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Treatment with anti-TGF-β antibody ameliorates chronic progressive nephritis by inhibiting Smad/TGF-β signaling

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Treatment with anti-TGF-β antibody ameliorates chronic progressive nephritis by inhibiting Smad/TGF-β signaling.

Background. Although short-term treatment with anti-transforming growth factor-β (TGF-β) antibody (αT) has been shown to prevent early glomerular lesions, its long-term effects and molecular mechanisms, including intracellular signaling, remain poorly understood. We examined whether αT treatment induces prevention of renal insufficiency and fibrosis, and affects the TGF-β/Smad signaling pathway in rats with chronic progressive anti-thymocyte serum (ATS) nephritis induced by repeated ATS injections on days 0 and 7.

Methods. Nephritic and non-nephritic rats were treated with either αT or control immunoglobulin (Ig)G twice weekly for 4 weeks from days 7 to 35 (each group, $N = 21$). Renal lesions and cortical expression of TGF-β1, TGF-β2, TGF-β3, type II TGF-β receptor (TβRII), Smads, type I collagen, and plasminogen activator inhibitor-1 were examined by immunohistochemistry, Western blot, and/or real-time reverse transcription polymerase chain reaction (RT-PCR). The binding of Smad3 in renal cortical cell nuclei to the Smad-binding element (SBE) was investigated by the electrophoretic mobility shift assay.

Results. Nephritic rats developed heavy proteinuria, renal insufficiency, and increased extracellular matrix deposition resulting in renal fibrosis. Cortical expression levels of TGF-β1, TGF-β2, TβRII, and Smad2, but not TGF-β3, Smad3, and Smad4 were increased. Expression and preferential localization of phosphorylated Smad2/3 in the glomerular and tubular cell nuclei, and Smad3-SBE complex-forming activity were also increased. Four-week αT treatment resulted in marked amelioration of chronic progressive ATS nephritis at 8 weeks.

Conclusion. In chronic progressive ATS nephritis, the TGF-β/Smad signaling was up-regulated. TGF-β blockade by αT suppressed the progression of renal scarring, at least in part, via inhibition of activated TGF-β/Smad signaling.

Key words: Transforming growth factor-β (TGF-β), anti-TGF-β antibody (αT), renal fibrosis, Smad, Smad-binding element (SBE), chronic progressive glomerulonephritis.

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Several lines of evidence indicate that overexpression of transforming growth factor-β (TGF-β) in the kidney underlies the development of renal fibrosis resulting from accumulation of extracellular matrix (ECM) in chronic progressive nephropathies [1]. Because the progression of renal fibrosis is the final common feature at the end-stage of various renal diseases and its level correlates with renal prognosis, TGF-β is thought to be a key molecule involved in the progression of chronic kidney diseases that lead to renal death [2].

TGF-β exerts its biologic effects through binding to specific cell surface receptor proteins, composed of two transmembrane serine/threonine kinases, designated type I (TβRI) and type II (TβRII) TGF-β receptors (TβR). Smad proteins are important signaling transducers from those receptors to the target genes in the nucleus. Three families of Smads have been identified: (*1*) receptor-regulated Smad2 and Smad3 (R-Smads); (*2*) common partner Smad4 (Co-Smad); and (*3*) inhibitory Smad6 and Smad7 (I-Smads). The activated TGF-β receptors induce phosphorylation of Smad2 and Smad3, which form hetero-oligomeric complexes with Smad4. In response to TβR activation, such complexes translocate to the nucleus, where they regulate transcriptional responses together with additional DNA binding cofactors [3].

Recently, several monoclonal antibodies to specific cytokines, such as interleukin-6 and tumor necrosis factor-α, have been clinically adopted for the treatment of various diseases, including rheumatoid arthritis and inflammatory bowel diseases. Similarly, inhibition of TGF-β has been proposed as a therapeutic strategy for prevention of renal fibrosis. To date, it has been reported that inhibition of TGF-β by neutralizing anti-TGF-β antibody (αT) [4–9], anti-sense oligonucleotide [10] and decorin [11] can suppress intrarenal ECM accumulation in rats with acute reversible mesangial proliferative glomerulonephritis induced by a single injection of anti-Thy-1.1 antibody [4, 10, 11], streptozotocin-induced diabetic mice [5], db/db

diabetic mice [6], rats with unilateral ureteral obstruction [7], and rats [8] and mice [9] with cyclosporin A nephropathy. However, most of these studies [4, 7, 8, 10–12] examined the short-term effects of TGF-β inhibition. Although two other studies investigated the long-term effects of αT in db/db diabetic mice [6] and mice with cyclosporin A nephropathy [9], the renal lesions in these models are dissimilar to those of human chronic progressive glomerulonephritis associated with tubulointerstitial lesions resulting in end-stage renal failure because no evident tubulointerstitial lesions developed in the former model, and the latter lacked glomerular damage and proteinuria. Therefore, at present, it remains unclear whether inhibition of TGF-β could exert long-term beneficial effects in chronic progressive glomerulonephritis. Furthermore, the molecular mechanisms involved in the profibrotic roles of TGF-β signaling system have been poorly documented.

To answer these questions, we adopted an experimental model of chronic progressive glomerulonephritis that was induced by repeated anti-thymocyte serum (ATS) injections. In this model, the animal develops irreversible glomerulosclerosis and tubulointerstitial fibrosis after the second ATS injection, associated with a gradual decline of renal function, and we also demonstrated previously a progressive increase in expression of TGF-β1 and types I, II, and III TGF-β receptors in the glomerular and tubulointerstitial lesions [13, 14]. Using this unique model, we studied the long-term effects of treatment with neutralizing αT in preventing proteinuria, renal insufficiency, renal fibrosis, and TGF-β/TβR/Smad signaling pathway. Treatment with αT significantly prevented proteinuria, renal insufficiency, and fibrosis. In addition, our findings present a complete picture of how the ligand, its receptor, and signaling components of the renal TGF-β system interact to promote the progression of glomerulonephritis with tubulointerstitial fibrotic lesions. To our knowledge, this is the first study that provides strong support for the premise that periodic inhibition of the biologic actions of TGF-β could prevent renal scarring, at least in part via down-regulation of pathologic TGF-β signaling in chronic progressive glomerulonephritis.

METHODS

Experimental animals and design

ATS was raised in non-pregnant adult female England Suffolk sheep as described previously [14]. All animals were treated according to the protocols approved by the Hamamatsu University School of Medicine Animal Care Committees at the Center Animal Care facility.

Male Wistar rats, weighing 150 g at the start of the experiment, were purchased from SLC (Hamamatsu, Shizuoka, Japan) and allowed free access to food and water. Eighty-four rats were divided into four groups including rats with chronic progressive ATS nephritis–treated with monoclonal neutralizing αT ($N = 21$), chronic progressive ATS nephritis rats treated with isotype-matched irrelevant immunoglobulin (Ig)G ($N = 21$), non-nephritic rats treated with αT ($N = 21$), and non-nephritic rats treated with irrelevant IgG ($N = 21$). Chronic progressive ATS nephritis was induced by repeated intravenous injections of 1.5 mL of ATS at days 0 and 7 [13, 14]. Rats that received intravenous injections of 1.5 mL of 0.15 mol/L phosphate-buffered saline (PBS, pH 7.4), at days 0 and 7, were adopted as non-nephritic animals. The murine IgG1 κ monoclonal αT neutralizes all three isoforms (-β1, -β2, and -β3) of TGF-β and has a circulating half-life of 15.2 hours in rat (1D11; ATCC HB-9849, Manassas, VA, USA). The efficacy and safety of this antibody has been reported in some in vivo studies conducted by other investigators [7, 9]. Therapeutic intervention using intraperitoneal injection of 2 mg of αT was started 6 hours after the second injection of ATS or PBS at day 7. Administration of αT was performed twice weekly for 4 weeks ending at day 35. As a control treatment, isotype-matched irrelevant IgG was administered instead of αT. Seven rats each in the four groups were sacrificed at day 0, and 4 and 8 weeks after the beginning of this protocol. The kidneys were perfused with ice-cold PBS and the renal cortical tissues were removed and reserved for the examinations described below.

Urinary protein excretion and blood urea nitrogen levels

Rats were housed in metabolic cages for a 24-hour urine collection under 12-hour light/12-hour dark cycle at days 0 and 3, and 1, 2, 4, 6, and 8 weeks after the beginning of this protocol. Urinary protein concentration was measured by a pyrogallol red method. The levels of blood urea nitrogen were measured as a marker of renal function at the end of the study period using the standard method.

Measurement of hydroxyproline (OH-proline)

The renal cortical tissues were fixed in acetone and frozen at −20°C until assayed. The OH-proline concentration (μg/mL) of the renal cortex was determined after dehydration and hydrolysis by hydrochloric acid, using the method of Woessner [15]. The concentration was corrected by tissue dry weight (μg/mg tissue).

