Hepatocyte Growth Factor Gene Therapy and Angiotensin II Blockade Synergistically Attenuate Renal Interstitial Fibrosis in Mice

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Abstract. Tubulointerstitial fibrosis is considered to be a common endpoint result of many forms of chronic renal diseases. Except for renal replacement, chronic renal fibrosis is presently incurable. This study demonstrates that the combination of hepatocyte growth factor (HGF) gene therapy with inhibition of the renin-angiotensin system produced synergistic beneficial effects leading to dramatic attenuation of renal tubulointerstitial fibrosis in obstructive nephropathy in mice. The combined treatment with human HGF gene and losartan, an angiotensin II (AngII) type I receptor blocker, preserved renal mass and gross morphology of the obstructed kidneys. Although HGF gene therapy alone inhibited the expression of α-smooth muscle actin (αSMA) by approximately 54% and 60% at day 7 and day 14 after surgery, respectively, its combination with losartan almost completely abolished αSMA induction in the obstructed kidneys. The combined therapy also synergistically inhibited the accumulation of interstitial matrix components, such as fibronectin and collagen I, and suppressed renal expression of transforming growth factor–β1 (TGF-β1) and its type I receptor. In vitro studies revealed that AngII by itself did not induce αSMA, but it drastically potentiated TGF-β1–initiated αSMA expression in tubular epithelial cells. Furthermore, HGF abrogated de novo αSMA expression induced by TGF-β1 plus AngII. These results suggest that many factors are implicated in the pathogenesis of renal interstitial fibrosis; therefore, a combined therapy aimed at simultaneously targeting multiple pathologic pathways may be necessary for halting the progression of chronic renal diseases. These findings may provide the basis for designing future therapeutic regimens for blocking progressive renal fibrosis in patients.

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Tubulointerstitial fibrosis is often recognized as an endpoint outcome of a wide range of chronic renal diseases, regardless of the underlying pathogenesis (1,2). Studies show that deterioration of renal function is largely determined by the extent of tubulointerstitial alterations in many forms of renal diseases both in experimental animal models and in patients (1–3). Although most patients with chronic renal diseases (CRD) are diagnosed well before they reach end-stage renal failure, the therapy aimed at complete blockade of progressive loss of renal function has proven to be enormously difficult (4). Current therapy with a strategy to reduce the activities of renin-angiotensin system (RAS) at best slows, but does not completely halt, the progression of chronic renal fibrosis in experimental and clinical conditions (5,6).

Angiotensin II (AngII), the central component of RAS, appears to be critical in initiating and sustaining the fibrogenic destruction of the kidney (7). Its actions are likely mediated by both hemodynamic and nonhemodynamic components. The contribution of AngII to the progression of chronic renal pathology has been elegantly illustrated by several genetic studies using knockout mouse models (8–10). Pharmacologic studies using angiotensin-converting enzyme (ACE) inhibitors and AngII receptor blockers also implicate the hyperactive RAS as a major mechanism leading to the development of chronically fibrotic lesions in the kidney (9,11–14). However, probably because many factors other than RAS contribute to the pathogenesis of chronic renal disease, blockade of the RAS alone has limited efficacy on prevention of renal fibrogenesis after persistent injury (15–18). Clearly, other novel therapeutic strategies must be developed, and perhaps the combination of new therapy with conventional RAS blockade may be necessary to achieve ideal therapeutic outcomes in patients.

Recent studies from our laboratory and others demonstrate that hepatocyte growth factor (HGF), a multiple functional protein with potent renotropic properties, may have therapeutic effects preventing chronic renal fibrosis (19–21). Blocking endogenous HGF signaling with neutralizing antibody markedly promotes renal tissue fibrosis and dysfunction in different models of CRD (22,23). Conversely, administration of recombinant HGF protein or its gene prevents the development and progression of renal lesions and dysfunction (24–27). Although the mechanism underlying HGF beneficial effects is not entirely understood, antagonizing the actions of profibrotic cytokine transforming growth factor–β1 (TGF-β1) probably
plays an important role. It has been shown that HGF specifically suppresses both TGF-β1 and its type I receptor (TβR-I) expression in the diseased kidneys (25,26). HGF also abolishes myofibroblastic transformation from tubular epithelial cells triggered by TGF-β1 in vitro (24). These observations suggest that the beneficial effects of HGF may be mediated, at least in part, by specifically targeting and effectively blocking the hyperactive TGF-β1 expression and signaling as seen in most, if not all, chronically diseased kidneys.

On the basis of the observations that TGF-β1 and AngII are imperative in initiating and promoting renal fibrosis, we hypothesized that a combined therapy with exogenous HGF and AngII blockade may have additive or synergistic effects on preventing renal fibrogenesis by simultaneously blocking the actions of pathogenic TGF-β1 and AngII. In this report, we show that whereas HGF gene transfer and AngII blockade individually display partially beneficial effects in slowing the progression of renal interstitial fibrosis in obstructive nephropathy, the combination of these two agents exhibits a synergistic efficacy that results in a superior therapeutic outcome.

