Inhibition of Renal Fibrosis by Gene Transfer of Inducible Smad7 Using Ultrasound-Microbubble System in Rat UUO Model

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Abstract. TGF-β is a key mediator in renal fibrosis. Kidney-targeted gene therapy with anti–TGF-β strategies is expected to have therapeutic potential, but this has been hampered by concerns over the safety and practicability of viral vectors and the inefficiency of nonviral transfection techniques. The present study explored the potential role of TGF-β/Smad signaling in renal fibrosis in vivo and developed a safe and effective gene therapy to specifically block TGF-β signaling and renal fibrosis in a rat unilateral ureteral obstruction (UUO) model by transferring a doxycycline-regulated Smad7 gene or control empty vectors using an ultrasound-microbubble (Optison)-mediated system. The Smad7 transgene expression was tightly controlled by addition of doxycycline in the daily drinking water. Groups of six rats were sacrificed at day 7, and the transfection rate, Smad7 transgene expression, and tubulointerstitial fibrosis including α-smooth muscle actin and collagen matrix mRNA and protein expression were determined. Compared with the non-ultrasound treatment, the combination of ultrasound with Optison largely increased the transfection rate of FITC-ODN and Smad7 transgene expression up to a 1000-fold, and this was found in all kidney tissues. Compared with normal rats, Smad7 expression within the UUO kidney was significantly reduced, and this was associated with up to a sixfold increase in Smad2 and Smad3 activation and severe tubulointerstitial fibrosis. In contrast, treatment with inducible Smad7 resulted in a fivefold increase in Smad7 expression with complete inhibition of Smad2 and Smad3 activation and tubulointerstitial fibrosis in terms of tubulointerstitial myofibroblast accumulation (85% ↓) and collagen I and III mRNA and protein expression (60 to 70% ↓). In conclusion, the ultrasound-mediated inducible Smad7 gene transfer is a safe, effective, and controllable gene therapy. TGF-β-mediated renal fibrosis is regulated positively by Smad2/3, but negatively by Smad7. Target blockade of TGF-β/Smad signaling by expression of Smad7 may provide a new therapeutic potential for renal fibrosis.

Tubulointerstitial fibrosis is a feature of end-stage renal disease (ESRD) and is a major determinant of progressive renal injury (1). This is characterized by the accumulation of myofibroblasts and extracellular matrix (ECM), including fibronectin and collagens (types I, III, IV, V, and VII) (1). There is increasing evidence that TGF-β is a key mediator of fibrosis in both experimental and human kidney diseases (2,3). This is clearly illustrated by the finding that renal fibrosis can be induced by the deliberate overexpression of TGF-β1 within the normal kidney (4). In addition, TGF-β is able to stimulate collagen synthesis and induce normal tubular epithelial cells to transform into a myofibroblast phenotype (5–7), contributing significantly to the progressive tubulointerstitial fibrosis in both experimental and human renal fibrosis. These studies suggest that TGF-β may play an important role in the pathogenesis of tubulointerstitial fibrosis.

The recent discovery of Smad proteins as intracellular mediators and regulators of TGF-β signaling has provided important insights into mechanisms that may determine the specific functional role of TGF-β in renal fibrosis (8,9). TGF-β exerts its biologic effects by signaling through the TGF-β receptor I and II (TβRI and TβRII) (8,9). TGF-β binds to receptor II and results in phosphorylation of TβRI. The activated TβRI then directly signals to downstream intracellular substrates, Smad2 and Smad3 (R-Smads). Activated R-Smads heterologimerize with the common partner Smad4 (C-Smad), and these complexes are translocated into the nucleus to regulate target gene expression. Activation of the TGF-β signaling pathway can also result in the expression of inhibitory Smads (I-Smads), including Smad6 and Smad7. These inhibitory Smads appear to act by specifically inhibiting Smad2 and Smad3 phosphorylation by blocking their access to TβRI or by causing degradation of TβRI (8–11).

The involvement of Smads in TGF-β-mediated renal fibrosis has been demonstrated in in vitro studies. It has been shown...
that Smads may play a role in TGF-β-induced collagen matrix synthesis in human mesangial cells and podocyte apoptosis during glomerulosclerosis (12–14). Overexpression of Smad7 by gene transfer is able to inhibit Smad2 and Smad3 activation in TGF-β-dependent lung fibrosis induced by bleomycin and in rat obstructive nephropathy (15,16). In addition, we and other investigators have also demonstrated that overexpression of Smad7 blocks heme oxygenase-1 expression and the fibrogenic effects of TGF-β on renal tubular epithelial cells and mesangial cells (17–19). The present study further extends these findings by presenting a novel, safe, effective, and inducible gene therapy to specifically block TGF-β/Smad signaling and renal fibrosis in the rat unilateral ureteral obstruction (UUO) model by transferring a doxycycline-regulated Smad7 using an ultrasound-microbubble mediated system.

