Signaling Mechanism of TGF-β1 in Prevention of Renal Inflammation: Role of Smad7

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TGF-β has been shown to play a critical role in anti-inflammation; however, the signaling mechanisms of TGF-β in anti-inflammatory response remains largely unclear. This study reported that mice that overexpress latent TGF-β1 on skin are protected against renal inflammation in a model of obstructive kidney disease and investigated the signaling mechanism of TGF-β1 in inhibition of renal inflammation in vivo and in vitro. Seven days after urinary obstruction, wild-type mice developed severe renal inflammation, including massive T cell and macrophage infiltration and marked upregulation of IL-1β, TNF-α, and intercellular adhesion molecule-1 (all P < 0.001). Surprising, renal inflammation was prevented in transgenic mice. This was associated with an increase in latent TGF-β1 in circulation (a 10-fold increase) and renal tissues (a 2.5-fold increase). Further studies showed that inhibition of renal inflammation in TGF-β1 transgenic mice was also associated with a marked upregulation of renal Smad7 and IkBα and a suppression of NF-κB activation in the diseased kidney (all P < 0.01). These in vivo findings suggested the importance of TGF-β–NF-κB cross-talk signaling pathway in regulating renal inflammation. This was tested in vitro in a doxycycline-regulated Smad7-expressing renal tubular cell line. Overexpression of Smad7 was able to upregulate IkBα directly in a time- and dose-dependent manner, thereby inhibiting NF-κB activation and NF-κB–driven inflammatory response. In conclusion, latent TGF-β may have protective roles in renal inflammation. Smad7-mediated inhibition of NF-κB activation via the induction of IkBα may be the central mechanism by which latent TGF-β prevents renal inflammation.

ing latent form of TGF-β1 may have protective roles in renal inflammation. This was investigated in a progressive model of obstructive kidney disease in mice that overexpress latent TGF-β1 in the circulation and in the skin. Furthermore, this study explored the underlying signaling mechanism of TGF-β in anti-inflammation in the kidney disease.

Materials and Methods

K5.TGFβ1transgenic Mice

The approximately 1.6-kb full-length wild type (Wt) human TGF-β1 cDNA was inserted into the bovine K5 expression vector (13). The K5.TGFβ1 plasmid then was microinjected into the pronuclei of mouse embryos that were obtained from strain ICR female mice. After birth, transgenic mice were genotyped by PCR analysis of their tail DNA using primers specific for human TGF-β1 cDNA, the sequence being forward 5'-TCTGCTGAGGAGGCTCAAGTT and reverse 5'-ACCTCAGGCGGCGGCGGTAG. The product size is 271 bp. All TGF-β transgenic mice develop psoriasis-like skin lesions at the age of 2 to 6 mo (14).

Obstructive Kidney Disease Model

A progressive kidney disease model was induced in both Wt and K5.TGFβ1transgenic mice (15 to 20 g body wt, 3 mo of age) by left ureter ligation as described previously (unilateral ureteral obstruction [UUO]) (15). Groups of six mice were killed on day 7 after the ligation. The experimental procedures were approved by Animal Experimental Committee at Baylor College of Medicine. Kidney tissue samples were collected for histology, immunohistochromistry, Western blot, real-time PCR, and quantitative ELISA analyses.

Measurement of TGF-β1 in Plasma and Renal Tissues

TGF-β1 levels in plasma and renal tissues including the active form, the latency-associated protein (LAP), and total TGF-β1 were analyzed quantitatively by the commercial ELISA kits (R&D System Inc., Minneapolis, MN), according to the manufacturer’s instructions. Briefly, protein samples were acidified with 1 N HCl and neutralized with 1.2 N NaOH/0.5 M HEPES to assay for the amount of total (the sum of latent and active) TGF-β1. The concentration of active TGF-β1 protein was analyzed on samples that were not acidified, whereas the levels of latent TGF-β1 protein were measured using a specific anti-LAP antibody.

Cell Culture

To test the hypothesis that TGF-β negatively regulates inflammatory response by inducing its negative signaling regulator, Smad7, we used a doxycycline-regulated Smad7-expressing tubular cell line (TEC). This cell line was established with a well-characterized normal rat kidney TEC line (NRK52E) by stably transfecting a Flag-tagged (M2) Smad7 gene, as described previously (16). Smad7 transgene expression by TEC was tightly controlled by the addition of doxycycline (0 to 2 μg/ml) in the culture medium. After an 18-h induction of Smad7, proinflammatory cytokines IL-1β (10 ng/ml) and TNF-α (10 ng/ml) were added into the cells for 0 to 24 h and the effects of Smad7 on IL-1β, TNF-α, and intercellular adhesion molecule-1 (ICAM-1) expression and on the activation of NF-κB were examined.