Materials and Methods

Animals

Male CD-1 mice weighing 18 to 22 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were housed in the animal facilities of the University of Pittsburgh Medical Center with free access to food and water. Animals were treated humanely using approved procedures in accordance with the guidelines of the Institutional Animal Use and Care Committee of National Institutes of Health at the University of Pittsburgh School of Medicine. Unilateral ureteral obstruction (UUO) was performed using an established procedure (25,28). Briefly, under general anesthesia, complete ureteral obstruction was carried out 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. Mice were randomly assigned into five groups (n = 6): (1) sham normal control; (2) UUO control; (3) UUO receiving HGF alone; (4) UUO receiving losartan alone; and (5) UUO receiving both HGF and Losartan. Delivery of human HGF was achieved by intravenous injection of naked HGF plasmid vector (see below), whereas AngII type I receptor (AT1) antagonist, losartan (Merck Research Laboratories, Rahway, NJ), was administrated immediately after surgery in the drinking water at 200 mg/L.

To demonstrate the reproducibility of the synergistic effects of HGF and losartan, the animal experiments were repeated and expanded. Briefly, 60 CD-1 mice were cloned as described previously (29). The empty expression plasmid vector pcDNA3 was purchased from Invitrogen (San Diego, CA). Plasmid DNA was administrated into mice by rapid injection of a large volume of DNA solution through the tail vein, as described previously (30,31). Briefly, 10 μg of plasmid DNA was diluted in 1.6 ml of saline and injected via the tail vein into the circulation within 5 to 10 s. Mice in groups 3 and 5 were injected twice with pCMV-HGF plasmid, one immediately after UUO (day 0) and another at day 7. Mice in groups 2 and 4 were injected with 10 μg of control empty vector pcDNA3 at the same time points in an identical manner. For animals sacrificed at day 7 after surgery.
surgery, only a single injection of plasmid vectors at day 0 was performed. Groups of mice ($n = 6$) were sacrificed at day 7 and day 14 after UUO, respectively, and the kidneys were removed. After the kidney was decapsulated and cut on paper towel to eliminate the urine pooled in dilated calyx, kidney weights were recorded. One part of the kidneys was fixed in 10% phosphate-buffered formalin for histologic studies after paraffin embedding. Another part was immediately frozen in Tissue-Tek OCT compound for cryosection. The remaining kidneys were snap-frozen in liquid nitrogen and stored at $-80^\circ$C for protein extraction.

**Determination of HGF Levels by Enzyme-Linked Immunosorbent Assay**

For measurement of tissue HGF level, kidneys from mice at day 14 after UUO were homogenized in the HGF extraction buffer containing 20 mM Tris-HCl, pH 7.5, 2 M NaCl, 0.1% Tween-80, 1 mM EDTA, and 1 mM PMSF, as described previously (31). After centrifugation at 19,000 × g for 20 min at 4°C, the supernatant was recovered for determination of HGF using an enzyme-linked immunosorbent assay (ELISA) method according to the protocols described previously (31). Briefly, the 96-well microtiter plates (Nunc-Immuno Module, Fisher Scientific, Pittsburgh, PA) were incubated with 50 µl of uncoupled monoclonal anti-HGF antibody (H14) per well diluted in 50 mM Tris-HCl, pH 8.0, at a final concentration of 1.5 µg/ml at room temperature for 16 h. H14 anti-human HGF antibody was prepared using a standard protocol of hybridoma technology and described previously (31). This antibody could detect human HGF protein, but it does not crossreact with the endogenous HGF in mouse and rat. Fifty-microliter aliquots of standard human HGF solution or tissue samples were added to the wells of the coated plates and incubated for 2 h at room temperature. After extensive washing, a 100-µl aliquot of biotinylated goat anti-human HGF polyclonal antibody (R & D Systems, Minneapolis, MN) at a dilution of 1:2000 was added, and the plates were incubated for another 2 h. After washing, they were then incubated with 100 µl of horseradish peroxidase (HRP)–conjugated streptavidin (Zymed Laboratories, South San Francisco, CA) at a dilution of 1:20,000 and subsequently with enzyme substrate solution containing 0.1 mg/ml of tetramethylbenzidine and 0.006% H$_2$O$_2$ in 0.1 M sodium citrate, pH 6.0. The plates were allowed to stand for 30 min at room temperature, and the reaction was stopped by addition of 50 µl of 4-N H$_2$SO$_4$. Absorbance was read at 405 nm by an automatic Emax Precision Microplate Reader (Molecular Devices Co., Sunnyvale, CA). Total protein levels were determined using a bicinecinic-acid (BCA) protein assay kit (Sigma, St. Louis, MO) with bovine serum albumin (BSA) as a standard. The concentration of HGF in kidneys was expressed as ng/mg total protein.