Materials and Methods
Ultrasound-Mediated Gene Transfer of Inducible Smad7 Gene-Bearing Microbubbles into the Kidney
The Dox-regulated Smad7 expressing plasmid was prepared as described previously (19,20). Briefly, a mouse Smad7 cDNA with a flag tag (m2) at its NH2 terminus in pcDNA3 (gift from Dr. P. ten Dijke) was subcloned into a tetracycline-inducible vector, pTRE (Clontech), to obtain pTRE-m2Smad7. An improved pTet-on vector (Clontech), pEFpurop-Tet-on, was generously provided by G. Varo (Cerylid, Melbourne, Australia). All plasmids were prepared using the EndoFree Plasmid kit (Qiagen Inc, Valencia, CA) following the manufacturer’s protocol. To achieve doxycycline (a tetracycline derivative)-induced Smad7 transgene expression, pTRE-m2Smad7 and pEFpurop-Tet-on were co-transfected into the kidney via the renal artery using an ultrasound-mediated system. Procedures of ultrasound-based gene transfer technique includes: (1) mixing pTRE-m2Smad7 and pEFpurop-Tet-on with Optison (echocardiographic contrast microbubbles; Mallinckrodt, St. Louis, Mo) in 1:1 vol/vol ratio and injecting the mixed solution containing 25 μg of designated plasmid in 0.5 ml into the left renal artery while temporarily clipping off the renal artery and vein (<5 min). We have found in the pilot studies in the normal kidney that the dose of 25 μg/kidney of the Smad7 plasmid produced optimal transfection rate and transgene expression without toxicity to the kidney as determined by undetectable local inflammation and proteinuria; (2) applying the ultrasound transducer (Ultrax UX-301; Celcom Medico Inc., Japan) directly onto one side of the left kidney with a continuous-wave output of 1-MHz ultrasound at 5% power output, for a total of 60 s with 30-s intervals; (3) turning over the kidney and treating the other side with ultrasound using the same procedure.

Assessment of Gene Transfection Rate, Transgene Expression, and Nephrotoxicity by Ultrasound-Mediated Destruction of Microbubbles
Two approaches were used to determine the efficiency or gene transfection rate of ultrasound-mediated system. First, after surgical exposure of the left kidney artery, we injected a mixture of FITC-oligodeoxynucleotides (5’-TTGCCGTACCTGACCTAGGC-3’, 3’-AACGGCATGACTGAATCGG-S’)/Optison (1:1 vol/vol) into the left renal artery at a dose of 5 μM/kidney (0.5 ml). The kidney was then treated with or without ultrasound as described above. Groups of four rats were euthanized at 45 min after ultrasound treatment, and the 4-μm cryostat sections were examined under a fluorescence microscope for FITC-nuclear positive cells within glomeruli, cortical and medullary tubulointerstitium, and vascular walls. Second, groups of four normal rats were injected via the renal artery with the mixture of pTRE-m2Smad7 plasmid (4.5 kD) and the pEFpurop-Tet-on plasmid (7.2 kD) with Optison (in 1:1 vol/vol) into the left kidney and were immediately treated with or without ultrasound as described above. Once the gene transfer procedure was completed, all animals were treated immediately with 1 ml of Dox (200 μg/ml) into the peritoneal cavity followed by the optimal dose of Dox (200 μg/ml) in the drinking water for 48 h to induce m2Smad7 transgene expression, according to the previously described experiment and the pilot studies (21). In our pilot studies, immunohistochemistry demonstrated that m2Smad7 transgene expression was significantly induced at 24 h and reached maximal expression at 48 h in a dose-dependent manner with the optimal dose of 200 μg/ml. This mimics our previous in vitro findings that Dox induces m2Smad7 transgene expression in a dose-dependent manner with an optimal dose of 2 μg/ml and with the m2Smad7 transgene expression reaching its maximal level at 24 h (19). Groups of four rats were euthanized at 48 h. The efficiency of the ultrasound-microbubble–mediated, doxycycline-induced m2Smad7 transgene expression was determined with the anti-flag m2 mAb (IBI, Eastman Kodak) by immunohistochemistry or Western blotting.

To detect m2Smad7 transgene expression by interstitial fibroblasts, a microwave-based two-color immunohistochemistry was applied (6,7,22). Briefly, after microwaving, sections were labeled with the anti-flag m2 mAb using a 3-layer APAAP method and developed with Fast Blue BB Salt (Ajax Chemicals, Melbourne, Australia) to give a blue product. A second round of microwave oven heating was used to denature bound immunoglobulins within the tissue, thereby preventing antibody crossreactivity (22). Sections then were labeled with a mouse monoclonal antibody to α-smooth muscle actin (α-SMA; Sigma, St. Louis, MO) using a three-layer PAP and developed with 3,3-diaminobenzidine to produce a brown product. Sections were mounted in an aqueous medium and examined under microscopy. Expression of m2Smad7 transgene (blue) by α-SMA+ interstitial myofibroblasts (brown), an active form of fibroblasts, was clearly identified (purple).