Histopathologic and immunohistochemical analysis

Kidney tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Three-μm thick tissue sections were stained with periodic acid-Schiff for histopathologic analysis. To assess the acute and chronic histopathologic changes, we employed the methods of

Austin et al [16] with minor modifications. In brief, a semiquantitative grade of severity (0, 1, 2, or 3+, indicating that the feature was absent, mild, moderate, or severe, respectively) was assigned to each of the following morphologic attributes: (*1*) glomerular cell proliferation; (*2*) leukocyte exudation; (*3*) the presence of cellular crescents; (*4*) interstitial mononuclear-cell infiltration; (*5*) glomerular sclerosis; (*6*) the presence of fibrous crescents; (*7*) tubular atrophy; and (*8*) interstitial fibrosis. The presence of cellular crescents was weighed by a factor of two because such lesion has been considered to be disproportionately ominous. Composite scores were calculated by summing individual scores for attributes 1 through 4 to arrive at an activity index and by summing individual scores for attributes 5 through 8 to arrive at a chronicity index.

Immunoreactivity for phosphorylated Smad2/3 proteins was determined using a standard biotin-streptavidin-peroxidase method as described previously [14]. The primary antibody was rabbit anti-human phosphorylated Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was affinity-purified biotinylated donkey anti-rabbit IgG (Cortex Biochem, San Leandro, CA, USA). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 20 minutes. Reaction products were visualized using a color solution that consisted of 20 mg 3,3′-diaminobenzidine (DAB), 40 mg sodium azide, and 35 μL of 30% hydrogen peroxide in 100 mL of 0.05 mol/L Tris-HCl buffer, pH 7.6. Sections incubated with nonimmune rabbit sera instead of the primary antibodies were used as negative controls. Nuclei were counterstained lightly with hematoxylin. All sections were stained under identical conditions together with control incubation.

Double immunostaining for TβRII and phosphorylated Smad2/3 was performed using the methods of Lan et al [17]. After immunostaining the sections with phosphorylated Smad2/3 using DAB, sections were heated in a microwave oven for 10 minutes in 0.01 mol/L sodium citrate, pH 6.0, set at 800 W, then incubated with anti-TβRII antibody (Santa Cruz Biotechnology), washed with PBS, and incubated with biotinylated donkey anti-rabbit IgG (Cortex Biochem). Then, Vectastain ABC-AP reagent (Vector Laboratories, Burlingame, CA, USA) was added and the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) was used as chromogen. Nuclei were counterstained lightly with hematoxylin.

For immunofluorescence analysis, 3-μm thick frozen sections were cut with a cryostat, air dried, and fixed in acetone for 15 minutes. The primary antibody was rabbit anti-rat type I collagen (Chemicon International, Temecula, CA, USA). The secondary antibody was fluorescein isothiocyanate-conjugated affinity-purified swine anti-rabbit IgG (Dako, Glostrup, Denmark). Renal localization of type I collagen was investigated with an immunofluorescence microscope (BX50, Olympus, Tokyo, Japan).

Preparation of nuclear extracts

To examine the nuclear localization of phosphorylated Smad2 and Smad3 by Western blot analysis and the binding of Smad3 in the renal cortical cell nuclei to the Smad-binding element (SBE) using electrophoretic mobility shift assay (EMSA), nuclear extracts from renal cortex were prepared according to the method of Jeremiah et al [18] with minor modifications. In brief, renal cortical tissues were minced and disrupted in 10 volumes (about 1 mL) of 10 mmol/L HEPES, pH 7.9, 10 mmol/L potassium chloride, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and 10 mmol/L β-glycerophosphate by 6 strokes in glass Teflon homogenizers on ice. Detergent Nonidet P-40 was added to a final concentration of 0.7% and the homogenates were centrifuged at 7000*g* for 30 seconds. The pellets were suspended in fresh homogenization buffer and re-centrifuged at 7000*g* for 30 seconds. The resulting pellets were re-suspended in 0.3 mL of 20 mmol/L HEPES, pH 7.9, 400 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and 10 mmol/L β-glycerophosphate. The re-suspended pellets were then incubated on ice for 20 minutes with periodic pipetting and centrifuged at 10,000*g* for 30 minutes. The supernatants were removed and aliquots were frozen at −70°C.

Western blot analysis

Kidney cortical tissues were dissolved in Triton X-100 lysis buffer [50 mmol/L Hepes (pH 7.5), 300 mmol/L NaCl, 0.5% Triton X-100] at 4°C containing 1 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and 10 mmol/L β-glycerophosphate. Equal amounts of proteins (40 μg) were loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [14]. The primary antibodies were rabbit anti-human TβRII, phosphorylated Smad2/3 and Smad4 (Santa Cruz Biotechnology), rabbit anti-human Smad2 (Zymed Laboratory, South San Francisco, CA, USA), rabbit anti-human Smad3 (Upstate Biotechnology, Lake Placid, NY, USA), rabbit anti-rat plasminogen activator inhibitor-1 (PAI-1) (American Diagnostica, Greenwich, CT, USA), and mouse monoclonal anti-β-actin

(Sigma, St. Louis, MO, USA). β-actin was used as an internal control. Quantification of the band intensity was performed using National Institutes of Health image software.

RNA isolation and quantitative analysis of mRNA by real-time RT-PCR

Total RNA was extracted from renal cortical tissue using ISOGEN (Nippon Gene, Inc., Tokyo, Japan) according to the instructions provided by the manufacturer. Reverse transcription (RT) of the RNA was performed using the first-strand cDNA synthesis kit (Roche, Mannheim, Germany) and 1 μg of total RNA. For real-time PCR, the Light Cycler PCR and detection system (Roche) were used for amplification and online quantification. All PCR experiments were performed using the QuantiTect^{TM} SYBR Green PCR kit purchased from Qiagen (Tokyo, Japan). The amplification program consisted of 1 cycle at 95°C with a 10-minute hold (hot start) followed by 50 cycles at 95°C with a 15-second hold, 58°C annealing temperature with a 5-second hold, and 72°C with a 20-second hold. Amplification was followed by a melting curve analysis to verify the accuracy of the PCR products. The PCR-primer sequences were as follows: rat TGF-β1 (sense: 5′-ACCGCAACAACGCAATCTAT-3′ and antisense 5′-ACGCCAGGAATTGTTGCTAT-3′), rat TGF-β2 (sense: 5′-TGGCTTCACCACAAAGAC AG-3′ and antisense 5′-CCATCGATACCTGCGAAT CT-3′), rat TGF-β3 (sense: 5′-CCAAGACCTGGAGCC TCT-3′ and antisense 5′-CCTCAGCTGCACTTACAC GA-3′), rat α2 type I collagen (COL1A2) (sense: 5′-TGTTCGTGGTTCTCAGGGTAG-3′ and antisense 5′-TTGTCGTAGCAGGGTTCTTTC-3′), rat PAI-1 (sense: 5′-CTTTATCCTGGGTC TCCCTG-3′ and antisense 5′-TGATGCCTCCCTGACATACA-3′), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: 5′-AGGACCA GGTTGTCTCCTGT-3′ and antisense 5′-TTACTCCTTGGAGGCCATGT-3′). As negative controls, RNA of the samples that were not reverse transcribed was run to assess the specificity of the reaction. To verify the accuracy of PCR amplification, PCR products were analyzed on an ethidium bromide–stained 2% agarose gel.

Data analysis was performed using Light Cycler software version 3.3.9 provided by Roche. To quantify the gene copy number of the target cDNA that was reverse transcribed from RNA in the samples, 1:10 serially-diluted PCR products of the target cDNA, which had been generated beforehand using the same primers as described above and purified by QIAquick PCR Purification Kit (Qiagen), were included in each run and the standard curves were obtained. The ratios of TGF-β1, TGF-β2, TGF-β3, COL1A2, and PAI-1 mRNA to GAPDH mRNA were calculated in each sample.

Table 1. Body weight of nephritic and non-nephritic rats treated with αT or irrelevant IgG

	Body weight *g*		
	0 week	4 week	8 week
Non-nephritic rats			
IgG	171.5 ± 2.5	259.8 ± 3.2	295.7 ± 4.5
αT	170.2 ± 1.9	255.6 ± 6.3	293.1 ± 5.1
Nephritic rats			
IgG	174.6 ± 1.8	234.9 ± 4.9[a]	266.8 ± 3.1[a]
αT	168.5 ± 2.5	232.4 ± 4.2[a]	270.5 ± 2.3[a]

Values represent mean ± SEM for 7 rats in each group.
[a] $P < 0.05$ vs. non-nephritic rats at the corresponding time period.