**Western Blot Analyses**

For detection of human HGF protein levels in vivo, total kidney protein extracts as described above for ELISA assay were used for Western blot analyses. For detection of other proteins such as αSMA by immunoblotting, kidney tissues were homogenized in RIPA lysis buffer (1% NP40, 0.1% sodium dodecyl sulfate [SDS], 100 µg/ml PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 µg/ml aprotinin, 2 µg/ml antipain, and 2 µg/ml leupeptin in phosphate-buffered saline [PBS]) on ice, and 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 tissue lysates were mixed with an equal amount 2 × SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, and 0.2% bromophenol blue), as described previously (32). Samples were heated at 100°C for 5 to 10 min before loading and separated on precasted 10%
antigen (PCNA), and TGF-
was obtained from Southern Biotechnology Associates, Inc. (Bir-
Lexington, KY). The goat polyclonal anti-type I collagen antibody
bronectin antibody was obtained from Transduction Laboratories

The membranes were then incubated with HRP-conjugated secondary
antibodies in blocking buffer containing 5% milk. The monoclonal
membranes were then incubated for 16 h at 4°C with various primary
antibodies after normalization with actin in the obstructed kidneys among different
groups at 14 d after UUO. Data (fold induction relative to sham
control) are presented as mean ± SEM of six animals per group (n = 6). *P < 0.01 versus sham control.

Figure 3. HGF does not further promote cell proliferation in the
obstructed kidneys. (a) Representative Western blot shows the levels
of proliferating cell nuclear antigen (PCNA) protein among various
groups in the obstructed kidneys at 14 d after UUO. The same blot
was stripped and reprobed with actin to confirm equal loading. 1 and
2 indicate two individual animals represented in each group. (b)
Graphic presentation of the relative abundance of PCNA after
normalization with actin in the obstructed kidneys among different
groups at 14 d after UUO. Data (fold induction relative to sham
control) are presented as mean ± SEM of six animals per group (n = 6). *P < 0.01 versus sham control.

or 5% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins
were electrophoresed to a nitrocellulose membrane (Amer-
sham, 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. Nonspecific binding to the membrane was blocked for 1 h at room
temperature with 5% Carnation nonfat milk in Tris-buffered saline
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. The monoclonal
anti-HGF antibody (H14) was diluted at a final concentration of 2
μg/ml. The αSMA antibody was purchased from Sigma. The antifi-
brocnictin antibody was obtained from Transduction Laboratories
(LEXINGTON, KY). The goat polyclonal anti-type 1 collagen antibody
was obtained from Southern Biotechnology Associates, Inc. (Bir-
mingham, AL). The antibodies against actin, proliferating cell nuclear
antigen (PCNA), and TGF-β type I receptor were purchased from
Santa Cruz Biochemicals (Santa Cruz, CA). After extensive washing,
the membranes were then incubated with HRP-conjugated secondary
antibody (Bio-Rad) for 1 h at room temperature in 1% nonfat milk. The signals were visualized by the enhanced chemiluminescence
system (ECL, Amersham).

**TUNEL Staining**

Apoptotic cell death was determined by using terminal deoxynu-
cleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining with Apoptosis Detection System (Promega, Madison, WI) (33). Briefly, 5-μm-thick kidney cryosections were washed by immersion into PBS and then treated with protease K at 20 μg/ml
in TE buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA) for 10 min. After preequilibration in 100 μl of buffer containing 200 mM potassium
cacodylate, 25 mM Tris-HCl, pH 6.6, 0.2 mM diithiothreitol, 0.25 mg/ml BSA, and 2.5 mM cobalt chloride, strands of DNA were
end-labeled by incubation at 37°C for 1 h in 50 μM fluorescein-12-
dUTP, 100 μM dATP, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, and
TdT. The reaction was stopped by adding 2× sodium chloride/sodium
chloride/sodium citrate hybridization buffer for 15 min. After being washed, the slides
were mounted and observed on Nikon Eclipse E600 Epi-fluorescence
microscope. Apoptotic cells were counted in at least five randomly
chosen nonoverlapping high power (×400) fields for each mouse and
expressed as apoptotic cells per field.

**Biochemical Measurement of Total Kidney Collagen Content**

To quantitatively measure collagen accumulation and deposition in
the kidney, total tissue collagen content was determined by biochem-
ical analysis of the hydroxyproline in the hydrolysates extracted from
kidney samples. This assay is based on the observation that essentially
all the hydroxyproline in animal tissues is found in collagen. Briefly,
accurately weighed portions of the obstructed kidneys were homog-
ennized in distilled H2O. The homogenates were hydrolyzed in 10 N HCl by incubation at 110°C for 18 h. The hydrolysates were dried by
speed vacuum centrifugation over 3 to 5 h and redissolved in a buffer
containing 0.2 M citric acid, 0.2 M glacial acetic acid, 0.4 M sodium
acetate, and 0.85 M sodium hydroxide, pH 6.0. Hydroxyproline concentrations in the hydrolysates were chemically measured according
to the techniques previously described (34,35). Total collagen was
calculated on the basis of the assumption that collagen contains 12.7%
hydroxyproline by weight. The results of total tissue collagen content
were expressed as μg of collagen per mg of kidney weight.