To investigate the potential nephrotoxicity caused by the ultrasound-microbubble procedure, both serum and urine were collected at the first 24 h after the ultrasound-microbubble procedure for measurement of urinary protein excretion and lactate dehydrogenase (LDH) using an in vitro toxicology assay kit (Sigma, St. Louis, MO). Changes in histology were examined on H&E-stained and PAS-stained kidney sections.

Animal Model
A UUO model was induced in male Sprague-Dawley rats (220 to 250 g) by ligation of the left ureter. Immediately after ligation of the left ureter, the pTRE-m2Smad7/Tet-on plasmids/Opitison, or the control empty vectors (pTRE/Tet-on/Opitison) were infused into the left kidney at a dose of 25 μg/kidney via the renal artery and treated with ultrasound as described above. To induce Smad7 transgene expression, a dose of Dox (1 ml) with a concentration of 200 μg/ml was administered into the peritoneal cavity immediately after ultrasound-microbubble gene transfer and followed by additional Dox in the daily drinking water (200 μg/ml) for 7 d. Groups of six rats were euthanized at day 7. In addition, a group of four rats was used as normal control.

Histology and Immunohistochemistry
Changes in renal morphology were examined in formalin-fixed, 4-μm paraffin sections by H&E and PAS staining. To detect activa-
tion of Smad2 and Smad3 and expression of Smad7, 4-μm paraffin sections were pretreated with trypsin (0.5%, pH 7.8) for 10 min at 37°C and then incubated with polyclonal antibodies to phosphorylated Smad2 (Cell Signaling Tech, Beverly, MA), Smad3, and Smad7 (Santa Cruz Biotechnology Inc.) using a three-layer PAP method (6–7). The degree of interstitial myofibroblast and collagen I and III accumulation were determined by a microwave-based antigen retrieval method with the anti-α-SMA mAb (Sigma) and goat anti-rat collagen I and III polyclonal antibodies (Southern Tech, Birmingham, AL). Sections were then developed with diaminobenzidine to produce a brown color and counterstained with hematoxylin and coverslipped in an aqueous mounting medium.

An isotype-matched mouse monoclonal antibody (73.5) that recognized human CD45R antigen and goat anti-rabbit IgG were used as negative controls throughout the study.

**RT-PCR Analyses**

Total RNA was isolated using the High Pure RNA Isolation Kit according to the manufacturer’s instructions (Roche). Contaminated DNA was removed by treating the samples with RNase-free DNase I (Promega, Madison, WI). RT-PCR was performed using a ThermoScript RT-PCR Kit following the manufacturer’s instruction (Life Technologies, BRL, Gaithersburg, MD). The first-strand cDNA was synthesized by using oligonucleotide primers and M-MLV reverse transcriptase (Promega), followed by PCR amplification (30 cycles) using primers specific for mouse Smad2, Smad3, Smad7, α-SMA, and rat collagen I and III as described previously (19). All samples were subjected to RT-PCR for the housekeeping gene GAPDH as a positive control and as an internal standard. Afterwards, RT-PCR products were resolved on 1.5% agarose gels in 1x Tris-Glycine buffer (Bio-Rad, Hercules, CA), and analyzed by densitometry.

**Western Blotting and Immunoprecipitation**

Western blot analysis was used for detection of phosphorylated Smad2, Smad7, α-SMA, and collagen I and III expression within the kidney as described (19), whereas immunoprecipitation was used for detection of phosphorylated Smad3. Briefly, kidney tissues were lysed in 1 ml of 1% Nonidet P-40, 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0, containing a 1-in-50 dilution of a protease inhibitor cocktail (P2714, Sigma) for 30 min on ice. Samples were centrifuged at 14,000 × g for 5 min to pellet cell debris. Samples (20 μg) were mixed with SDS-PAGE sample buffer, boiled for 5 min, electrophoresed on a 10% SDS polyacrylamide gel, and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham International, Buckinghamshire, UK). The membrane was blocked in PBS containing 5% skimmed milk powder, 0.02% Tween 20 and then incubated for 1 h with mouse monoclonal antibodies to flag m2 and α-SMA or goat (rabbit) polyclonal antibodies to Smad7 (Santa Cruz), p-Smad 2 (Upstate Biotechnology, Inc.), and collagen I and III (Santa Cruz). After washing, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated goat anti-mouse IgG or swine anti-goat (rabbit) IgG in PBS containing 1% normal goat serum and 1% FCS. Detection of phosphorylated Smad3 was subjected to immunoprecipitation with the rabbit anti-Smad3 antibody (Zhymed Laboratories, South San Francisco, CA) followed by rabbit anti-phosphoserine antibody (Zhymed). The blots were then developed using the ECL detection kit (Amersham) to produce a chemiluminescence signal that was captured on x-ray film.