Electrophoretic mobility shift assay (EMSA)

Double-strand oligonucleotides that contain three repetitions of the AGCCAGAC Smad-binding element (SBE) in PAI-1 promoter were purchased from Santa Cruz Biotechnology, and this SBE has been shown to bind specifically with Smad3/4 complex [19]. EMSA was performed as described previously [20]. Binding interaction of the nuclear proteins, which had been isolated from the renal cortical tissues of nephritic and non-nephritic rats treated with either αT or IgG to the SBE oligonucleotides, was performed in a final volume of 13 μL containing 2 μL of nuclear extracts containing 4 μg of proteins, 3 μL of binding buffer (100 mmol/L HEPES, 1 mmol/L EDTA, 20 mmol/L $MgCl_2$, 250 mmol/L KCl, 25 mmol/L DTT, and 50% glycerol), 1 ng of radiolabeled probes resuspended in 1 μL, 2 μg of poly-dIdC in 2 μL, and 5 μL of antibodies (5 μg) where necessary. The mixtures were incubated for 60 minutes at 30°C and DNA protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris/borate/EDTA (TBE).

Statistical analysis

All values are shown as mean ± SEM. Differences between groups were examined for statistical significance using analysis of variance (ANOVA). When a significant difference was present, statistical analysis was further performed using the Scheffe F test between the two groups. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Body weight of rats treated with αT or irrelevant IgG

Regardless of treatment with αT or isotype-matched irrelevant IgG, the mean body weight of nephritic rats treated with repeated ATS injections was significantly less than that of non-nephritic animals at 4 and 8 weeks of the experimental period (Table 1). However, treatment with αT did not significantly change the weight of nephritic or non-nephritic rats.

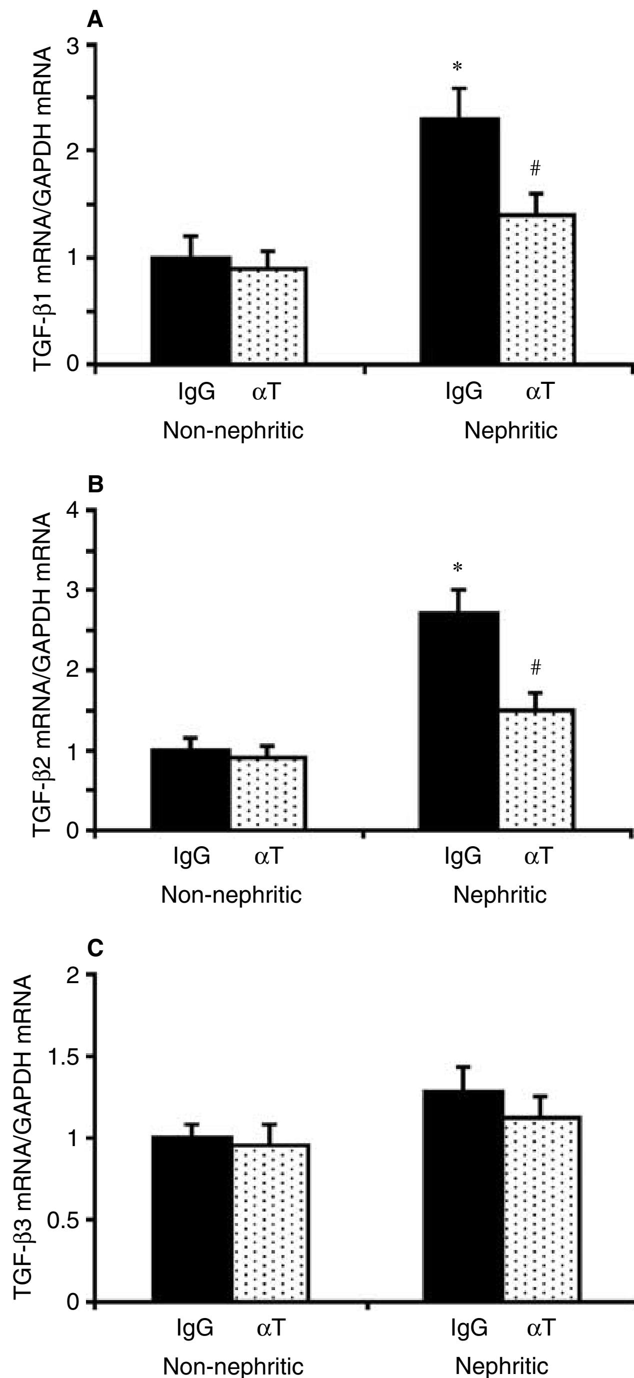

Fig. 1. Renal cortical expression of (*A*) TGF-β1, (*B*) TGF-β2, and (*C*) TGF-β3 mRNA at 8 weeks in nephritic and non-nephritic rats treated with αT or irrelevant IgG. Expression of TGF-β1 and TGF-β2 mRNA, but not TGF-β3 mRNA, in the renal cortex of nephritic rats treated with irrelevant IgG was significantly higher than that of IgG-treated non-nephritic rats at 8 weeks. Twice weekly administration of αT for 4 weeks, from days 7 to 35, significantly suppressed the elevated expression of TGF-β1 and TGF-β2 mRNA in nephritic kidneys. Neither marked increases of TGF-β1, TGF-β2, and TGF-β3 mRNA expression, nor effects of αT treatment on their expression were noted in non-nephritic kidneys. Data analysis was performed using Light Cycler software. To quantify the gene copy number of the target cDNA that was reverse transcribed from RNA in the samples, 1:10 serially-diluted PCR products of the target cDNA, which had been generated beforehand using the same primers, were included in each run and the standard curves were obtained. The ratios of TGF-β1, TGF-β2, and TGF-β3 mRNA to GAPDH mRNA were calculated in each sample. *$P < 0.01$ vs. non-nephritic groups; #$P < 0.05$ vs. nephritic rats treated with irrelevant IgG. Values are mean ± SEM calculated from the data of 7 rats in each group.

Renal cortical TGF-β expression

Expression levels of TGF-β1 and TGF-β2 mRNAs in the renal cortex of IgG-treated nephritic rats were significantly higher than those of IgG-treated non-nephritic rats at 4 (data not shown) and 8 weeks (2.3-fold and 2.7-fold increases in TGF-β1 and TGF-β2 mRNA, respectively) (Fig. 1). Twice weekly administration of αT for 4 weeks, from days 7 to 35, significantly suppressed the elevated expression of these TGF-β isoforms in nephritic kidneys at 4 (data not shown) and 8 weeks (Fig. 1). On the other hand, the levels of TGF-β3 mRNA expression were not significantly different among the groups.

αT treatment reduced proteinuria and prevented renal insufficiency

As shown in Figure 2A, proteinuria progressively worsened in rats treated with repeated ATS injections, while administration of αT from days 7 to 35 resulted in significant improvement of proteinuria on and after 4 weeks. No significant proteinuria was observed in non-nephritic rats, regardless of treatment with αT or irrelevant IgG throughout the experimental period of 8 weeks.

At baseline, the concentration of blood urea nitrogen was identical in each group (data not shown). Significant increases in blood urea nitrogen levels were noted in IgG-treated nephritic rats compared with IgG-treated non-nephritic rats at 8 weeks (35.5 ± 2.0 vs. 18.4 ± 1.0 mg/dL, respectively; $P < 0.05$). Four-week treatment with αT significantly prevented the increase in blood urea nitrogen in nephritic rats at 8 weeks (Fig. 2B).

Histopathologic changes. Consistent with our previous reports [13, 14], two ATS injections, given 1 week apart, induced chronic progressive glomerulonephritis with tubulointerstitial lesions in rats (Fig. 3C). In addition to proliferation of mesangial cells with increased matrix accumulation, periglomerular and focal tubulointerstitial lesions included increases in ECM deposition, mononuclear cell infiltration, and tubular atrophy at 8 weeks.