**Morphologic Studies**

Paraffin-embedded kidney sections from the mice were prepared at
4 μm thickness by a routine procedure. Sections were stained with
hematoxylin/eosin for general histology. Renal morphologic injury, as
characterized by tubular dilution with epithelial atrophy and intersti-
tial expansion with matrix deposition, were scored in a blind fashion
on the basis of a scale of 0 (absent), 1 (mild), 2 (moderate), and 3
(severe), as previously reported (22). Five random, nonoverlapping
fields at high magnification (×400) were selected for scoring in the
cortical region from each animal. The overall injury index was calcu-
lated on the basis of individual values determined per mouse and
expressed as mean ± SEM of six animals per group.

**Immunofluorescence Microscopy**

Kidney cryosections were prepared at 5 μm thickness and fixed for
5 min in PBS containing 3% paraformaldehyde. After being blocked
with 1% normal donkey serum in PBS for 30 min, the sections were
incubated with primary antibodies against fibronectin and collagen I,
respectively, in PBS containing 1% BSA overnight at 4°C. Sections
were then incubated for 1 h with fluorescein-conjugated secondary antibodies at a dilution of 1:200 in PBS containing 5% BSA before being extensively washed with PBS. As a negative control, the primary antibody was replaced with nonimmune IgG, and no staining occurred. Slides were mounted with anti-fade mounting media (Vector Laboratories, Burlingame, CA) and viewed with a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY).

For colocalization of αSMA and the proximal tubular marker in the kidney, cryosections 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 purpureas* (Sigma). Stained slides were viewed and photographed as described above.

**Determination of Tissue TGF-β1 Levels by ELISA**

For measuring renal TGF-β1 levels, mouse kidneys were homogenized in the extraction buffer containing 20 mM Tris-HCl, pH 7.5, 2 M NaCl, 0.1% Tween-80, 1 mM EDTA, and 1 mM PMSF, 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 ELISA kit in accordance with the protocol specified by the manufacturer (R & D Systems). This kit detects active TGF-β1 protein that binds to its soluble type II receptor precoated onto a microplate. Total protein levels were determined using a BCA protein assay kit as described above. The concentration of TGF-β1 in kidneys was expressed as pg/mg total protein.

**Cell Culture and Treatment**

Human proximal tubular epithelial HKC cells (clone 8) were provided by Dr. Lorraine Racusen of Johns Hopkins University and maintained in DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS) (Life Technologies, Inc., Grand Island, NY), as described previously (36, 37). The HKC cells were seeded on 6-well culture plates to 60 to 70% confluence in complete medium containing 5% FBS for 16 h and then changed to serum-free medium after washing twice with medium. Recombinant human TGF-β1 (R&D Systems) was added to the culture at the concentrations as indicated. AngII was purchased from Sigma and used at the concentrations ranging from 10⁻⁹ to 10⁻⁶ M. Recombinant human HGF (provided by Genentech Inc. South San Francisco, CA) was also added at the same time at the concentrations as indicated. The cells were typically incubated for 72 h after addition of growth factors and hormone before harvesting and subjecting to Western blot analyses. For control experiments, the cells were incubated with vehicle (PBS). The entire experiments were repeated at least three times.

**Statistical Analyses**

Animals were randomly assigned to control and treatment groups. Quantitation of the Western blot data were performed by measuring the intensity of the hybridization signals using NIH Image analysis software (25). Statistical analyses of the data were performed by using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by the Student-Newman-Kuels test. *P* < 0.05 was considered significant.

**Results**

*Combined HGF Gene Therapy and AngII Blockade Preserve Renal Mass and Gross Morphology after Ureteral Obstruction*

Mice were injected through the tail vein with pCMV-HGF plasmid or control vector pcDNA3 at day 0 and day 7, respectively, after unilateral ureteral obstruction (UUO). Exogenous
HGF protein expression in the obstructed kidneys was determined by ELISA and Western blot using a specific anti-human HGF monoclonal antibody. As shown in Figure 1a, more than 150 ng of human HGF per mg total protein was detected in whole kidney lysates at 14 d after ureteral obstruction. Western blot also exhibited that human HGF protein was readily detected at 14 d in the obstructed kidneys after pCMV-HGF plasmid injections. No detectable human HGF protein was found in whole kidney lysates receiving empty pcDNA3 plasmid (Figure 1). These results confirm a sustained expression of HGF gene therapy and AngII blockade synergistically inhibit α-smooth muscle actin (αSMA) expression and myofibroblast activation in the obstructed kidneys. (a and c) Representative Western blot shows the levels of αSMA protein among various groups in the obstructed kidneys at day 7 (a) and day 14 (c) after UUO. The same blot was stripped and reprobed with actin to confirm equal loading. 1 and 2 indicate two individual animals represented in each group. (b and d) Graphic presentation of the relative abundance of αSMA after normalization with actin in the obstructed kidneys among different groups at day 7 (b) and day 14 (d) after UUO. Data (fold induction relative to sham control) are presented as mean ± SEM of either six (b, n = 6) or twelve (d, n = 12) animals per group. *P < 0.01 versus sham; †P < 0.05 versus UUO control; **P < 0.01 versus HGF alone. (e through h) Representative micrographs show double immunofluorescence staining for αSMA (red) and proximal tubular marker, lectin from Tetragonolobus purpures (green), in the obstructed kidneys among different groups at day 14 after UUO. Co-localization (yellow) of αSMA and tubular marker was visible in the obstructed kidneys at day 14 (arrowheads). (e) UUO control, (f) HGF alone, (g) losartan alone, (h) HGF plus losartan. Scale bar, 20 μm.
exogenous HGF in the obstructed kidneys after intravenous injection of naked plasmid vector.