**Quantitative Analyses**

Smad2 and Smad3 activation was identified by the nuclear location. Total numbers of positive cells for activated Smad2 and Smad3 (as identified by nuclear staining) and transgene Smad7 (flag m2+) within the glomerulus was counted in 20 consecutive glomeruli and expressed as cells/glomerular cross-section (gcs) or percent positive cells (%), while positive nucleus for activated Smad2 and Smad3 in tubulointerstitium was counted under 20-high power-field (×40) by means of a 0.02-mm2 graticule fitted in the eyepiece of the microscope, and expressed as cells per mm2. Smad7 expression in both glomeruli and tubulointerstitium was scored as follows: 0.5, weak; 1, mild; 2, moderate; 3, strong. Areas of positive staining for α-SMA and collagen types I and III in the entire cortical tubulointerstitium (a cross-section of the kidney) were determined using quantitative Image Analysis System (Optima 6.5, Media Cybernetics, Silver Spring, MD). Briefly, the examined area of tubulointerstitium was outlined, the positive staining patterns were identified, and the percent positive area in the examined tubulointerstitium was then measured. The glomeruli including Bowman’s space and large arterial wall and lumen space were excluded from the study. Data were expressed as percent positive area examined. All scoring was performed blinded on coded slides.

**Statistical Analyses**

Data obtained from this study are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, Inc. San Diego, CA). Differences in Smad2 and Smad3 activation, Smad7 and flag-m2Smad7 expression, α-SMA+ cells and collagen matrix accumulation were assessed by one-way ANOVA or by t test.

**Results**

**Efficacy of Ultrasound-Microbubble Mediated Gene Transfer and Smad7 Transgene Expression within the Kidney**

To determine the efficacy and distribution of ultrasound-microbubble mediated gene transfer into the kidney, the left kidney in normal rats was infused with FITC-ODN/Optison and treated with or without ultrasound. As shown in Figures 1A through 1C, kidney with ultrasound treatment for 2 min exhibited strong nucleated FITC-ODN accumulation in almost all of the glomerular cells (>95%), vascular and perivascular cells (>90%), and medullary tubular and interstitial cells (>95%), while cortical tubular epithelial and interstitial cells were less strongly positive (70 to 80%). In contrast, the kidney without ultrasound treatment exhibited fewer nucleated FITC-ODN positive cells (<0.1%) with abundance within the cytoplasm of the cortical tubular epithelial cells (re-absorption), but with absence in the glomeruli, vascular walls, interstitial, and medullary areas (Figure 1, D and E). Thus, the combination of ultrasound and Optison largely enhances the gene transfer into the kidney, resulting in more than a 1000-fold increase in the transfection rate, compared with the nonultrasound treatment.

We next examined the efficacy of ultrasound-microbubble-mediated, Dox-induced m2Smad7 transgene expression within the kidney with the anti-flag m2 antibody. Unfortunately, in the normal rat kidney, immunohistochemistry demonstrated that the majority of tubular epithelial cells and vascular smooth muscle cells were labeled with the anti-flag m2 monoclonal
smooth muscle cells by immunohistochemistry or by Western blotting. Interestingly, all glomerular cells and interstitial cells including resident mononuclear cells, elongated fibroblasts, and capillary endothelial cells in normal kidneys were completely negative for flag m2 staining (Figure 2A). Thus, m2Smad7 transgene expression was examined in glomerular cells, interstitial cells, and vascular endothelial cells. As shown in Figure 2, after 48-h induction of m2Smad7 expression with Dox in the drinking water (200 µg/ml), the kidneys injected with a mixture of pTRE-m2Smad7/Tet-on plasmids and Optison and then treated with ultrasound exhibited marked flag m2 expression in all glomeruli (Figure 2B) and accounted for up to 95% of glomerular cells, while the kidneys treated with either empty vectors (pTRE and Tel-on plasmids)/Optison and ultrasound (Figure 2D) or m2Smad7/Tet-on and Optison without ultrasound (Figure 2E) showed completely negative or little if any (<0.1%) of the anti-flag m2 antibody within glomeruli. In the interstitium, one-color or two-color immunohistochemistry demonstrated that ultrasound treatment resulted in strong expression of m2Smad7 transgene by all capillary endothelial cells (Figure 2C) and all interstitial fibroblasts/myofibroblasts as identified by α-SMA expression (Figure 3A). In contrast, no m2Smad7-positive cells were found in capillary endothelial cells (Figure 2F) and interstitial fibroblasts/myofibroblasts (Figure 3B) within the kidney without ultrasound treatment. Quantitative analysis showed that the combination of ultrasound and Optison treatment increased glomerular m2Smad7 transgene expression up to a 1000-fold compared with those without ultrasound or control vector treatment (Figure 4).