Western Blot Analysis

Protein from kidney tissues and cultured cells was extracted with RIPA lysis buffer (1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride in PBS). After protein concentrations were determined, 20 μg of the protein was mixed with an equal amount of 2× SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, and 0.2% bromophenol blue) for Western blot analysis. Briefly, samples were heated at 99°C for 5 min and then transferred to a polyvinylidene difluoride membrane. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% BSA in Tris-buffered saline buffer (TBST; 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes then were incubated overnight at 4°C with primary antibodies against Smad7 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Flag-M2 (Sigma, St. Louis, MO), and NF-κB p50 or p65 (Santa Cruz); IκBα (Santa Cruz); phospho-IκBα (Cell Signaling, Beverly, MA); and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon Inc., Temecula, CA). After being washed extensively, the membranes then were incubated with horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature in 1% BSA/TBST. The signals were visualized by an enhanced chemiluminescence system (Amer sham, Piscataway, NJ).

Real-Time PCR

Total kidney RNA was isolated using the RNeasy kit, according to the manufacturer’s instructions (Qiagen Inc., Valencia, CA). The cDNA was synthesized as described previously (17). Real-time PCR was run with the Opticon real-time PCR machine (MJ Research Inc., Waltham, MA). The specificity of real-time PCR was confirmed via routine agarose gel electrophoresis and Melting-curve analysis. Housekeeping gene GAPDH was used as an internal standard. The primers used in this study are as follows: Smad7, forward 5'-GTGCTATGTCCTCGTGCACCTTG and reverse 5'-ATGAAAGATG-GCTGGAAGAGGTCTC; ICAM-1, forward 5'-CTCAGATATGCCATCCACACAGAGAGA and reverse 5'-AGCTCATCATGACCCATCTGTC; IL-1β, forward 5'-CTTCCAGGCGACCTAACATCAT and reverse 5'-TCTAATGGAAACCTGACCAACAG; TNF-α, forward 5'-CATGACACAGAAGCATGATCC and reverse 5'-AAAGCAGAAATGAGAAGAGGCT; TGF-β, forward 5'-CAACATTTCCGCGTGTACCTG and reverse 5'-GAAAAGGCTGTATCTGCTCCT; GAPDH, forward 5'-CTGCTAGATGTGCGGTGAGTCTA and reverse 5'-AGTGGAGTGTGCTCTTGAAAATC.

Transient Transfection and Promoter Activity Assay

Doxycycline-regulated Smad7-expressing NRK52E cells were transiently transfected by the Lipofectamine (Invitrogen Inc., Carlsbad, CA) with the specific NF-κB responsive promoter 241RM1-SEAP (secreted alkaline phosphatase) plasmid (gift from Dr. Bharat B. Aggarwal, Department of Molecular Oncology, University of Texas MD Anderson Cancer Center) (18). A control plasmid, pCMV-βGal (Clontech, Palo Alto, CA), was co-transfected into the cells for transfection efficiency. The transfection procedure was carried out according to the manufacturer’s instruction. The SEAP and β-galactosidase activities were analyzed by SEAP reporter gene assay kit and β-gal reporter gene assay kit, respectively (Roche Inc., Indianapolis, IN), according to the manufacturer’s instructions. NF-κB SEAP activity was reported as the SEAP activity normalized to β-gal activity.

Electrophoretic Mobility Shift Assay

Nuclear proteins from cultured NRK52E cells were isolated as described previously (19). Aliquots of 5 μg of nuclear extracts were incubated with a 19bp double-strand oligonucleotide probe that contained consensus sequence that binds to NF-κB/c-Rel homodimeric and heterodimeric complexes (Santa Cruz) in binding buffer for 30 min at 4°C. The probe is end-labeled with 32P-dATP by using T4 polymerase (Invitrogen Inc.) according to the manufacturer’s instructions. After the binding reaction, the samples were loaded on a 5% native polyacryl-
amide gel and run for 3 to 4 h at 4°C. The gel then was dried and developed on an x-ray film.