Figure 2 shows representative gross morphology and renal mass in five different treatment groups at day 14 after surgical operation. As expected, prolonged ureteral obstruction for 14 d caused severe reduction of renal mass. Treatment with AT1 receptor blocker, losartan, alone displayed no appreciable beneficial effects on renal mass, although it improved the gross morphology (Figure 2g). Combined therapy with HGF gene and losartan not only completely reversed the reduction of renal mass but also dramatically preserved the gross morphology of obstructed kidneys after persistent, complete ureteral obstruction. Similar results were obtained in five different treatment groups at day 7 after UUO (Figure 2f).

We next investigated the mechanism underlying the preservation of renal mass after combined therapy. Because renal mass presumably results from the balance of cell growth and cell loss, we examined the cell proliferation and apoptosis in the obstructed kidneys among various treatment groups. As shown in Figure 3, ureteral obstruction caused an increased cell proliferation in the diseased kidneys at 14 d after UUO, as shown by a marked increase in proliferating cell nuclear antigen (PCNA) expression. To our surprise, HGF gene therapy alone did not further induce cell proliferation. In fact, PCNA levels in the kidneys receiving HGF plasmid injections tended to decline compared with pcDNA3 groups (Figure 3).

Marked apoptosis was detected in the obstructed kidneys at 14 d after UUO, as demonstrated by TUNEL assay. Interestingly, HGF gene therapy alone significantly prevented cells from apoptotic death. Although losartan alone only marginally inhibited apoptosis, the combination of HGF and losartan dramatically prevented cell death (Figure 4). Thus, the preservation of renal mass after combined therapy is mainly mediated by inhibition of apoptosis, rather than by promoting cell proliferation.

**Synergistic Effects of HGF Gene Transfer and AngII Blockade on Inhibition of αSMA Expression and Myofibroblast Activation**

Figure 5 demonstrates the levels of αSMA protein in the obstructed kidneys among five different treatment groups at day 7 and day 14 after UUO, respectively. Compared with the sham-operated animals, the obstructed kidneys displayed a dramatic upregulation of αSMA protein. Quantitative determination revealed that the levels of αSMA in the obstructed kidney at day 7 and day 14 were approximately 26-fold and 58-fold higher than that in sham control animals, respectively (Figure 5, a through d), manifesting a marked activation of the
matrix-producing myofibroblast cells as typically seen in this model.

HGF gene therapy significantly inhibited αSMA expression in the obstructed kidneys (Figure 5). Approximately 54% and 60% inhibition of αSMA expression was observed at day 7 and day 14, respectively, in the obstructed kidneys receiving intravenous injections of pCMV-HGF plasmid. Administration of losartan alone at 200 mg/L in the drinking water for 7 d also inhibited αSMA expression to a lesser extent in the obstructed kidney. However, at day 14 after UUO, losartan alone only exhibited marginal suppression of αSMA expression in the obstructed kidney. Combination of both HGF gene transfer and losartan strikingly suppressed renal αSMA expression in a very dramatic fashion. As shown in Figure 5, the levels of αSMA protein in the obstructed kidneys receiving both HGF gene and losartan were essentially similar to that in sham-operated group either at day 7 or at day 14 after surgery. Quantitative determination exhibited that combined therapy abrogated αSMA expression by more than 90% in the obstructed kidney at 7 and 14 d after ureteral obstruction, respectively, suggesting a marked synergistic effect of exogenous HGF and AngII blockade on preventing αSMA induction and myofibroblast activation.

Consistent with the Western blot results, immunofluorescence staining also revealed a synergistic effect of HGF gene therapy and AngII blockade on inhibition of myofibroblast activation. There was marked induction of αSMA staining in the obstructed kidneys at 14 d after UUO. Double fluorescence staining for both αSMA (red) and a proximal tubular marker (green), lectin from *Tetragonolobus purpureas*, demonstrated the presence of co-localization (yellow) in some areas (Figure 5e), suggesting that tubular epithelial cells acquire a phenotypic marker of myofibroblasts by a process of epithelial to myofibroblast transition (EMT) in the obstructed kidney. Of note, at this stage of obstructive nephropathy, tubular marker largely disappeared (Figure 5e), presumably due to EMT. However, mice receiving intravenous injection of pCMV-HGF plasmid alone displayed a weak αSMA staining. The cells at the transitional stage containing both epithelial and myofibroblast markers were absent in the kidneys; and the αSMA-positive cells were primarily confined to the interstitial compartments (Figure 5f). Consistently, combined therapy with HGF gene and losartan markedly inhibited αSMA staining in the obstructed kidneys (Figure 5h).