Rats with chronic progressive ATS nephritis treated twice weekly with αT from days 7 to 35 showed marked amelioration of these glomerular and tubulointerstitial changes at 8 weeks (Fig. 3C vs. 3D). No notable glomerular or tubulointerstitial lesions were observed in non-nephritic animals treated with either αT or irrelevant IgG (Fig. 3A and 3B). Both activity and chronicity indexes were significantly higher in nephritic rats treated with irrelevant IgG compared with non-nephritic rats. αT

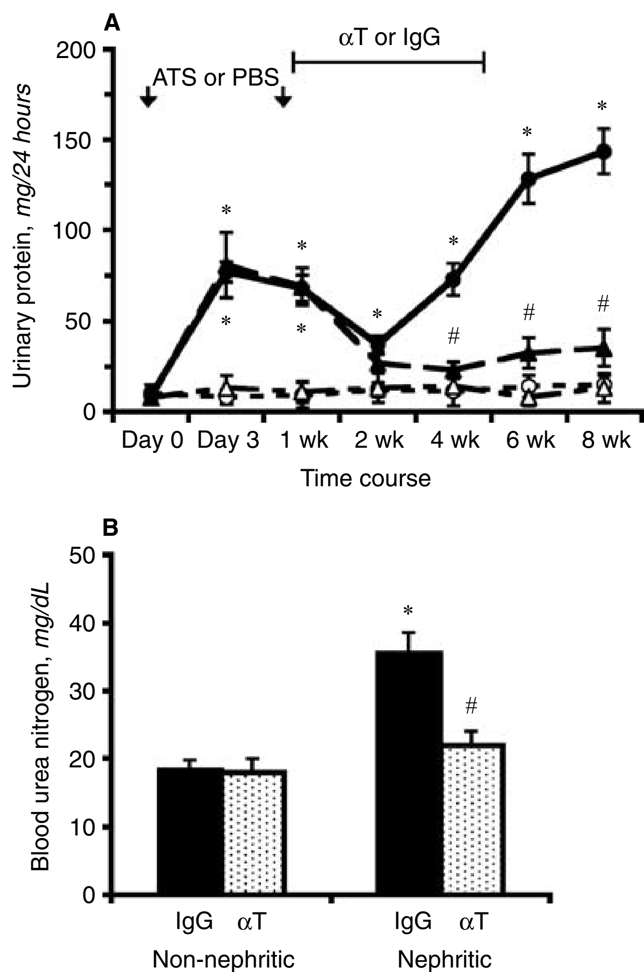

Fig. 2. Levels of urinary protein excretion and blood urea nitrogen at 8 weeks. (*A*) Levels of proteinuria increased progressively in rats receiving repeated ATS injections (closed circles) and administration of αT from days 7 to 35 (closed triangles) resulted in significant decreases in levels of proteinuria on and after 4 weeks. No significant proteinuria was observed in non-nephritic rats regardless of treatment with αT (open triangles) or irrelevant IgG (open circles) throughout the experimental period of 8 weeks. (*B*) Blood urea nitrogen was measured at 8 weeks after the first injection of ATS. Blood urea nitrogen levels of nephritic rats treated with IgG were significantly higher than those of non-nephritic rats. Treatment with αT significantly reduced blood urea nitrogen levels in nephritic rats, although no remarkable changes were noted in non-nephritic animals. $^*P < 0.05$ vs. non-nephritic groups; #$P < 0.05$ vs. nephritic rats treated with irrelevant IgG. Values are mean ± SEM calculated from the data of 7 rats in each group.

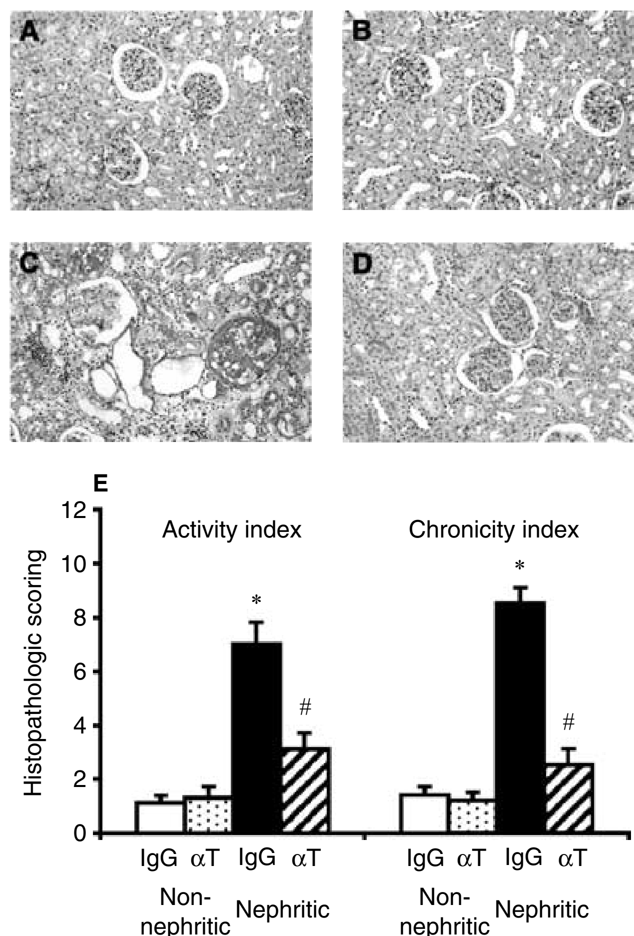

Fig. 3. Light microscopic images of kidneys at 8 weeks in (*A*) non-nephritic rats treated with irrelevant IgG, (*B*) non-nephritic rats treated with αT, (*C*) nephritic rats treated with irrelevant IgG, and (*D*) nephritic rats treated with αT, and (*E*) semi-quantitative histopathologic evaluation of renal lesions. Marked mesangial matrix expansion, interstitial fibrosis, tubular atrophy, and interstitial mononuclear infiltration were noted in the renal cortex of nephritic rats treated with irrelevant IgG (C). In nephritic rats treated with αT, these glomerular and tubulointerstitial lesions were markedly ameliorated at 8 weeks (D). No remarkable renal changes were noted in non-nephritic rats treated with IgG or αT (A and B, respectively). (Periodic acid-Schiff stain, magnifications ×200) (E) Both activity and chronicity indexes were significantly increased in nephritic rats treated with irrelevant IgG compared to non-nephritic rats. αT treatment significantly ameliorated both indexes in nephritic rats. $^*P < 0.05$ vs. non-nephritic rats; # $P < 0.05$ vs. nephritic rats treated with irrelevant IgG. Values are mean ± SEM calculated from the data of 7 rats in each group.

treatment resulted in significant amelioration of both of these indexes in nephritic rats (Fig. 3E).

Renal cortical OH-proline levels and expression of COL1A2 and PAI-1. Renal accumulation of OH-proline–containing proteins such as collagens is a feature of increased ECM deposition in nephritis associated with glomerulosclerosis and tubulointerstitial fibrosis. In addition, PAI-1 is known to be induced by the action of TGF-β and is a potent inhibitor of plasminogen activator that takes part in matrix degradation [21, 22]. To evaluate the effect of αT on ECM deposition in chronic progressive ATS nephritis, we investigated the renal cortical OH-proline levels and expression of COL1A2 and PAI-1.

In non-nephritic rats, the renal OH-proline concentration at 8 weeks was not significantly different between rats treated with αT and those treated with irrelevant IgG (Fig. 4A). In contrast, the renal OH-proline concentration was markedly increased in nephritic rats treated with IgG at 8 weeks ($P < 0.01$ vs. IgG-treated non-nephritic

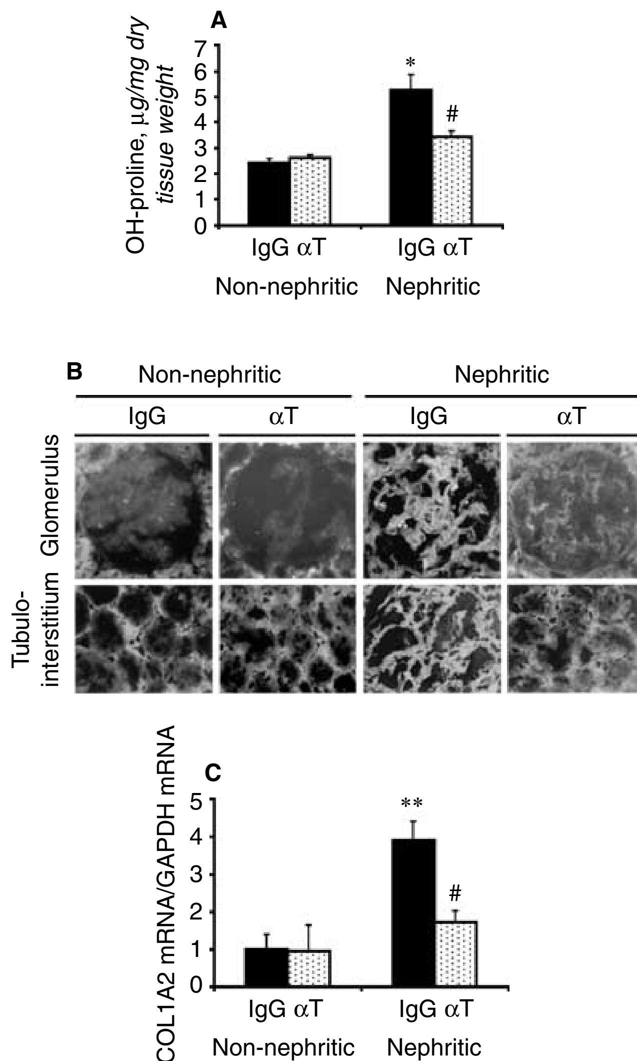

Fig. 4. Levels of (*A*) hydroxyproline (OH-proline), (*B*) immunofluorescence images of type I collagen, and (*C*) levels of COL1A2 mRNA measured by real-time RT-PCR in renal cortical tissues of nephritic and non-nephritic rats at 8 weeks. (A) Levels of OH-proline were significantly increased in the renal cortex of chronic progressive nephritic rats induced by repeated ATS injections, and αT treatment significantly decreased the levels in nephritic rats. (B) Type I collagen protein was noted scarcely in glomeruli and moderately in interstitium of non-nephritic rats treated with αT or IgG. In IgG-treated nephritic rats, increased deposition of type I collagen was noted in the glomerular and tubulointerstitial lesions compared with those of IgG-treated non-nephritic animals, and treatment with αT markedly suppressed its glomerular and tubulointerstitial deposition. (C) COL1A2 mRNA was significantly increased in IgG-treated nephritic rats compared with non-nephritic animals. Four-week treatment with αT prevented the increase of COL1A2 mRNA expression at 8 weeks, while αT treatment did not significantly change its expression in non-nephritic kidneys. Data analysis was performed using Light Cycler software. To quantify the gene copy number of the target cDNA that was reverse transcribed from RNA in the samples, 1:10 serially-diluted PCR products of the target cDNA, that had been generated beforehand using the same primers, were included in each run and the standard curves were obtained. The ratios of COL1A2 mRNA to GAPDH mRNA were calculated in each sample. $^{*}P < 0.01$ vs. non-nephritic rats; $^{**}P < 0.05$ vs. non-nephritic rats; $\#P < 0.05$ vs. nephritic rats treated with irrelevant IgG. Values are mean ± SEM calculated from the data of 7 rats in each group.