**Histology and Immunohistochemistry**

Changes in renal morphology were examined in methyl Carnoy’s-fixed, paraffin-embedded tissue sections (4 μm) stained with hematoxylin and eosin or periodic acid-Schiff. Infiltration of CD3⁺ T cells and macrophages; expression of IL-1β, TNF-α, ICAM-1; and activation of NF-κB p50/p65 were determined by three-layer immunohistochemistry with mAb to macrophages (M170); CD3⁺ T cells (OKT3); and rabbit polyclonal antibodies to IL-1β, TNF-α, p65, and p50 NF-κB subunits. mAb were generated in our laboratory from standard cell lines (M170 and OKT3; gifts from Dr. David Nikolic-Paterson, Monash University, Melbourne, Australia), whereas polyclonal rabbit antibodies were purchased from Santa Cruz Biotechnology. Immunostaining for CD3⁺ T cells, macrophages, IL-1β, TNF-α, and ICAM-1 was performed in 2% paraformaldehyde-lysine-periodate-fixed frozen sections as described previously (20), whereas detection of NF-κB/p50 and p65 subunits was performed in methyl Carnoy’s fixed paraffin sections using a microwave-based antigen retrieval technique (21). After being developed with 3,3-diaminobenzidine, sections were counterstained with hematoxylin. Tubulointerstitial NF-κB activated cells, infiltrating CD3⁺ T cells, and macrophages were counted and expressed as positive cells per cm² as described previously (20). The quantification of IL-1β, TNF-α, and ICAM-1 expression was analyzed by using Optima program, and the results were expressed as percentage of positive area examined.

**Statistical Analyses**

Data obtained from this study are expressed as the mean ± SEM. Statistical analyses were performed using one-way ANOVA from GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

**Results**

**TGF-β1 Transgenic Mice Have an Elevated Level of Latent TGF-β1 in Plasma and Renal Tissues but Exhibit a Normal Renal Function and Histology**

The K5.TGFβ1wt transgenic mice were generated successfully in the ICR background mice, as demonstrated by the genotypic PCR (Figure 1A). These mice displayed psoriasis-like skin lesions with overexpression of TGF-β1 in keratinocytes (14). Cir-

![Figure 1](image_url) Pertinent features of K5.TGFβ1wt transgenic (Tg) mice. (A) DNA from the tails of K5.TGFβ1wt Tg mice is amplified with specific primers showing the 271-bp delivered gene. (B) Circulating levels of active, latency-associated protein (LAP), and total TGF-β1 in Tg mice are significantly higher than that of wild-type (Wt) mice (ng/ml). (C) The local renal levels of active, LAP, and total TGF-β1. Both LAP and total TGF-β1 in the kidney tissues are significantly higher in Tg mice than in Wt mice. (D) The 24-h urinary protein excretion is within normal levels in both Tg and Wt mice (<1 mg/24 h). □, Tg mice; ▣, Wt mice; each bar represents the mean ± SEM for groups of six mice. *P < 0.05, **P < 0.01, ***P < 0.001 versus Wt mice.
culating levels of latent TGF-β1 were significantly elevated in the transgenic mice when compared with the Wt mice (a 10-fold increase; \( P < 0.001 \)), and this was associated with a 1.7-fold increase (\( P < 0.05 \)) in the levels of circulating active TGF-β1 (Figure 1C). In the kidney, the levels of total and latent TGF-β1 but not the active form of TGF-β1 were elevated in the transgenic mice when compared with the Wt mice (two- and three-fold increases; \( P < 0.05 \), respectively; Figure 1D). The circulating levels of active, latent (LAP), and total TGF-β1 were significantly elevated in the transgenic mice when compared with the Wt mice (two- and three-fold increases; \( P < 0.05 \), respectively; Figure 1D). However, TGF-β transgenic mice exhibited a normal renal function as determined by a normal level of 24-h urine protein excretion (Figure 1B) and histology (Figure 2, A and B).