**Figure 7.** Combined therapy with HGF and losartan suppresses total collagen accumulated in the obstructed kidney. Total kidney collagen contents in the obstructed kidneys at day 7 (a) and day 14 (b) after surgery were determined by biochemical assay. Results in various groups are presented as mean ± SEM (n = 6). *P < 0.01 versus sham control. **P < 0.01 versus sham control. #P < 0.05 versus sham control.

HGF Gene Therapy and AngII Blockade Synergistically Attenuate Renal Interstitial Fibrosis

The effects of combined therapy with HGF gene and losartan on the progression of renal morphologic lesions and interstitial fibrosis were examined in the obstructed kidneys. Figure 6 shows representative micrographs of the obstructed kidneys among various treatment groups. The obstructed kidneys receiving empty pcDNA3 vector developed severe renal lesions at 14 d after UUO, characterized by tubular dilation with epithelial atrophy, interstitial expansion with myofibroblast activation and matrix deposition (Figure 6b). HGF gene therapy alone attenuated these morphologic injuries in the obstructed kidneys (Figure 6c). Although administration of losartan for 14 d alone only slightly improved the morphologic appearance of the obstructed kidneys, combination of both HGF gene and losartan resulted in a remarkable inhibition of morphologic injuries seen in this model (Figure 6, e and f).

We next examined the accumulation and deposition of total collagen in the obstructed kidneys after various treatments by quantitative, biochemical measurements. Figure 7 shows the total kidney collagen contents in the obstructed kidneys at day 7 and day 14 after surgery, respectively. Ureteral obstruction significantly increased kidney collagen contents; approximately 2.5-fold and 5.6-fold induction of collagen deposition was observed in the obstructed kidneys at day 7 and day 14, respectively (Figure 7a and b). Although HGF alone inhibited collagen accumulation, combine therapy with losartan exhibited a dramatic suppression of collagen contents in the obstructed kidneys at different time points (Figure 7). In fact, the level of total collagen in the obstructed kidney treated with
HGF plus losartan at day 7 was compatible to that in sham control (6.40 ± 0.33 versus 5.43 ± 0.98; P > 0.05; n = 6). Similar results were obtained when the expression and accumulation of collagen I was examined. As shown in Figure 8, sustained obstruction led to marked induction of collagen I expression. Whereas HGF or losartan only partially inhibited collagen I expression and its interstitial deposition, combination of both resulted in marked suppression in the diseased kidneys (Figure 8). Synergistic effects of HGF and losartan were also observed on inhibition of collagen IV expression (data not shown).

The expression and deposition of fibronectin, another major component of interstitial matrix, was also investigated in the obstructed kidneys after various treatments. Figure 9 illustrates the results of fibronectin expression in the obstructed kidneys as determined by Western blot as well as an indirect immunofluorescence staining. Compared with the sham control, the obstructed kidneys receiving empty pcDNA3 vector exhibited a more than ninefold increase in renal expression of fibronectin at 14 d after UUO (Figure 9, a and b). Administration of either HGF gene or losartan resulted in substantial inhibition of fibronectin expression in the obstructed kidneys (Figure 9, a and b). Of interest, the combined therapy with both almost totally abolished the fibronectin induction in the obstructed kidneys (Figure 9, a and b). Similar results were obtained by using immunofluorescence staining (Figure 9, c through f).

**Suppression of TGF-β1 Axis Expression In Vivo by HGF Gene and AngII Blockade**

We investigated the expression of TGF-β1 in the obstructed kidneys after administration of HGF gene or losartan individually or in combination. Figure 10 shows the levels of active TGF-β1 protein determined by a specific ELISA in the obstructed kidneys at day 7 (Figure 10a) and day 14 (Figure 10b) after UUO, respectively. Ureteral obstruction induced a dramatic increase in renal TGF-β1 expression. Both HGF gene and losartan significantly inhibited the TGF-β1 expression in the obstructed kidneys when they were administrated individually (Figure 10, a and b). In combination, they apparently exhibited a more effective suppression of TGF-β1 expression. The expression of TβR-I was also examined in the obstructed kidneys. Western blot analyses exhibited that TβR-I was significantly increased in the obstructed kidney at 14 d after UUO compared with control sham kidney (Figure 10, c and d). This induction of TβR-I in the obstructed kidneys was inhibited by delivery of HGF gene. Administration of losartan alone did not significantly suppress renal TβR-I expression. However, combined therapy with HGF gene and losartan

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**Figure 8.** HGF gene therapy and AngII blockade inhibit interstitial collagen I expression and deposition. (a) Representative Western blot shows collagen I expression in obstructed kidney among various groups at 14 d after UUO. The same blot was reprobed with actin to confirm equal loading. (b) Graphic presentation of the relative abundance of collagen I after normalization with actin in the obstructed kidneys. Data (fold induction relative to sham control) are presented as mean ± SEM of six animals per group (n = 6). *P < 0.01 versus sham; †P < 0.05 versus UUO control; **P < 0.05 versus HGF or losartan alone. (c through f) Representative micrographs show collagen I deposition in the obstructed kidneys at day 14 after UUO. (c) UUO control, (d) HGF alone, (e) losartan alone, (f) HGF plus losartan. Scale bar, 20 μm.
markedly repressed TβR-I expression by approximately 70% in the obstructed kidneys (Figure 10, c and d).