rats). αT treatment significantly decreased the levels in nephritic rats ($P < 0.05$ vs. IgG-treated nephritic rats).

Immunofluorescence analysis showed that type I collagen protein was scarce in glomeruli and moderate in interstitium in non-nephritic rats treated with αT or IgG (Fig. 4B). In IgG-treated nephritic rats, increased deposition of type I collagen was noted in the glomerular and tubulointerstitial lesions compared with those in IgG-treated non-nephritic animals, and treatment with αT markedly suppressed its deposition in the glomeruli and tubulointerstitium. To confirm this phenomenon, we also examined COL1A2 mRNA expression in renal cortical tissues. As shown in Figure 4C, COL1A2 mRNA was significantly increased in IgG-treated nephritic rat kidneys compared with non-nephritic ones ($P < 0.05$). Four-week treatment with αT prevented the increase of COL1A2 mRNA expression at 8 weeks, indicating that αT treatment inhibited the enhanced COL1A2 production in chronic progressive ATS nephritic kidneys.

Both PAI-1 protein and mRNA expression were markedly increased in IgG-treated nephritic rat kidneys, and αT treatment almost completely prevented the expression at 8 weeks (Fig. 5). These results suggested that αT treatment efficiently suppressed renal accumulation of ECM in chronic progressive ATS nephritis.

Cortical expression of TGF-β/Smad signaling components. Recent studies have demonstrated that Smad proteins are important intracellular transducers of TGF-β signaling, and that activated TGF-β receptors induce phosphorylation of Smad2 and Smad3, which translocate into the nucleus and bind to their target genes [23]. Therefore, we next examined the TGF-β/Smad signaling pathway to elucidate the molecular mechanisms of renoprotective effects of αT.

No differences in the cortical levels of TβRII and Smad2 proteins were noted between non-nephritic rats treated with IgG and those treated with αT at 8 weeks (Fig. 6A and C). The levels of TβRII and Smad2 proteins were increased in the renal cortical tissues of IgG-treated nephritic rats; however, αT treatment markedly decreased their levels. In contrast, no remarkable differences in the renal cortical levels of Smad3 and Smad4 proteins were observed among nephritic and non-nephritic rats treated with either αT or IgG (Fig. 6A and C).

To elucidate the signaling activity of R-Smads, we then investigated the levels of phosphorylated Smad2 and Smad3 in the nuclear protein fractions prepared from renal cortical tissues (Fig. 6B and C). There were no differences in the levels of nuclear phosphorylated Smad2 and Smad3 in non-nephritic rats regardless of treatment with αT or IgG at 8 weeks. In contrast, both nuclear phosphorylated Smad2 and Smad3 were increased markedly

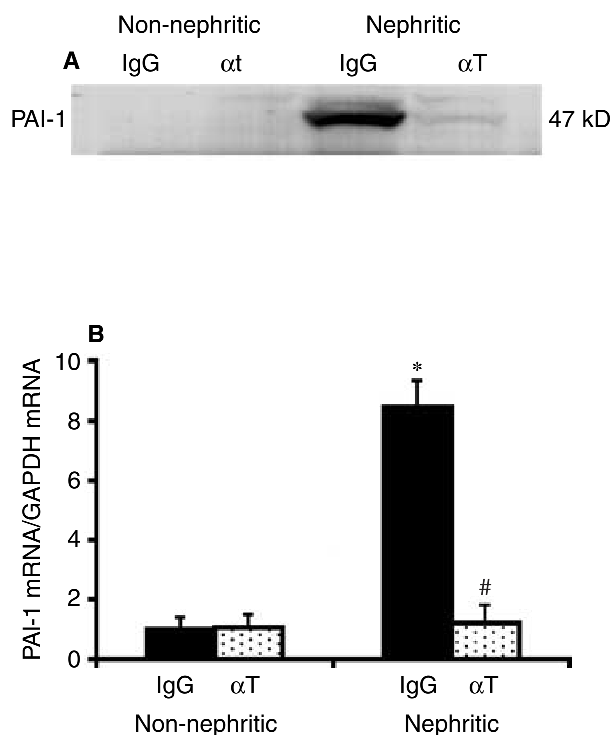

Fig. 5. Western blotting of (*A*) PAI-1 protein and levels of PAI-1 (*B*) mRNA measured by real-time RT-PCR in renal cortical tissues of nephritic and non-nephritic rats at 8 weeks. Both (A) PAI-1 protein and (B) mRNA expression were markedly increased in IgG-treated nephritic rat kidneys at 8 weeks and αT treatment almost completely prevented the expression. Data analysis was performed using Light Cycler software. To quantify the gene copy number of the target cDNA that was reverse transcribed from RNA in the samples, 1:10 serially-diluted PCR products of the target cDNA, that had been generated beforehand using the same primers, were included in each run and the standard curves were obtained. The ratios of PAI-1 mRNA to GAPDH mRNA were calculated in each sample. $^*P < 0.05$ vs. non-nephritic rats; $\#P < 0.05$ vs. nephritic rats treated with irrelevant IgG. Values are mean ± SEM calculated from the data of 7 rats in each group.

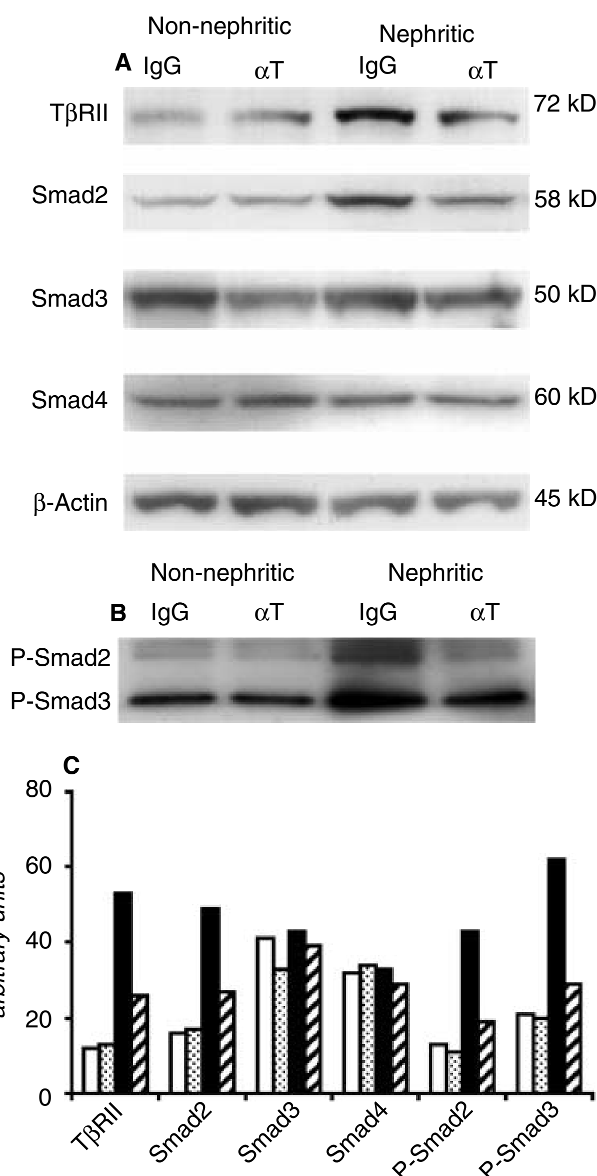

Fig. 6. Western blotting of TGF-β/Smad signaling components in the whole (*A*) cellular and (*B*) nuclear extracts obtained from renal cortical tissues of nephritic and non-nephritic rats at 8 weeks, and their (*C*) densitometric intensity. (A) Levels of TGF-β type II receptor (TβRII) and Smad2 were increased in the renal cortical tissues of nephritic rats treated with IgG compared with those of non-nephritic rats, whereas no significant changes were observed in the levels of Smad3 and Smad4. Treatment with αT reduced the levels of TβRII and Smad2 in nephritic rats. (B) No remarkable differences in the levels of nuclear phosphorylated Smad2 (P-Smad2) and Smad3 (P-Smad3) were noted in non-nephritic rats regardless of treatment with αT or IgG at 8 weeks. In contrast, both nuclear P-Smad2 and P-Smad3 were markedly increased in the renal cortical tissues of IgG-treated nephritic rats compared with levels in non-nephritic animals. Treatment with αT markedly suppressed the nuclear accumulation of P-Smad2 and P-Smad3 in the nephritic kidneys. In addition, nuclear P-Smad3 was more abundant than P-Smad2 in the cortices of nephritic and non-nephritic rat kidneys. (C) Symbols are: (□) non-nephritic rats treated with irrelevant IgG; (▩) non-nephritic rats treated with αT; (■) nephritic rats treated with irrelevant IgG; (▨) nephritic rats treated with αT.