**Figure 2.** Histologic features of kidneys of K5.TGF/1wt Tg and Wt mice in normal and obstructive kidney. (A and B) Kidneys from both K5.TGF/1wt Tg and Wt mice show normal renal histology. (C) A representative obstructive kidney from a Wt mouse shows severe renal damage, including interstitial fibrosis, tubular atrophy, and mononuclear cell infiltration, at day 7 after urethral ligation. (D) A representative obstructive kidney from a Tg mouse shows relatively normal histology at day 7 after the ureteral ligation. (E) Circulating levels of total TGF-β1, active form of TGF-β1, and LAP in both Tg and Wt mice at day 7 after induction of obstructive kidney disease. (F) The local renal levels of active, LAP, and total TGF-β1 in both Tg and Wt mice at day 7 after induction of obstructive kidney disease. Note that active TGF-β1 within the diseased kidney is significantly increased in Wt mice but not in Tg mice. \( P < 0.05 \), Tg mice; \( P < 0.01 \), Wt mice; \( P < 0.001 \) versus Wt mice. Tissue sections are stained with periodic acid-Schiff. Magnification, \( \times 200 \).

**TGF-β1 Transgenic Mouse Are Protected against Renal Histologic Damage and Leukocytic Infiltration in Obstructive Kidney Disease**

Seven days after the left ureteral ligation, Wt mice developed moderate to severe tubulointerstitial damage, including focal tubular atrophy, mononuclear inflammatory cell infiltration, and fibrosis. In contrast, TGF-β1 transgenic mice exhibited a relatively normal renal histology (Figure 2, C and D). The circulating levels of active, latent (LAP), and total TGF-β1 remained unchanged in both Wt and transgenic mice after the ureteral ligation (Figure 2F). However, Wt mice exhibited a significant increase in active TGF-β1 within the diseased kidney compared with the TGF-β1 transgenic mice in which a latent form of TGF-β1 remained dominant (Figure 2F). As shown in Figure 2, B and E, Wt mice developed severe renal inflammation as demonstrated by a marked increase in the number of T cells and macrophages in the tubulointerstitium, which was prevented in TGF-β1 transgenic mice (Figure 2, C and F). Quantitative analysis showed that T cell and macrophage infiltration in the tubulointerstitium was significantly inhibited in TGF-β1 transgene mice (Figure 3, G and H).

**Upregulation of Inflammatory Cytokines and Adhesion Molecule in Obstructive Kidney Is Prevented in TGF-β Transgenic Mice**

It has been well accepted that inflammatory cytokines and adhesion molecules, such as IL-1β, TNF-α, and ICAM-1, play an important role in the pathogenesis of kidney diseases (22,23). As shown in Figure 4, real-time PCR demonstrated that Wt mice exhibited a marked increase in mRNA levels of IL-1β, TNF-α, and ICAM-1 in the diseased kidney (all \( P < 0.001 \)), which was significantly inhibited in TGF-β1 transgenic mice. Similar results were also obtained at the protein level by immunohistochemistry, as shown in Figure 5.

**NF-κB Activation in Obstructive Kidney Is Inhibited in TGF-β1 Transgenic Mice**

NF-κB, a critical transcriptional factor for controlling inflammatory response, has been shown to play a central role in inflammatory diseases, including kidney diseases (24). As shown in Figure 6, immunohistochemistry revealed that the NF-κB/p65 subunit (Figure 6, E and H) but not the p50 subunit (Figure 6, B and G) was markedly activated in Wt mice as evidenced by its nuclear location in the areas of severe tubulointerstitial damage. In contrast, activation of the NF-κB/p65 subunit was abrogated in TGF-β1 transgenic mice (Figure 6, F and H).

**Smad7 Is Upregulated in Both Normal and Diseased Kidney in TGF-β1 Transgenic Mice**

We then investigated the potential mechanism by which TGF-β transgenic mice exhibited a protective role in renal inflammation. It has been reported that Smad7 is capable of inhibiting transcriptional activity of NF-κB (25), and it is well documented that Smad7 is induced by TGF-β1 and acts as a negative regulator of the TGF-β1 signaling pathway (26).
Hence, we hypothesized that prevention of renal inflammation in TGF-β1 transgenic mice may be associated with upregulation of Smad7. As shown in Figure 7, both real-time PCR and Western blot analyses revealed that, compared with Wt mice, a marked increase in renal Smad7 mRNA and protein was found in both normal and diseased kidney in the TGF-β1 transgenic mice. This observation poses the question of whether upregulation of renal Smad7 contributes to the prevention of renal NF-κB activation and inflammatory responses in obstructive kidney disease, which is investigated further below.