Smad7 transgene expression was also examined in UUO kidneys. As shown in Figure 5, serial sections showed that rats treated with an inducible Smad7 plasmid DNA at day 7 exhibited a strong flag m2 expression in all glomeruli and tubulointerstitial cells, which were co-localized with strong Smad7 expression as demonstrated by the anti-Smad7 antibody (Figure 5A and B). However, rats that were treated with control vectors showed a strong flag m2 expression in all tubular cells, but negative in both glomerular and interstitial cells (Figure 5C), while Smad7 expression was found in some glomerular and interstitial cells with absence or weak expression by tubular cells (Figure 5D). These observations indicate that the majority of flag m2-positive cells in the diseased kidneys treated with the m2Smad7 plasmid DNA are presumably Smad7 transgene expression and also suggest that the combination of ultrasound and Optison make an equal high transfection rate to both glomeruli and tubulointerstitium as seen in FITC-ODN transfection (Figure 1).

It should be pointed out that the ultrasound treatment did not cause any abnormal histologic and functional changes demonstrated by normal urinary protein excretion (4 to 8 mg/24 h), normal glomerular and tubulointerstitial morphology, no cellular and interstitial edema, and no local inflammation. In addition, there was no difference between normal and ultrasound-microbubble–treated rats in the serum and urinary LDH levels (OD: 0.449 ± 0.02 versus 0.447 ± 0.01 in serum and 0.062 ± 0.004 versus 0.056 ± 0.002 in urine), suggesting no major toxicity. However, because the use of the ultrasound resulted in

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antibody (Figure 2A). This immunoreactivity is also demonstrated by Western blotting (data not shown) and is due to the crossreactivity of the monoclonal anti-flag m2 antibody to an isoform of rat Mg2+-dependent protein phosphatase β (23). It is therefore difficult to accurately examine the m2Smad7 transgene expression in both tubular epithelial cells and vascular

Figure 1. Immunofluorescence demonstrates the distribution and transfection rate of FITC-ODN within the normal rat kidney by ultrasound-Optison–mediated system. Groups of four rats were injected with Optison and FITC-ODN via renal artery and then treated with or without ultrasound. After 45 min, kidney sections were examined under fluorescent microscope for FITC-ODN transfection as described in Materials and Methods. (A through C) Ultrasound-Optison–mediated FITC-ODN transfer in the cortex (A) and medullary tissues (C). The FITC-nucleated positive cells are found in both the cortex (A) and medullas (C) and show stronger in the blood-rich areas such as glomeruli and vascular walls (B, high power illustration from the area indicated in panel A), and medullary tissues (C), accounting for more than 95% of cells. Note that most tubular epithelial cells and interstitial cells (arrows) are FITC-nucleated positive (B, insert picture). (D and E) Non–ultrasound-mediated FITC-ODN/ Optison transfection. By contrast, there are no detectable FITC-nucleated positive cells in glomeruli, vascular walls, and medullary areas, although all cortical tubules exhibit moderate to strong FITC-cytoplasmic labeling (FITC-ODN absorption). (E) Negative control showing no autofluorescence in the normal rat kidney. g, glomerulus; a, artery; t, tubulus. Magnifications: ×100 in A, C, and D; ×400 in B.
Figure 2. Immunohistochemistry demonstrates the distribution and transfection rate of m2Smad7 transgene expression within the normal rat kidney by ultrasound-Optison-mediated system. Groups of four rats were injected with the mixture of Optison and Dox-regulated m2Smad7/Tet-on plasmids or empty vector control (pTRE/Tet-on plasmids) via renal artery and then treated with or without ultrasound. After 48 h following the injection and Dox (200 μg/ml) in the drinking water, kidney sections were examined for m2Smad7 transgene expression with the anti-flag m2 antibody as described in Materials and Methods. (A) Normal rat kidney without any treatments; (B and C) pTRE-m2Smad7/Tet-on plasmids + Optison + ultrasound; (D) empty vectors + Optison + ultrasound; (E and F) pTRE-m2Smad7/Tet-on plasmids + Optison without ultrasound. It is noted that all tubular cells and vascular smooth muscle cells, but not glomerular, interstitial, and vascular endothelial cells, are crossreactive with the flag-m2 antibody (brown) in the normal rat kidney (A). Similar results are also found in vector control (D) and m2Smad7/Tet-on plasmids + Optison without ultrasound treatment (E and F). Indeed, all glomerular cells (D and E), interstitial cells (D and E, arrows), and capillary endothelial cells (F, inner medulla, *) are negative for the anti-flag m2 antibody staining. In contrast, ultrasound-Optison-mediated m2Smad7/Tet-on plasmid transfer results in strong anti-flag m2 antibody staining in more than 90% of glomerular cells (B) and capillary endothelial cells (C, inner medulla, *). Arrows, interstitial cells; * capillaries. Cell nuclei are counter-stained with hematoxylin (blue). Magnifications: ×250 in A; ×400 in B through F.
Figure 3. Two-color Immunohistochemistry demonstrates the distribution and transfection rate of m2Smad7 transgene expression in interstitial myofibroblasts in normal rat kidney by ultrasound-Optison–mediated system. Cells with m2Smad7 expression are labeled as blue, while α-smooth muscle actin (α-SMA⁺) interstitial myofibroblasts are labeled as brown. Cells co-expressed m2Smad7 (blue) and α-SMA (brown) are shown in purple (two-mixed color). Note that all tubular epithelial cells (t) are also crossreactive with the anti-flag m2 mAb (blue). (A) Normal rat kidney treated with pTRE-m2Smad7/Tet-on plasmids + Optison + ultrasound. Note that all α-SMA⁺ interstitial myofibroblasts strongly co-express m2Smad7 as shown in purple (arrows). (B) Normal rat kidney treated with pTRE-m2Smad7/Tet-on plasmids + Optison without ultrasound. Note that there is no m2Smad7 expression by all α-SMA⁺ interstitial myofibroblasts as demonstrated by brown staining only (arrows). Magnification, ×400.
Each bar represents the mean in 20 glomeruli per animal as described in Materials and Methods.