in the renal cortical tissues of IgG-treated nephritic rats compared with those in non-nephritic animals, indicating the enhanced TGF-β/Smad signaling in the nephritic kidneys. In addition, the levels of nuclear phosphorylated Smad3 were more abundant than those of phosphorylated Smad2 in the cortices of nephritic and non-nephritic kidneys. Moreover, treatment with αT markedly suppressed the nuclear accumulation of phosphorylated Smad2 and Smad3 in nephritic kidneys to levels almost identical to those in non-nephritic kidneys. These biochemical data were also confirmed by the immunohistochemical study as shown in Figure 7. Phosphorylated Smad2 and Smad3 in IgG-treated nephritic rat kidneys were preferentially localized in the nuclei of glomerular and tubular cells when compared with non-nephritic rat kidneys (Fig. 7A vs. C and 7E vs. G). Furthermore, double immunostaining for TβRII and phosphorylated Smad2/3 in nephritic kidney specimens at 8 weeks demonstrated

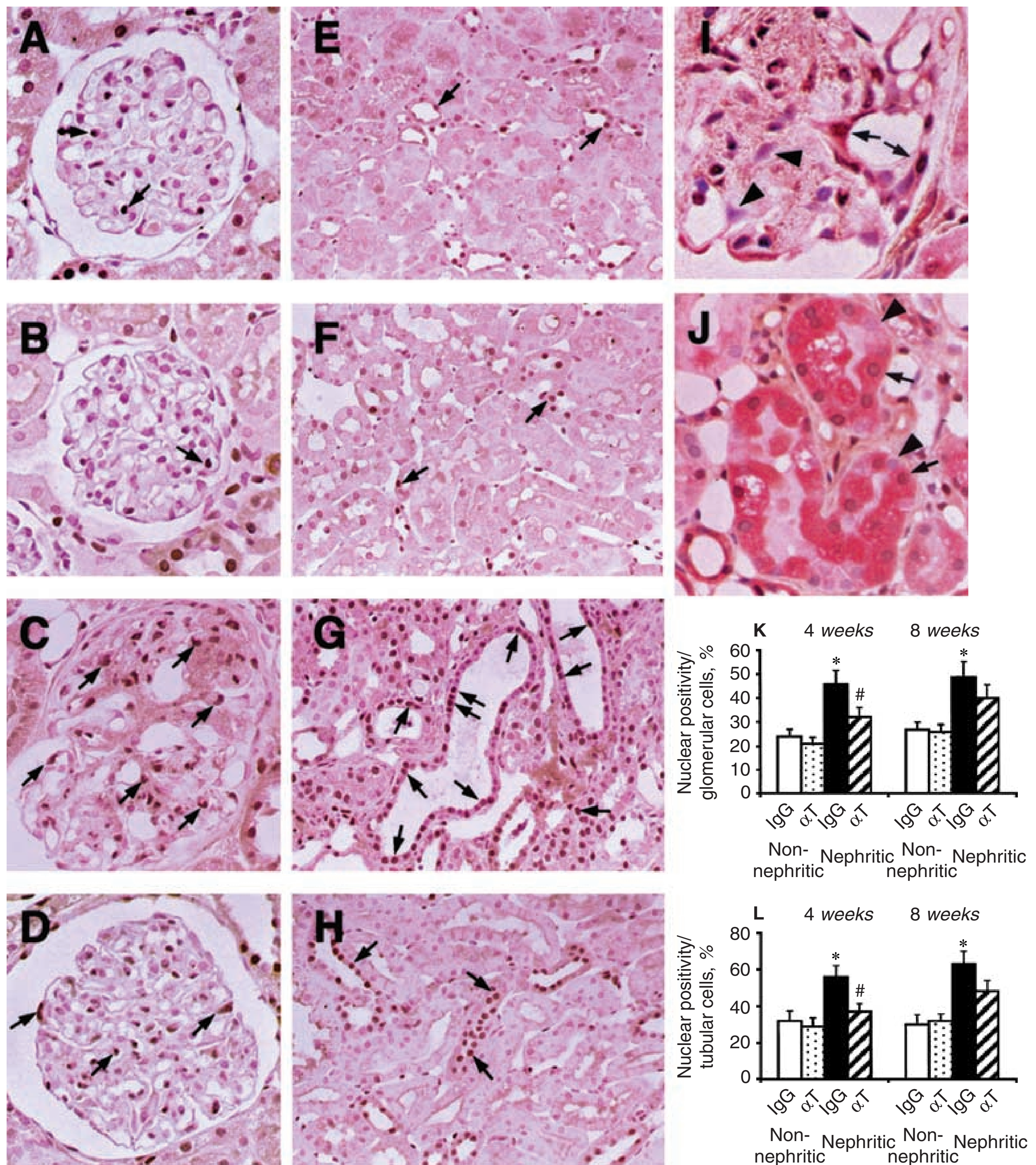

Fig. 7. Immunohistochemical demonstration of phosphorylated Smad2 and Smad3 (P-Smad2/3) in the glomeruli (*A* to *D*) and tubulointerstitium (*E* to *H*) of non-nephritic rats treated with irrelevant IgG (A and E), non-nephritic rats treated with αT (B and F), nephritic rats treated with irrelevant IgG (C and G), and nephritic rats treated with αT (D and H) at 8 weeks, and higher magnification pictures of double immunostaining for TβRII (stained red by Vector Red) and P-Smad2/3 (stained brown by 3,3′-diaminobenzidine) in the glomerular (*I*) and tubulointerstitial lesions (*J*) of nephritic rats treated with irrelevant IgG at 8 weeks. Percentages of P-Smad2/3-positive nuclei in the glomerular and tubular cells at 8 weeks are shown in (*K*) and (*L*), respectively. Note the weak nuclear staining of P-Smad2/3 in a few glomerular and distal tubular cells of IgG-treated (arrows in A and E, respectively) and αT-treated (arrows in B and F, respectively) non-nephritic rat kidneys. Increased nuclear immunoreactivity for P-Smad2/3 was noted in the glomerular endothelial, epithelial, mesangial, Bowman's capsular epithelial, tubular cells, and interstitial infiltrating mononuclear cells of IgG-treated nephritic rat kidneys (arrows in C and G). Treatment with αT markedly decreased the number of P-Smad2/3-positive nuclei in nephritic rat kidneys (arrows in D and H). Double immunostaining for TβRII and P-Smad2/3 demonstrated that increased nuclear accumulation of P-Smad2/3 in glomerular and tubular cells was associated with increased TβRII expression (arrows in I and J, respectively), and cells without significant TβRII expression were negative for nuclear immunoreactivity for P-Smad2/3 (arrowheads in I and J). Percentages of P-Smad2/3-positive nuclei in the glomerular and tubular cells are shown in panels K and L, respectively. $^*P < 0.05$ vs. non-nephritic groups; $\#P < 0.05$ vs. nephritic rats treated with irrelevant IgG. Values are mean ± SEM calculated from the data of 7 rats in each group.