Upregulation of Smad7 Suppresses IL-1β- or TNF-α-Induced NF-κB Activation and Inflammatory Response In Vitro

It is known that TGF-β1 can induce Smad7 expression (27). Activation of the NF-κB pathway has been shown to play a critical role in renal inflammation (24). Thus, we hypothesized that upregulation of renal Smad7 may block NF-κB-mediated renal inflammation. This hypothesis was tested in a doxycycline-regulated Smad7-expressing normal rat kidney tubular epithelial cell line (NRK52E) in which Smad7 expression is tightly regulated by the concentrations of doxycycline (16). As
shown in Figure 8A, two-colored immunohistochemistry demonstrated that cells that were cultured in the presence of doxycycline substantially induced Smad7 expression. Importantly, cells with marked Smad7 expression completely blocked IL-1β/H9252–induced NF-κB/p65 nuclear translocation, whereas cells without or with minimal Smad7 expression exhibited NF-κB/p65 nuclear location. This is demonstrated further by Western blot analysis of the nuclear protein. Addition of doxycycline completely inhibited IL-1β/H9252– or TNF-α/H9251–induced NF-κB/p65 but not p50 nuclear translocation (Figure 8B), indicating that upregulation of Smad7 is capable of inhibiting IL-1β or TNF-α–mediated NF-κB activation. This observation was confirmed further by electrophoretic mobility shift assay. As shown in Figure 8C, cells that were cultured with doxycycline suppressed NF-κB DNA binding activity in response to IL-1β and TNF-α stimulation.

Figure 4. Real-time PCR shows IL-1β, TNF-α, and intercellular adhesion molecule-1 (ICAM-1) mRNA expression in the K5.TGFβ1wt Tg and Wt UUO mice kidneys. Total renal RNA extracted from normal control (■), Wt UUO (■), and K5.TGFβ1wt Tg UUO (■) were reverse-transcribed and subjected to real-time PCR for IL-1β (A), TNF-α (B), and ICAM-1 (C). Each bar represents the mean ± SEM for a group of six mice. *P < 0.05, **P < 0.01, ***P < 0.001 versus normal control; #P < 0.05, ##P < 0.01 versus Wt UUO.

Figure 5. Immunohistochemistry shows that upregulation of IL-1β, TNF-α, and ICAM-1 expression is prevented in the K5.TGFβ1wt Tg mice. (A through C) IL-1β, TNF-α, and ICAM-1 expression in the normal Wt control. (D through F) IL-1β, TNF-α, and ICAM-1 expression in the Wt UUO mice. (G through I) IL-1β, TNF-α, and ICAM-1 expression in the K5.TGFβ1wt Tg UUO mice. (J through L) Semiquantitative analysis of IL-1β, TNF-α, or ICAM-1 expression in the kidney tissues (percentage of positive staining) using Optimum Imaging Analysis System as described in the Materials and Methods section: □, normal control; ■, Wt UUO; ◇, Tg UUO. Each bar represents the mean ± SEM for a group of six mice. *P < 0.05, **P < 0.01, ***P < 0.001 versus normal control; #P < 0.05, ##P < 0.01 versus Wt UUO.
The effect of overexpression of Smad7 on the transcriptional activities of NF-κB was investigated further in this doxycycline-regulating Smad7-expressing tubular cell line by transient transfection with an NF-κB reporter, 241RMI-SEAP (18). As shown in Figure 9, A and B, doxycycline-induced Smad7 expression significantly inhibited IL-1β- or TNF-α–induced NF-κB promoter SEAP activities. This was associated with inhibition of IL-1β–induced, NF-κB–driven proinflammatory cytokine (IL-1β and TNF-α) and adhesion molecule (ICAM-1) expression (Figure 9, C through E).

**Upregulation of IκBα by Smad7 Is a Key Mechanism by Which TGF-β1 Suppresses NF-κB Activation and Renal Inflammation In Vitro and in Obstructive Kidney Disease**