mediated system. Percentage of flag m2 positive cells were counted – within glomeruli in the normal rat kidney by ultrasound-Optison – Smad3, identified by their nuclear location with anti-phospho-

myofibroblasts and collagen types I and III accumulation, (Figure 9A), animals that received empty control vectors exhibited severe tubular atrophy, tubulointerstitial mononuclear cell accumulation, and fibrosis (Figure 9B), which were significantly inhibited by the ultrasound-mediated Smad7 gene transfer (Figure 9C).

Effects of Smad7 Gene Transfer on Renal Histology in Rat UUO Model

Effects of induced m2Smad7 transgene expression on renal histologic changes in rats with obstructive nephropathy were analyzed in H&E-stained and PAS-stained paraffin sections. As shown in Figure 9, compared with the normal kidney (Figure 9A), animals that received empty control vectors exhibited severe tubular atrophy, tubulointerstitial mononuclear cell accumulation, and fibrosis (Figure 9B), which were significantly inhibited by the ultrasound-mediated Smad7 gene transfer (Figure 9C).

Overexpression of Smad7 Inhibits Extracellular Matrix (ECM) Production

The suppressive effects of Dox-induced Smad7 expression on tubulointerstitial fibrosis including α-SMA and collagen I and III mRNA and protein expression were further determined by RT-PCT, Western blotting, and immunohistochemistry. As shown in Figure 10, renal α-SMA and collagen I and III mRNA expression were markedly upregulated in UUO kidneys compared with normal kidneys, which were significantly inhibited by Smad7 treatment. Similarly, Western blot analysis also demonstrated that treatment with inducible Smad7 gene therapy resulted in a substantial inhibition of renal α-SMA and collagen I and III protein expression compared with the control vector treatment (Figure 11). In addition, treatment with Smad7 also resulted in a substantial inhibition of renal fibrosis as demonstrated by immunohistochemistry. As shown in Figure 12, rats with control vector treatment exhibited severe tubulointerstitial fibrosis as evidenced by numerous α-SMA+ myofibroblasts and collagen types I and III accumulation, which were virtually blocked by treatment with Smad7.

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Figure 4. Semiquantitative analysis of m2Smad7 transgene expression within glomeruli in the normal rat kidney by ultrasound-Optison-mediated system. Percentage of flag m2-positive cells were counted in 20 glomeruli per animal as described in Materials and Methods. Each bar represents the mean ± SEM for a group of four rats. *** p < 0.001 compared with all other groups.
Discussion

This study provides the first demonstration that the ultrasound-mediated disruption of gas-filled microbubbles can be used to effectively transfet the naked plasmid DNA(s) into the kidney. In addition, the study has also demonstrated that inducing Smad7 transgene expression is able to block Smad2 and Smad3 activation and inhibit tubulointerstitial fibrosis in UUO model in rats. Furthermore, by using this powerful technique, we are able to co-transfect the inducible gene with the Tet-on plasmid into the same cell in vivo and control the transgene expression within the kidney at the therapeutic level without undesirable side effects.

With the human genome project and continuing advances in molecular biology, gene therapy is expected to have a major impact on medical treatments in the future. All gene therapy strategies have three essential technical requirements, which include: (1) the efficient introduction of the relevant genetic material into the target cell; (2) the expression of the transgene.