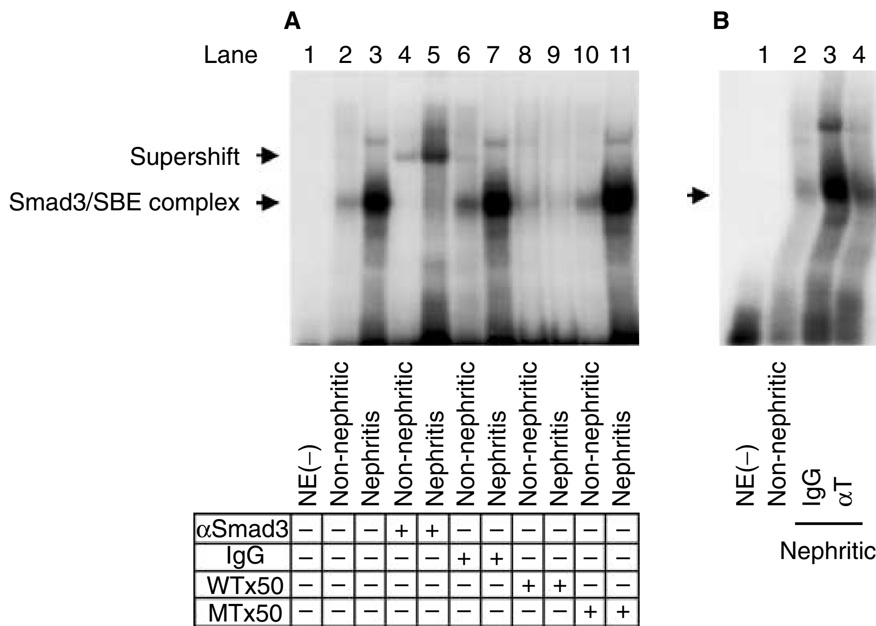

Fig. 8. Electrophoretic mobility shift assay (EMSA) of Smad3-Smad binding element (SBE) complex in nuclear extracts from renal cortical tissues of non-nephritic and nephritic rats at 8 weeks. EMSA was performed using ^{32}P-labeled Smad-binding element (SBE) of the PAI-1 promoter as a probe. The specific shifted bands were observed in nuclear extracts (NE) from renal cortical tissue of non-nephritic and nephritic rats (*A*, lanes 2 and 3). The shifted band was considered to comprise Smad3-SBE complexes, because it was super-shifted by incubation with anti-Smad3 antibody (αSmad3) (A, lanes 4 and 5) but not with control IgG (IgG) (A, lanes 6 and 7), and was diminished by incubation with excess amounts of cold wild-type probes (WT, ×50) (A, lanes 8 and 9) but not with excess amounts of cold mutant probes (MT, ×50) (A, lanes 10 and 11). Levels of Smad3-SBE complex were increased in nuclear extracts from IgG-treated nephritic rat kidneys compared with those from non-nephritic rat kidneys (A, lanes 2 vs. 3, and *B*, lanes 2 vs. 3). Treatment with αT resulted in significant decreases of binding activity for the SBE (B, lanes 3 vs. 4). Representative data of three independent experiments are shown.

that increased nuclear accumulation of phosphorylated Smad 2/3 was noted in glomerular, tubular, and interstitial infiltrating mononuclear cells with increased TβRII expression (Fig. 7I and J). αT-treated nephritic kidneys showed decreased nuclear localization of phosphorylated Smad2/3 in the glomeruli and tubulointerstitium compared with those in IgG-treated nephritic kidneys (Fig. 7C vs. D and 7G vs. H). These data indicated that αT treatment suppressed not only the quantities but also the accumulation of phosphorylated Smad2 and Smad3 in the renal cortical cell nuclei in rats with chronic progressive ATS nephritis.

Activation of Smad3 binding to Smad-binding element (SBE). Following activation by TβR in response to TGF-β stimuli, activated Smad3 couples with Smad4, translocates into the nucleus, and binds to certain DNA sites such as SBE for the Smad3/4 complex in the target gene promoters [24]. Finally, we examined whether nuclear protein binding to SBE is enhanced in nephritic rat kidneys using EMSA.

^{32}P-labeled SBE probe of the PAI-1 promoter was clearly shifted by the addition of nuclear extracts obtained from non-nephritic and nephritic rat cortical tissues (Fig. 8A, lanes 1 vs. 2 and 1 vs. 3, respectively). However, the binding level was markedly increased in nuclear extracts obtained from nephritic rat kidneys (lane 3) compared with non-nephritic kidneys (lane 2). The band was further shifted following incubation with anti-Smad3 antibody (lanes 2 vs. 4 in non-nephritic kidneys and lanes 3 vs. 5 in nephritic kidneys), whereas incubation with control IgG did not affect the band (lanes 2 vs. 6 in non-nephritic kidneys and lanes 3 vs. 7 in nephritic kidneys). Incubation with anti-Smad2 antibody caused a trivial change of the band (data not shown). The shifted band disappeared in the presence of excess amounts of cold wild-type SBE (lanes 8 and 9) but not mutant SBE (lanes 10 and 11). This suggested that the shifted band mostly consisted of Smad3-SBE complexes and that the binding of Smad3 to SBE was markedly increased in chronic progressive ATS nephritic kidneys.

Using this EMSA assay, we evaluated the effects of 4-week αT treatment on Smad3-SBE complex formation in the nephritic kidneys at 8 weeks. We found that αT treatment significantly decreased the formation of Smad3-SBE complex in the nephritic kidneys (Fig. 8B, lanes 3 vs. 4). Taken together, these data strongly suggested that the activated TGF-β/Smad signaling pathway plays a pathogenic role in the development of proteinuria, renal insufficiency, and intrarenal ECM accumulation in chronic progressive ATS nephritis. We also demonstrated that blockade of TGF-β/Smad signaling pathway by 4-week αT treatment markedly reduced these functional and morphologic derangement involved in chronic progressive ATS nephritis at 8 weeks.

DISCUSSION

In the present study, we evaluated the effects of neutralizing αT, administered twice weekly for 4 weeks from days 7 to 35, on renal insufficiency and fibrosis, and attempted to define the intrarenal TGF-β/Smad signaling system involved in chronic progressive ATS nephritis induced by repeated ATS injections. Our results demonstrated that 4-week administration of αT, which neutralizes the activity of all three mammalian isoforms of TGF-β, TGF-β1, -β2, and -β3, ameliorated proteinuria, renal dysfunction, and pathologic glomerular and tubulointerstitial ECM deposition at 8 weeks in chronic progressive ATS nephritis. αT treatment significantly suppressed the elevated expression of TGF-β1 and

-β2 in nephritic kidneys at 4 as well as 8 weeks. Interruption of TGF-β autoinduction [25, 26] by αT treatment may be involved in the suppression of TGF-β1 and -β2 mRNA expression in the nephritic kidneys of rats treated with αT. On the other hand, there were no differences in TGF-β3 mRNA expression levels among the groups. Although the reason for the different TGF-β3 mRNA expression relative to that of TGF-β1 and -β2 mRNAs in chronic progressive ATS nephritic kidneys is not clearly understood, a distinct role for TGF-β3 in the progression of fibrosis compared with TGF-β1 and β2 has been reported [27].

Not only decreases of ECM production as evidenced by the decreases of renal cortical OH-proline content, type I collagen deposition and COL1A2 mRNA expression, but also increased ECM degradation attributable to decreased PAI-1 expression could be involved in the antifibrotic effects of αT. In addition to direct inhibition of TGF-β, which was expressed in the glomerular and tubulointerstitial lesions in chronic progressive ATS nephritis, the associated decrease in proteinuria might have also participated in amelioration of fibrotic tubulointerstitial lesions in αT-treated nephritic rats [28].

Although the ameliorative effects of long-term administration of αT were reported in db/db diabetic mouse nephropathy [6] and mouse cyclosporin A nephropathy [9], αT was administered until the end point of the experimental period (8 weeks) in these studies. In our study, however, αT was administered twice weekly only from days 7 to 35 and the ameliorative effects were noted 3 weeks after the cessation of its administration. These data indicate that the initial 4-week suppression of pathogenic TGF-β activity by αT is therapeutically potent in preventing the development of chronic ATS nephritis, which itself has a progressive potential.

Recently, Smad proteins have been identified as predominant components in the TGF-β signaling pathway [3, 24]. TGF-β signals through the heteromeric complex of TβRI and TβRII, transmembrane serine/threonine kinase receptors. Activation of the receptor complex occurs when type II receptor kinase transphosphorylates the GS domain of type I kinase. The activated type I kinase associates transiently with, and also phosphorylates such R-Smads as Smad2 and Smad3. Once phosphorylated, R-Smads dissociate from the receptor, bind to Smad4, and then enter the nucleus. We have previously shown that TGF-β1 and TβRs were up-regulated in chronic progressive ATS nephritis [13, 14]. However, the role of Smad signaling components involved in the model has not been identified.