Next we studied the signaling mechanism by which overexpression of Smad7 inhibits NF-κB activation. NF-κB is inactivated by binding to its inhibitor, IκBα, which traps NF-κB in the cytoplasm and prevents its nuclear translocation. We hypothesized that induction of IκBα by Smad7 may be a key mechanism of anti-inflammatory effects of TGF-β1. Results shown in Figure 10 demonstrated that doxycycline induced Smad7 expression by tubular cells in a time- and dose-dependent manner. This was tightly associated with an increase in IκBα expression (Figure 10, A and B). However, overexpression of Smad7 did not alter the levels of IκBα phosphorylation/degradation induced by IL-1β and TNF-α (Figure 10C). These observations were tested further in the diseased kidney in both Wt and TGF-β1 transgenic animals. A marked increase in renal Smad7 in TGF-β1 transgenic mice was associated with a substantial upregulation of IκBα and inhibition of IκBα phosphorylation (p-IκBα). In contrast, Wt mice showed a
decreased renal Smad7 in the obstructive kidney, which was associated with the reduction of renal IκBα and an increase in p-IκBα (Figure 7, B and C).

**Discussion**

We report here that increased circulating latent TGF-β has protective roles in renal inflammation in obstructive kidney disease induced in TGF-β1 transgenic mice. Upregulation of renal Smad7 may be a central mechanism of the anti-inflammatory effects of TGF-β1 in this disease model. Induction of IκBα by Smad7, thereby inhibiting NF-κB activation and NF-κB–driven inflammatory response, may be a key signaling pathway whereby TGF-β1 exerts its anti-inflammatory properties in vivo and in vitro. Thus, this study demonstrates the
protective roles of circulating latent TGF-β in inflammatory kidney disease and delineates the signaling mechanism of TGF-β in anti-inflammation.

TGF-β1 is produced and secreted in vivo as a latent complex, consisting of mature dimeric TGF-β1, LAP, and a latent TGF-β binding protein (LTBP). LAP binds to the N-terminal of TGF-β, rendering TGF-β latent, thereby preventing TGF-β from binding to its receptor, whereas LTBP-1 binds the LAP-TGF-β complex and prevents TGF-β from interacting with local matrix protein. TGF-β must be liberated from LAP to become activated. The mechanisms involved in the liberation of TGF-β from LAP include plasmin, thrombospondin-1, reactive oxygen species, and acid (28,29). TGF-β is a pleiotropic cytokine with significant anti-inflammatory and immunosuppressive properties. Mice that are deficient in TGF-β1 develop a lethal multi-organ inflammation at 3 wk of age (7). TGF-β inhibits the production of proinflammatory cytokines from macrophages and T cells (30,31). Administration of TGF-β attenuates autoimmune diseases, including collagen-induced arthritis (32), allergic encephalomyelitis (33), and experimental colitis (34). Various experimental approaches have shown convincingly the importance of LAP-TGF-β as targeting molecules of inflammation and immune diseases. Indeed, CD4+ T helper cells engineered to produce latent TGF-β1 reverse allergen-induced airway hyperactivity and inflammation, which is associated with an increase in the levels of activated TGF-β (35). T cells that are engineered to produce latent TGF-β1 downregulate Th1-mediated autoimmune and Th2-mediated allergic inflammatory processes (36). CD4+CD25+ T cells that express LAP on the surface suppress CD4+CD45RB+ high-induced colitis by a TGF-β–dependent mechanism (37). All of these studies suggest that overexpression of latent TGF-β1 on immune effector cells produces protective roles in inflammation and immune-mediated diseases. In this study, transgenic expression of the latent TGF-β1 in epidermal keratinocytes also resulted in prevention of renal inflammation in obstructive kidney disease, which was associated with a significant increase in the circulating levels of a latent form of TGF-β1. This finding further demonstrates that latent TGF-β1 derived from nonimmune cells also plays a protective role in inflammatory response in the kidney disease.

A most significant finding in our study is that upregulation of renal Smad7 may be a central mechanism whereby TGF-β1 transgenic mice are protected against renal injury. It is now clear that TGF-β signals through two downstream proteins, Smad2 and Smad3, to induce Smad7 expression, which in turn inhibits TGF-β signaling (27). Thus, overexpression of Smad7 can block TGF-β–induced renal fibrosis in vivo and in vitro (15,16). Beyond the antifibrotic effect of Smad7 in renal fibrosis, we have now shown that upregulation of Smad7 can also inhibit renal inflammation. This is consistent with the previous report that overexpression of Smad7 in T cells prevents cell-mediated renal injury in anti–glomerular basement membrane glomerulonephritis and overexpression of Smad7 within the kidney inhibits renal inflammation in remnant kidney disease (38,39). Therefore, upregulation of Smad7 may be a key mechanism by which TGF-β exerts its protective role in renal fibrosis and inflammation.

