathologic conditions. Indeed, significant changes in cell phenotypes, such as loss of E-cadherin and de novo expression of αSMA and vimentin, take place in renal tubules in the obstructed kidneys. Cells at the transitional stage with both αSMA and tubular marker are abundant in vivo (Figure 5), which is consistent with a previous observation demonstrating the presence of cells still positive for epithelial markers in the widened interstitium of end-stage diseased kidney in patients (25). It is conceivable to speculate that once cells progress through this transitional stage, they will lose tubular marker and retain the αSMA and myofibroblastic phenotypes. In view of the fact that TGF-β1 initiates renal epithelial to myofibroblast transition in vitro and that tubule-specific elevation of TGF-β1 and its receptor protein occurs in the diseased kidney, our results likely provide novel insights into the mechanisms of this potent profibrogenic cytokine in promoting chronic renal fibrosis.

Given that, except for the collecting duct cells, all renal epithelial cells are developmentally derived from the metanephrogenic mesenchyme via mesenchymal to epithelial conversion (19,34), it is not surprising to find that tubular epithelial cells possess the ability to transdifferentiate back into a mesenchymal phenotype under certain pathologic conditions. This process, in essence, is a reverse embryogenesis. In agreement with this view, we found that the renal collecting duct cell line mIMCD-3, which is developmentally derived from ureteric bud epithelium, cannot undergo phenotypic conversion after incubation with TGF-β1, suggesting that renal epithelial to myofibroblast transition likely is limited to the tubular epithelia that developmentally derived from metanephrogenic mesenchyme. HGF is obviously a key regulator of renal cell transdifferentiation both during normal nephrogenesis and un-

Figure 8. Semiquantitative analysis of immunohistochemical findings. Kidney sections from various groups were stained as described in Figure 7. Data are presented as mean ± SEM from six animals per group (n = 6). **P < 0.01, sham versus UUO. *P < 0.01, UUO versus UUO + HGF.
Under pathologic circumstances. Previous studies showed that HGF and its specific receptor c-met are coexpressed in early metanephrogenic mesenchyme, leading to formation of an autocrine loop essential for promoting mesenchymal to epithelial transition during early nephrogenesis (35,36). Furthermore, coexpression of both HGF and c-met in vitro by transfection of fibroblasts induces a phenotypic conversion into epithelium (37), and HGF has been shown to be responsible for facilitating the conversion of meta-
ephrogenic mesenchymal cells into epithelium (38). In light of the role of HGF in initiating/promoting mesenchymal to epithelial conversion during nephrogenesis, it is reasonable to speculate that HGF is also essential for preserving the tubular epithelial cell phenotype by blocking an epithelial to myofibroblast transition under pathologic conditions.

It should be noted that given the pleiotropic nature of its actions, HGF inhibition of renal fibrogenesis in the obstructed kidneys could be mediated by other mechanisms as well. Studies from our laboratory and others demonstrate that HGF protects tubular epithelial cells from apoptosis and promotes matrix degradation by upregulating proteinases (7,39–41). Therefore, inhibition of apoptosis and activation of matrix degradation by HGF could be two additional pathways that lead to the suppression of renal fibrogenesis in vivo. Of note, the presence of these potential multiple mechanisms by no means suggests that they are mutually exclusive. In fact, it is possible that the multiple pathways triggered by exogenous HGF may work in concert to lead to ultimate amelioration of renal fibrosis in the obstructed kidneys in vivo.

The observation that HGF blocks myofibroblast activation and fibrosis in animals after chronic renal injury may have significant implications for developing clinically relevant therapeutic strategies for renal diseases. It is tempting to propose that administration of HGF may provide a novel and effective treatment for chronic renal diseases by specifically inhibiting the activation of myofibroblasts, the principal cells responsible for the accumulation and deposition of extracellular matrix seen in the diseased kidney. Although the antifibrogenic effect of HGF in vivo remains to be verified in other animal models with different causes, delivery of exogenous HGF protein or its gene (42,43) seems to have the potential to block the progression of chronic renal fibrosis, one of the devastating diseases that is otherwise incurable.

Acknowledgments

This work was supported by the National Institutes of Health Grants DK-54922 and DK-02611 (Y.L.). J.Y. was supported by a postdoctoral fellowship from the American Heart Association, Pennsylvania-Delaware Affiliate. We thank Drs. G. K. Michalopoulos and W. M. Mars for stimulating discussion and critical review of this manuscript. We are grateful to Dr. R. Schwall of Genentech for the generous gift of human recombinant HGF.

References