To investigate the Smad signaling involved in chronic progressive ATS nephritis, we first examined the whole cellular as well as nuclear expression and localization of TGF-β/Smad signaling components in the renal cortical tissues of both nephritic and non-nephritic rats. In agreement with previous reports [29, 30], TβRII and Smad2 proteins were increased in the renal cortical cells of rats with chronic progressive ATS nephritis compared with those in non-nephritic animals, while no significant differences in the cortical cellular levels of Smad3 and Smad4 proteins were noted between nephritic and non-nephritic rat kidneys. On the other hand, the nuclear localization of phosphorylated Smads 2 and 3 was markedly increased in nephritic compared with non-nephritic rat kidneys. Although Ostendorf et al [30] recently reported that phosphorylated Smad2 staining predominantly localized to nuclei in the glomerular endothelial cells in acute reversible anti-Thy-1.1 nephritis, nuclear accumulation of phosphorylated Smads 2 and 3 was noted in glomerular endothelial, epithelial, mesangial, Bowman's capsular epithelial, tubular, and interstitial infiltrating mononuclear cells in chronic ATS nephritis with tubulointerstitial lesions in the present study. This discrepancy may be attributable to the fact that the antibody used in our study reacts with both phosphorylated Smads 2 and 3, and also to the difference in kidney cells with accelerated TGF-β/Smad signaling between acute reversible anti-Thy-1.1 nephritis and chronic progressive ATS nephritis with tubulointerstitial lesions.

Smad3 and Smad4 can bind to the specific nucleotide sequence CAGA box, called the consensus SBE. A number of such Smad3/Smad4 binding elements have been characterized in the promoters of some TGF-β1 responsive genes. These include genes encoding PAI-1, types I and IV collagens, Smad7, c-jun, and junB [19, 21, 31, 32]. Using EMSA for the SBE in the PAI-1 promoter that binds specifically to the Smad3/Smad4 complex [19], we also demonstrated that the SBE binding activity of Smad3 in nuclear proteins extracted from the renal cortical tissues of rats with chronic progressive ATS nephritis were markedly increased. Moreover, Smad3 binding activity to SBE was markedly suppressed in nephritic rat kidneys treated with αT compared with that in IgG-treated nephritic kidneys. To the best of our knowledge, these observations are the first to prove that the specific downstream TGF-β signaling to the PAI-1 promoter mediated by Smad3 is activated in kidneys with chronic progressive glomerulonephritis and could be suppressed effectively by αT treatment. Considering these findings together with the increased nuclear localization of phosphorylated R-Smads, Smad2 and Smad3, in nephritic rat kidneys, it is plausible to consider that overactivation of TGF-β/Smad signaling plays a pathogenic role in the functional and structural derangement that develops in chronic progressive ATS nephritis. However, in the present study, we could not determine whether the binding of Smad2 to its specific target genes was activated similarly in the nephritic kidneys, because, in contrast to Smad3, Smad2 does not bind to DNA directly [33]. Further studies are needed to investigate whether Smad2 and Smad3 each has a different subset of target genes and thus regulates distinct gene expressions in the nephritic kidneys.

CONCLUSION

Our study demonstrated the following: (*1*) the expression of type I collagen, PAI-1, and TGF-β/Smad signaling components including TGF-β1, TGF-β2, TβRII, and Smad2; (*2*) nuclear localization of phosphorylated Smad2 and Smad3; and (*3*) binding of Smad3 to SBE in PAI-1 promoter, were increased in the renal cortical tissues of rats with chronic progressive ATS nephritis associated with progressive glomerulosclerosis and tubulointerstitial fibrosis; and *(4)* four-week αT treatment caused amelioration of chronic progressive ATS nephritis at 8 weeks, which was associated with decreases of intrarenal TGF-β/Smad signaling activity. These data suggest that strategies to down-regulate TGF-β/Smad signaling could provide a valuable adjunctive therapy in patients with chronic progressive glomerulonephritis who are destined for end-stage renal failure.

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REFERENCES

1. Border WA, Ruoslahti E: Transforming growth factor-beta in disease: The dark side of tissue repair. *J Clin Invest* 90:1–7, 1992
2. Eddy AA: Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol* 7:2495–2508, 1996
3. Derynck R, Zhang Y, Feng XH: Smads: Transcriptional activators of TGF-beta responses. *Cell* 95:737–740, 1998
4. Border WA, Okuda S, Languino LR, *et al*: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. *Nature* 346:371–374, 1990
5. Sharma K, Jin Y, Guo J, *et al*: Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45:522–530, 1996
6. Ziyadeh FN, Hoffman BB, Han DC, *et al*: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020, 2000
7. Miyajima A, Chen J, Lawrence C, *et al*: Antibody to transforming growth factor-beta ameliorates tubular apoptosis in unilateral ureteral obstruction. *Kidney Int* 58:2301–2313, 2000
8. Islam M, Burke JF, Jr, McGowan TA, *et al*: Effect of anti-transforming growth factor-beta antibodies in cyclosporine-induced renal dysfunction. *Kidney Int* 59:498–506, 2001
9. Ling H, Li X, Jha S, *et al*: Therapeutic role of TGF-beta-neutralizing antibody in mouse cyclosporin A nephropathy: Morphologic improvement associated with functional preservation. *J Am Soc Nephrol* 14:377–388, 2003
10. Akagi Y, Isaka Y, Arai M, *et al*: Inhibition of TGF-beta 1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 50:148–155, 1996
11. Border WA, Noble NA, Yamamoto T, *et al*: Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 360:361–364, 1992
12. Sharma K, Ziyadeh FN: The emerging role of transforming growth factor-beta in kidney diseases. *Am J Physiol* 266:F829–842, 1994
13. Yamamoto T, Noble NA, Miller DE, *et al*: Sustained expression of TGF-beta 1 underlies development of progressive kidney fibrosis. *Kidney Int* 45:916–927, 1994
14. Watanabe T, Yamamoto T, Ikegaya N, *et al*: Transforming growth factor-beta receptors in self-limited vs. chronic progressive nephritis in rats. *J Pathol* 198:397–406, 2002
15. Woessner JB: The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch Biochem Biophysics* 93:440–447, 1961
16. Austin HA, 3rd, Muenz LR, Joyce KM, *et al*: Prognostic factors in lupus nephritis. Contribution of renal histologic data. *Am J Med* 75:382–391, 1983
17. Lan HY, Mu W, Nikolic-Paterson DJ, *et al*: A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: Use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J Histochem Cytochem* 43:97–102, 1995
18. Morrissey JJ, Klahr S: Rapid communication. Enalapril decreases nuclear factor kappa B activation in the kidney with ureteral obstruction. *Kidney Int* 52:926–933, 1997
19. Dennler S, Itoh S, Vivien D, *et al*: Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J* 17:3091–3100, 1998
20. Martin M, Vozenin MC, Gault N, *et al*: Coactivation of AP-1 activity and TGF-beta1 gene expression in the stress response of normal skin cells to ionizing radiation. *Oncogene* 15:981–989, 1997
21. Chen SJ, Yuan W, Lo S, *et al*: Interaction of smad3 with a proximal smad-binding element of the human alpha2(I) procollagen gene promoter required for transcriptional activation by TGF-beta. *J Cell Physiol* 183:381–392, 2000
22. Stroschein SL, Wang W, Luo K: Cooperative binding of Smad proteins to two adjacent DNA elements in the plasminogen activator inhibitor-1 promoter mediates transforming growth factor beta-induced smad-dependent transcriptional activation. *J Biol Chem* 274:9431–9441, 1999
23. Massague J, Wotton D: Transcriptional control by the TGF-beta/Smad signaling system. *Embo J* 19:1745–1754, 2000
24. Heldin CH, Miyazono K, ten Dijke P: TGF-beta signaling from cell membrane to nucleus through SMAD proteins. *Nature* 390:465–471, 1997
25. Miyazono K: Positive and negative regulation of TGF-beta signaling. *J Cell Sci* 113:1101–1109, 2000
26. Cutroneo KR, Phan SH: TGF-beta1-induced Smad 3 binding to the Smad 7 gene: Knockout of Smad 7 gene transcription by sense phosphorothioate oligos, autoregulation, and effect on TGF-beta1 secretion: bleomycin acts through TGF-beta1. *J Cell Biochem* 89:474–483, 2003
27. Hosokawa R, Nonaka K, Morifuji M, *et al*: TGF-beta 3 decreases type I collagen and scarring after labioplasty. *J Dent Res* 82:558–564, 2003
28. Remuzzi G, Bertani T: Pathophysiology of progressive nephropathies. *N Engl J Med* 339:1448–1456, 1998
29. Morath C, Dechow C, Lehrke I, *et al*: Effects of retinoids on the TGF-beta system and extracellular matrix in experimental glomerulonephritis. *J Am Soc Nephrol* 12:2300–2309, 2001
30. Ostendorf T, Kunter U, van Roeyen C, *et al*: The effects of platelet-derived growth factor antagonism in experimental glomerulonephritis are independent of the transforming growth factor-beta system. *J Am Soc Nephrol* 13:658–667, 2002
31. Nagarajan RP, Zhang J, Li W, *et al*: Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *J Biol Chem* 274:33412–33418, 1999
32. von Gersdorff G, Susztak K, Rezvani F, *et al*: Smad3 and Smad4 mediate transcriptional activation of the human Smad7 promoter by transforming growth factor beta. *J Biol Chem* 275:11320–11326, 2000
33. Labbe E, Silvestri C, Hoodless PA, *et al*: Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol Cell* 2:109–120, 1998