Although it is now clear that Smad7 acts by inhibition of Smad2/3 activation to exert its antifibrotic effect of TGF-β, it remains unknown the signaling mechanism by which Smad7 prevented renal inflammation in obstructive kidney disease. It
is well accepted that NF-κB is a key transcriptional factor to regulate a variety of inflammatory responses (24). NF-κB is composed of p50 and p65 subunits, among which p65 is a potent transcriptional activator, strongly promoting inflammatory reaction in kidney diseases (40). In this study, marked activation of p65, not p50, was closely correlated with the renal inflammation, consistent with the previous observation in experimental diabetic nephropathy (41). A putative NF-κB regulatory site has been found in the mouse Smad7 promoter, suggesting a functional link between the NF-κB and Smad7 (42). In our study, by using a doxycycline-regulated Smad7-expressing tubular epithelial cell line, we were able to show that doxycycline-induced overexpression of Smad7 substantially suppresses NF-κB activation as demonstrated by inhibition of IL-1β- and TNF-α–induced NF-κB/p65 nuclear translocation, NF-κB transcriptional activity (reporter assay), and NF-κB DNA binding activity. These findings are consistent with the previous reports that induction of Smad7 inhibits transcriptional activity of NF-κB in MDCK cells and in conditionally immortalized mouse podocytes (25,43).

It is well established that NF-κB activation is regulated by its inhibitor, IκB. Under basal conditions, IκB binds to NF-κB p50/p65 subunits to prevent their nuclear translocation. Once IκB is phosphorylated or degraded, p50/p65 subunits become activated and translocate into the nucleus to activate the target genes. TGF-β is able to induce IκB expression (44); however, its intracellular mechanism remains unclear. In our study, we demonstrated that Smad7 was able to induce directly the ex-

Figure 9. Overexpression of Smad7 inhibits NF-κB transcriptional activity and the IL-1β, TNF-α, ICAM-1 mRNA expression induced by IL-1β in a dose- and time-dependent manner in a Dox-regulated Smad7 expressing NRK52E TEC cell line. (A and B) NF-κB reporter assay (241RMJ-SEAP). Dox-regulated Smad7-expressing NRK52E TEC are transfected with 241RMJ-SEAP (NF-κB responsive construct), and pCMV-βGal with lipofectamine and Smad7 expression is induced by 1 μg/ml Dox for 18 h. Cells then are stimulated with IL-1β (10 ng/ml; A) or TNF-α (10 ng/ml; B) for 24 h for the analysis of SEAP and β-galactosidase activity.

(C through E) Real-time PCR. Results show that Dox-induced (1 μg/ml for 18 h) overexpression of Smad7 blocks IL-1β-induced (10 ng/ml) mRNA expression of IL-1β (C), TNF-α (D), and ICAM-1 (E) at 3 h. Each bar represents the mean ± SEM for three independent experiments. **P < 0.001 versus basal control; *<0.05, ***<0.001 versus IL-1β or TNF-α treatment group.
pression of IκBα. This suggests that TGF-β may act by stimulating Smad7 to induce IκBα expression, thereby preventing NF-κB from activation. This \textit{in vitro} finding was supported further by the \textit{in vivo} finding. Upregulation of renal Smad7 in TGF-β1 transgenic mice was associated with an increase in renal IκBα expression, resulting in suppression of NF-κB-driven inflammatory response in the diseased kidney (Figures 4 through 6). It was noted that doxycycline-induced overexpression of renal Smad7 could not prevent IκBα phosphorylation (Figure 10C) and produced a modest effect on inhibition of NF-κB promoter activity (Figure 9) induced by high concentrations of IL-1β (10 ng/ml) and TNF-α (10 ng/ml) \textit{in vitro}. In contrast, in TGF-β transgenic mice, increased renal Smad7 was associated with a significant inhibition of IκBα phosphorylation, which was associated with the low expression levels of IL-1β and TNF-α and significant inhibition of NF-κB activation and NF-κB–dependent renal inflammation (Figures 3 through 7). This discrepancy between \textit{in vitro} and \textit{in vivo} may be associated with the difference in the levels of Smad7 and proinflammatory cytokines in the cell culture system and in the diseased kidney in TGF-β transgenic mice. In addition, it is possible that latent TGF-β1 may act \textit{via} different mechanisms than Smad7 to regulate NF-κB activation or that Smad7 functions differently \textit{in vivo} in the kidney or \textit{in vitro} in cultured tubular cells. Nevertheless, inhibition of IκBα phosphorylation in the diseased kidney may be one mechanism by which increased latent TGF-β1 prevents NF-κB–driven renal inflammation.