Figure 5. Immunohistochemistry demonstrates the co-expression of flag m2 and Smad7 in both glomeruli and tubulointerstitium in serial sections in day 7 rat unilateral ureteral obstruction (UUO) model. (A and B) Kidney serial sections from a rat treated with m2Smad7 plasmid and stained with antibodies to flag m2 (A) and Smad7 (B). Note that strong m2-positive cells are co-localized with strong expression of Smad7 in both the glomerulus and tubulointerstitium (*). (C and D) Serial sections from a kidney treated with control vectors. Note that strong m2 expression is found in tubular cells, but negative in the glomerulus (C). In contrast, Smad7 expression is seen in some glomerular and interstitial cells, but negative or weak expression by tubular cells (D). Magnification, ×400.
at therapeutic levels; and (3) ensured safety and minimal toxicity. Although biologic gene delivery using viral vectors or nonbiologic approaches, including gene gun, electroporation, liposome, and naked DNA injection, have been developed (24–30), the clinical use of gene therapy today remains problematic due to the disadvantages in the aspects of sufficiency and safety. In addition, controlling the transgene expression at a therapeutic level without causing side effects is another important challenge.

Recently, the use of ultrasound with echocontrast agents, including Optison, has resulted in improved gene delivery with minimal cytotoxicity (31–42). Ultrasound itself is harmless to the body and is widely used clinically for many purposes such as physical therapy, diagnosis, guidance for deep organ biopsy, and local drug and genetic material delivery (31,36–39). Optison contrast, like most microbubble contrast agents, is also a safe agent and widely used clinically (31–33). Optison is liquid at room temperature, but it becomes a gas-filled microbubbles with the average of 3 μm in diameter at body temperature. Microbubbles are elastic, compressible, and efficient reflectors of ultrasound. Microbubbles work by resonating in an ultrasound beam, rapidly contracting and expanding in response to the pressure changes of the sound wave (31–33). Microbubbles can aid drug delivery in themselves and as agents to carry drugs or genetic materials for site-specific treatment and gene therapy (31–35,39). The principle of the ultrasound-based strategy is that the use of ultrasound contrast agents lowers the threshold for cavitation by ultrasound energy (31–35). Using physical properties of microbubbles and coating materials, genes can be incorporated into ultrasound contrast agents. Gene-bearing microbubbles can be injected intravenously or locally and ultrasound energy applied to the target region. As the microbubbles enter the region of insonation, they cavitate,
locally releasing DNA. Cavitation also likely causes a local shockwave that increases cell permeability and thus improves cellular uptake of DNA (34,35). Scanning electron microscopy also demonstrates that ultrasound with microbubble (Optison) causes a transient formation of holes (< 5 μm) in the cell surface (40,41). In vitro, ultrasound alone enhances transgene expression in vascular cells by up to tenfold after naked DNA transfection (34), which is greatly enhanced by Optison with a 300-fold higher than with naked DNA alone (35). Strikingly, the combination of ultrasound with Optison largely increases luciferase plasmid DNA by more than 7000-fold in human vascular endothelial cells and muscle cells as compared with naked plasmid alone (40,41). In vivo, ultrasound-mediated destruction of microbubbles has also resulted in a tenfold increase in an adenovirus reporter gene expression in rat myocardium (42). Interestingly, while transfection of the naked DNA with or without ultrasound or Optison alone fails to increase the transgene expression as determined by luciferase activity, the combination of ultrasound with Optison results in a marked increase in luciferase activity > 1000-fold (40).

In the past, many gene delivery strategies including viral and nonviral methods such as electroporation and liposome systems have been shown to effectively transfer the target gene into glomeruli and interstitium via renal arterial, pelvic, or ureteric routes (24-30). However, many of them have resulted in low-level expression (< 30% of glomeruli), although a
recent study has shown that electroporation increases glomerular transfection up to 75% (29). The present study has demonstrated that the use of the ultrasound in the presence of gene-bearing microbubbles largely enhances both transfection rate and transgene expression, resulting in more than 95% of glomeruli and 80 to 95% of glomerular, vascular, tubular, and interstitial cells with nucleated FITC or cytoplasmic Smad7 transgene expression. Compared with naked pTRE-Smad7/Tet-on plasmid transfection, the ultrasound-mediated destruction of gene-bearing microbubbles causes up to a 1000-fold increase in FITC-ODN transfection and the Smad7 transgene expression within the kidney. Furthermore, the ability of ultrasound-mediated destruction of microbubble technique to co-transfect the pTRE-m2Smad7 plasmid (4.5 kb) and the pEFpurop-Tet-on plasmid (7.2 kb) into the cells without significant damage to the cells indicates that this gene transfer system is a powerful, safe, and effective strategy and is capable of transfection of all sizes of target genes or genetic products.