AngII Does Not Initiate, but Synergistically Promotes, αSMA Expression and Tubular EMT In Vitro

To understand the potential mechanism underlying the synergistic effect of HGF and AngII blockade on inhibition of renal fibrosis, we investigated the interplay among AngII, TGF-β1, and HGF in the regulation of de novo αSMA expression in tubular epithelial cells, a key event known as epithelial to mesenchymal transition during renal interstitial fibrogenesis. As presented in Figure 11a, TGF-β1 induced de novo αSMA expression in human kidney tubular epithelial HKC cells at a concentration as low as 0.1 ng/ml. Under the same conditions, incubation of HKC cells with AngII at a concentration as high as 10^{-6} M did not induce αSMA expression, suggesting that AngII by itself does not initiate de novo expression of αSMA. However, the combination of TGF-β1 and AngII drastically stimulated αSMA expression in a highly synergistic way (Figure 11b). The αSMA protein level in HKC cells after combined treatment with TGF-β1 and AngII was about threefold of that induced by TGF-β1 alone, suggesting that AngII synergistically promotes TGF-β1-initiated αSMA expression and myofibroblastic transition.

We next examined the effects of HGF on αSMA expression in HKC cells. Treatment of HKC cells with HGF alone did not induce any αSMA expression (24). However, simultaneous co-incubation of HKC cells with HGF markedly blocked de novo αSMA expression triggered by the combination of TGF-β1 and AngII in a dose-dependent manner. At the concentration of 10 ng/ml, HGF almost completely abrogated the αSMA expression in HKC cells (Figure 11c). Collectively, these results indicate that for αSMA expression and myofibroblastic transition, TGF-β1 is an initiator, AngII works as a promoter, and HGF functions as a potent inhibitor.

Discussion

The initiation and progression of chronic renal fibrosis after persistent injury is a complicated process in which many factors may play an active role (1,15,18). In this regard, it is not surprising to find out that targeting hyperactive RAS alone as in current clinical therapy may only have limited efficacy in preventing the progressive loss of renal function. The present study was undertaken to test the hypothesis that a combined therapy by simultaneously targeting multiple pathogenic pathways may be more effective in preventing the progression of chronic renal fibrosis. Our results clearly demonstrate that delivery of exogenous HGF gene together with losartan, an
AT_1 blocker, synergistically inhibits renal αSMA expression and myofibroblast activation and attenuates renal interstitial fibrosis in obstructive nephropathy in mice. These studies suggest that combined treatment regimens by supplement of exogenous HGF with conventional pharmacologic agent may potentially lead to superior therapeutic outcomes for the treatment of chronic renal fibrosis in a clinical setting.

In mice with continuous, complete ureteral obstruction for 14 d, reduction of the RAS activities by AT_1 receptor blocker alone induced only partial attenuation of matrix deposition and morphologic lesions, with little improvement of renal mass and αSMA expression. Because the dose of losartan given is sufficient for functionally blocking AT_1 receptor (7,38), these results likely highlight a partial contribution of the RAS to the pathogenesis of chronic renal fibrosis. This notion is supported by several recent studies using sophisticated experimental approaches by reduction and augmentation of endogenous RAS activities via genomic manipulation of angiotensinogen (Agt) expression _in vivo_ (8,10). In mice lacking Agt, though reduced, renal interstitial fibrosis persists in the complete absence of AngII generation after ureteral obstruction (10). Likewise, interstitial fibrosis in UUO mice with Agt duplication (four-copy) is not further exacerbated (10). Significant renal interstitial fibrosis is also observed in _AT_1_α_ null mutant mice, especially in the late stage after ureteral obstruction (8). Furthermore, _in vitro_ studies reveal that AngII does not initiate αSMA expression and myofibroblastic activation from renal tubular epithelial cells (Figure 11). Altogether, these observations indicate that there is/are pathogenic pathway(s) other than the RAS leading to the development of chronic renal fibrosis. Thus, solely aiming at the RAS by pharmacologic inhibition of ACE or AT_1 receptors may miss other potentially major therapeutic targets for the treatment of chronic fibrotic lesions in the kidney.

Among many potential targets, TGF-β1 is probably a principal one because extensive studies implicate it in tissue fibrogenesis (15,39–41). TGF-β1 induction is found in many forms of chronic tissue injury. Inhibition of TGF-β1 expression via diverse strategies reduces renal pathology, and its overexpression leads to significant renal fibrosis (42,43). Intriguingly, although AngII-induced renal injury is classically attributable to the systemic and renal hemodynamic consequences of its vasoconstrictor action, increasing evidence suggests that AngII may induce renal fibrotic alterations in a hemodynamic-independent fashion, probably by upregulating TGF-β1 axis expression (15,44,45). Because many AngII blockade therapies...
inhibition of TGF-β1 expression and signaling using different approaches only partially ameliorates renal fibrosis (43,47). These observations imply that targeting TGF-β1 alone may also not completely halt renal fibrosis in clinical diseases.