An interesting observation in this study is that although K5.TGFβ1\textsuperscript{wt} transgenic mice were protected against renal inflammation, they developed inflammatory skin lesions, including epidermal hyperproliferation, massive infiltration of neutrophils, T lymphocytes, and macrophages (14). The discrepancy between kidney and skin lesions may be associated with the local levels of latent and active TGF-β1. The latent TGF-β1 can function to inhibit bioactivities of active TGF-β1 \textit{in vitro} and \textit{in vivo} (45,46). Administration of LAP-TGF-β1 completely reverses suppression of early proliferative response in the remnant liver in mice with overexpression of a bioactive form of TGF-β1 and prevents skin fibrosis in a murine model of sclerodermatous graft-versus-host disease (46,47). In this study, K5.TGFβ1\textsuperscript{wt} transgenic mice developed severe skin inflammation at the site where latent TGF-β1 was massively produced. It is likely that the excessive latent TGF-β1 may prevent biologic functions of TGF-β1 in anti-inflammatory response, resulting in massive macrophage and T cell infiltration and production of proinflammatory cytokines and chemokines (14). In addition, the distinct action of TGF-β1 on various tissue types may partially account for the discrepancy in the skin and the diseased kidney in K5.TGFβ1\textsuperscript{wt} mice. K5.TGFβ1\textsuperscript{wt} transgenic mice also secrete latent TGF-β1 from the skin, resulting in a significant increase in the levels of latent TGF-β1 in circulation (a 10-fold increase) and renal tissues (a two-fold increase compared with Wt mice). The latent TGF-β1 can be activated by plasmin and thrombospondin-1 within the circulation at certain levels, resulting in a moderate activation of circulating TGF-β1 (a 1.7-fold increase compared with Wt mice). It is highly possible that the persistent high levels of latent versus low levels of active forms of TGF-β1 within circulation and renal tissues may counterregulate each other to balance the biologic effects of TGF-β1 on extracellular matrix production and inflammatory response in the kidney by upregulating renal Smad7. Thus, an excessive latent TGF-β1 in the disease status may be in favor of the anti-inflammatory effect of TGF-β1. In contrast, normal Wt mice have equal levels of latent and active TGF-β1 in both circulation and renal tissue; however, active form of TGF-β1 was significantly increased in the diseased kidney (a three-fold increase compared with the latent form of TGF-β1). This was associated with a marked reduction of renal Smad7 and the development of severe renal inflammation in obstructive kidney. Thus, upregulation of Smad7 within the normal and diseased kidney in K5.TGFβ1\textsuperscript{wt} transgenic mice may be a key mechanism responsible for prevention of renal inflammation in response to high levels of latent TGF-β1 in obstructive kidney.
disease. However, several fundamental questions regarding latent versus active TGF-1 in induction of renal Smad7 expression remain unsolved: (1) Why is an increase in latent TGF-β1 but not an active form of TGF-β1 associated with upregulation of renal Smad7? (2) Does latent TGF-β1 act to stimulate Smad7 expression via the conventional TGF-β/Smad signaling pathway or an alternative pathway independent of Smad? Although the latent TGF-β1 has been shown to counterregulate bioactivities of active TGF-β1 in vitro and in vivo (45,46), the mechanisms are yet unclear. These questions are complicated but will require further investigation because they are potentially important for understanding the roles of latent versus active TGF-β1 in renal inflammation.

In summary, we demonstrated that increased circulating and local levels of latent TGF-β1 could prevent renal inflammation in a murine model of obstructive kidney disease in K5.TGFβ1wt transgenic mice. Upregulation of renal Smad7, which inhibits NF-κB activation by inducing IκBα expression, might be the central mechanism by which K5.TGFβ1wt transgenic mice are protected against renal inflammation. In contrast to the finding that overexpression of an active hepatic TGF-β1 largely elevates circulating bioactive TGF-β1 and results in severe renal damage (3), the renoprotective effects of latent TGF-β1 on obstructive kidney disease suggest that latent TGF-β may have therapeutic potential for kidney diseases.

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