The mechanism by which ultrasound-microbubble enhances both the transfection rate (by FITC-ODN) and transgene expression (by m2Smad7) in all cell types within the kidney may largely be attributed to the ultrasound-mediated microbubble cavitation. It has been shown that the direct mechanical effect of ultrasound-mediated microbubble cavitation not only results in a rapid release of DNA, but it also causes a transient formation of holes on the cell surface (40,41). It is possible that the cavitation may also largely increase the permeability of capillary and tubular basement membranes, which allows the locally released DNA to cross through the capillary (and tubular) basement membrane and enter onto the cells such as glomerular, interstitial, and tubular epithelial cells. These may explain the finding of the relative lower transfection rate in tubular epithelial cells (70 to 80%) compared with that in blood-rich tissues such as glomerular cells, capillary endothelial cells, and interstitial cells (>90%).

Renal fibrosis is a final common pathway leading to ESRD. It has been shown clinically that urinary obstruction causes irreversible renal failure by inducing chronic tubulointerstitial nephritis (45). TGF-β has long been considered as a key mediator of the fibrotic process. Blockade of TGF-β with the neutralizing antibody, antisense, or decorin strategies has provided strong evidence for the therapeutic potential by targeting this molecule (24,25,43,44). Recent discovery of TGF-β/Smad
signaling has allowed to further delineate the intracellular mechanism whereby TGF-β mediates fibrosis. Signaling by TGF-β is regulated in both positive and negative pathways, and it is tightly controlled temporally and spatially through multiple mechanisms at the extracellular, membrane, cytoplasmic, and nuclear levels (8–11). Positive regulation could be critical for amplification of signaling by TGF-β through the activation of Smad2 and Smad3, whereas negative regulation may play an important role in restriction and termination of TGF-β signaling by activation of Smad7. Activation of Smad signaling has been shown in both human and experimental kidney diseases (46–48). In vitro, induced overexpression of Smad7 transgene in a Dox-regulated Smad7 expressing renal tubular epithelial line (NRK52E) is able to block TGF-β-induced Smad2 activation, resulting in inhibition of tubular epithelial-myofibroblast transdifferentiation and collagen I, III, and IV production (19). Similarly, overexpression of Smad7 has been shown to inhibit TGF-β-induced Smad2 and Smad3 activation, heme oxygenase-1 expression, and collagen promoter activity by glomerular mesangial and epithelial cells and TEC (17,18,48). Although Smad signaling in cell biology has been extensively studied in vitro, limited information is available in the disease states. In vivo, overexpression of Smad7 transgene blocks Smad2 phosphorylation induced by bleomycin in mouse lung, and gene transfer of Smad7, but not Smad6, prevents bleomycin-induced lung fibrosis (15). Most recently, gene transfer of Smad7 using electroporation of adenovirus has been shown to partially prevent renal fibrosis in the postobstructed kidney (16). However, the majority of Smad7 transgene expression has been limited to the renal medullary tissue of rats given AdCMV-Smad7 with electroporation (16). In the present study, we found that the combination of ultrasound-microbubble with FITC-ODN or Dox-inducible m2Smad7/Tet-on plasmids resulted in more than 90% of kidney cells in both cortex and medullary areas with strong nucleated FITC labeling and significant expression of Smad7 transgene (up to a 1000-fold increased) within the kidney, although RT-PCR demonstrated only a small, but significant increase in Smad7 mRNA expression. This discrepancy between Smad7 protein and mRNA may reflect a rapid Smad7 synthesis and export to cytoplasm or prolonged degradation of Smad7 within the cells. Induced Smad7 transgene expression is also associated with a complete blockade of Smad2 and Smad3 activation and substantial inhibition of tubulointerstitial fibrosis, including myofibroblast accumulation (80%) and collagen matrix expression (80%) and collagen matrix expression and accumulation (60 to 70%) (16). Data from this study strongly indicate that TGF-β/Smad signaling is a critical pathway leading to progressive tubulointerstitial fibrosis and implicate that renal fibrosis is regulated positively by Smad2 and Smad3 but negatively by Smad7.

It should be noted that inducing overexpression of Smad7 with higher dose of Dox (400 μg/ml, twice daily, intraperitoneally) also caused massive glomerular and tubular cell death through apoptosis, which is consistent with previous reports that Smad7 is an inducer of kidney cell apoptosis (14,48,49). This may be associated with the inhibition of a survival factor NF-κB (50,51) and the activation of the JNK pathway (52). Thus, it is critical to control the degree of Smad7 transgene expression to maintain a physiologic balance within the TGF-β, NF-κB, and JNK signaling pathways when attempting to target TGF-β signaling with overexpression of Smad7. We have previously shown that the degree of Smad7 transgene expression could be controlled by varying the concentrations of Dox in vitro (19). We have now demonstrated that the degree of Smad7 transgene expression can also be successfully controlled in vivo. These in vitro and in vivo data implicate that it may be more safe and advantageous to use a Dox-inducible naked Smad7, rather than naked Smad7 gene alone, to prevent or treat renal fibrosis in vivo.
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References