The present work validates a hypothesis that a combined therapy targeting multiple pathogenic pathways may be necessary for an effective treatment of chronic renal disease. Although delivery of exogenous HGF gene alone attenuates largely renal fibrotic lesions, its combination with AT1 blocker dramatically improves its efficacy. The effectiveness of the combined therapy is quite striking, as exemplified by the near nullification of renal mass reduction, αSMA induction, and interstitial matrix deposition in the kidneys after complete obstruction for 14 d. Several potential mechanisms may explain for the efficacy of the combined therapy. One is obviously the reduction of the hyperactive RAS activities by the virtue of AT1 blocker administration. In vitro studies show that, although AngII does not per se induce αSMA expression, it immensely potentiates TGF-β1’s action. Another is the decreased expression of TGF-β1 axis. Of many biologic actions of exogenous HGF, the simultaneous inhibition of both TGF-β1 and TβR-I in vivo is perhaps most relevant to the suppression of renal fibrosis (23–25). More importantly, HGF specifically blocks the TGF-β1-triggered transdifferentiation of tubular epithelial cells into matrix-producing myofibroblasts (24), suggesting that it precisely targets a key event during renal fibrogenesis (3,48). Finally, whereas current clinical therapy only focuses on reducing the effects of the pathogenic mediators such as AngII or TGF-β1, the combined therapy with HGF potentially not only diminishes these pathogenic effects, but it also instructs the kidney cells, leading to a constructive repair process to restore the nephron structure and function (20,24,49–51). This potential is best illustrated by the complete preservation of renal mass after continuous ureteral obstruction for 14 d in mice. Therefore, the combined therapy may have the real potential to halt, rather than merely slow, the onset and progression of chronic renal disease.

Although TGF-β1 and AngII have been individually identified as major pathologic factors, our study demonstrates that there are dramatic interactions among themselves as well as to others (such as HGF) in the process of renal fibrogenesis. The novel finding that AngII markedly potentiates TGF-β1–initiated de novo αSMA expression underscores that these two separate signaling pathways are somehow converged in activating myofibroblast cells. Such convergence likely takes place at some points in their distinctive signaling circuits, rather than occurring through upregulation of TGF-β1 axis expression by AngII. This view is supported by the fact that incubation of AngII alone at 10^{-6} M for 3 d failed to induce trivial amount of αSMA in tubular epithelial cells. Of note, tubular expression of αSMA is considered as a key event known as EMT, which leads to activation of matrix-producing myofibroblast cells in the diseased kidneys. Indeed, the αSMA induction triggered by TGF-β1 is accompanied by induction of mesenchymal marker vimentin, loss of epithelial marker E-cadherin, production of interstitial matrix fibronectin, and morphologic transformation (24,40,52). The identification of the
transitional cells co-expressing both epithelial and myofibroblast markers in the obstructed kidney (Figure 5e) suggests that EMT plays an active role in obstructive nephropathy. Of note, the contribution of EMT to renal fibrogenesis in this study, as demonstrated by co-localization of αSMA and tubular marker, is underestimated because tubular marker is lost already at this stage (day 14) of obstructive nephropathy, presumably owing to EMT. Consistent with a critical role of TGF-β1 and AngII in EMT, administration of HGF, losartan, or both in vivo predominantly blocks EMT, as evidenced by the absence of the transitional cells and preservation of tubular structure and phenotypes (Figure 5). Therefore, our findings provide novel insights into the mechanism by which multiple pathologic signals work in concert to initiate and promote EMT in vivo that leads to renal interstitial fibrogenesis.

The inability of AngII alone to induce αSMA expression suggests that perhaps it is not responsible for initiating the pathologic activation of myofibroblasts. Instead, AngII likely promotes renal myofibroblast activation through its complex interactions with TGF-β1 in vivo. Remarkably, HGF clearly blocks αSMA expression triggered by TGF-β1 plus AngII in a dose-dependent manner (Figure 11c). Thus, we have identified three key factors in regulating the αSMA expression and myofibroblastic transition from tubular epithelial cells, with TGF-β1 as an initiator, AngII as a promoter, and HGF as an inhibitor. This observation reinforces the notion that multiple factors such as TGF-β1 and AngII participate in the pathologic process of renal fibrogenesis in a synergistic way. Hence, simultaneously targeting more than one pathologic pathway may be crucial for effective therapy aimed at halting the progression of chronic renal fibrosis in patients.

In summary, we have demonstrated that a combined therapy with HGF gene transfer and RAS blockade synergistically attenuates renal interstitial fibrosis in obstructive nephropathy in mice. The rationale behind this therapeutic strategy is to specifically and simultaneously target multiple pathogenic pathways leading to chronic renal fibrosis. Although it remains to be determined whether this combined therapy could display any superior efficacy than conventional RAS blockade alone in patients, our current study may provide a foundation for designing future therapeutic regimens for ultimately halting the progression of chronic renal diseases in clinical settings.

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References