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TGF-β isoforms in renal fibrogenesis

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Background. Transforming growth factor-β1 (TGF-β1) is generally considered to be the major or predominant isoform involved in fibrosis, with the roles of TGF-β2 and -β3 being less clear. Because anti-TGF-β–specific isoform treatment is in development, it is important to know more precisely about isoform action. Here we compared the actions of each isoform on production and degradation of extracellular matrix proteins by cultured rat mesangial cells, renal fibroblasts, and tubular epithelial cells. We investigated endogenous production of each isoform, the effect of adding one isoform on the production of the other isoforms, and the response to addition of isoform combinations on matrix protein production. Isoform-specific antibodies were used to determine the relative contribution of these isoforms to matrix protein production.

Methods. Each cell type was treated with TGF-β (0.01 to 10 ng/mL) alone or in different combinations. Living cell number was determined by 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay. Supernatant fibronectin and TGF-β isoform concentration were measured by enzyme-linked immunosorbent assay (ELISA). Collagen and proteoglycan production were measured by [^{3}H]-proline and [^{35}S]-sulfate incorporation, respectively. Matrix protein and TGF-β isoform gene expression were determined by Northern blot. Release of ^{3}H from preformed radiolabeled matrix by fibroblasts was used as a measure of matrix degradation.

Results. Each isoform increased matrix protein synthesis and reduced matrix degradation by renal cells similarly. Combination of TGF-β isoforms showed additive effects. No antifibrotic effect was observed with TGF-β3. TGF-β1 increased -β2 and -β3 production in a small and inconsistent manner. In contrast, TGF-β2 and -β3 stimulated TGF-β1 in all three cell types. Eighty percent of TGF-β3's fibrogenic effect was mediated by TGF-β1. A pan-specific antibody to TGF-β most effectively blocked plasminogen activator inhibitor type 1 (PAI-1) synthesis by epithelial cells under oxidative stress.

Conclusion. All three TGF-β isoforms have fibrogenic effects on renal cells. TGF-β2 and TGF-β3 effects may be partially mediated by TGF-β1. These data suggest that blockade of all isoforms together may yield the best therapeutic effect in reducing renal fibrosis.

Key words: fibrosis, TGF-β, isoform, kidney.

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Transforming growth factor-β1 (TGF-β1) is a key mediator of fibrosis. The original observation that TGF-β1 overexpression is involved in fibrosis in an acute model of mesangioproliferative glomerulonephritis has been confirmed in many fibrotic disorders of the kidney and other organs [1, 2]. There has been much emphasis on blockade of TGF-β as a therapeutic strategy [3]. However, TGF-β1 overexpression does not dominate all fibrogenic processes. TGF-β2 has been reported to be dominant in the acute phase of nephropathy in streptozotocin (STZ)-induced type I diabetes in the rat [4]. Subsequently, administration of neutralizing antibody to TGF-β2 during the acute phase of diabetes in the STZ rat significantly reduced renal fibrosis [5]. TGF-β2 is also the predominant isoform in ocular scarring and again, TGF-β2 antibody therapy reduces scarring following glaucoma surgery [6–8]. However, in general, compared with TGF-β1, the roles of TGF-β2 and TGF-β3 in fibrosis are not precisely known.

While much is known about developmental expression of TGF-β isoforms, differences in the three isoforms in terms of fibrogenesis is less well studied. The limited publications in this field generally show similar profibrotic effects of all three isoforms [9, 10]. However, controversial results have been reported for TGF-β3. Shah et al [11] found that TGF-β3 applied to dermal wounds had antifibrotic effects. Later, Murata et al [12] observed that the increases in α1(I) procollagen and TGF-β1 mRNA levels induced by TGF-β1 were decreased when both TGF-β3 and TGF-β1 were added to dermal fibroblasts. These studies led us to ask whether we could demonstrate an antifibrotic effect of TGF-β3 on extracellular matrix production in renal cells. We also provided new data of relevance to renal fibrosis on isoform actions and interactions in terms of TGF-β effects on matrix protein synthesis and degradation.

METHODS

Materials

Recombinant human TGF-β1, -β2, and -β3 were obtained from R&D Systems, Inc. (Minneapolis, MN, USA)

and reconstituted with sterile 4 mmol HCl containing 1mg/mL bovine serum albumin (BSA).

TGF-β isoform-specific neutralization antibodies were also obtained from R&D Systems, including anti-TGF-β1 antibody (AF-101-NA); anti-TGF-β2 antibody (AF-302-NA); and anti-TGF-β3 antibody (AF-243-NA). Pan-specific TGF-β antibody, 1D11, which blocks isoforms TGF-β1, -β2, and -β3 was kindly provided by Genzyme (Cambridge, MA, USA).

Unless otherwise indicated, materials, chemicals, or culture media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

Rat mesangial cells (RMC) were obtained from intact glomeruli of four- to six-week-old Sprague-Dawley rats and characterized according to published methods [1, 13, 14]. Cells were used between passages 6 and 9 and were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratory, Logan, UT, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 U/mL insulin, 25 mmol N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer at 37°C in a 5% CO_2 incubator.

Rat tubular epithelial cells (NRK-52E) and renal fibroblasts (NRK-49F) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS at 37°C in a 5% CO_2 incubator.

Subconfluent mesangial cells and confluent renal fibroblast and tubular cells seeded on 6-well plates were made quiescent by serum-free medium (containing 1 mg/mL BSA) for 24 hours. For matrix protein measurements, TGF-β isoforms were added to cell cultures in doses from 0.01 to 10 ng/mL and incubated for 24 hours (RMC, NRK-49F) or 72 hours (NRK-52E).

In order to study the effects of combining TGF-β isoforms, equal amounts of two isoforms were added together to cells in total doses from 0.01 to 10 ng/mL and compared with the effect of the same dose of one isoform alone. In addition, 1 ng/mL of one isoform was combined with different dosages of other isoforms to see if different ratios produced different effects. Finally, to simulate the in vivo situation in which TGF-β1 is present early, and TGF-β3 later, in tissue injury, renal fibroblasts were pretreated with TGF-β1 (0∼5 ng/mL) for 16 hours, and then TGF-β3 (0∼5 ng/mL) was added for another 24 hours.

MTT assays

The number of living cells was determined by 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) assay based on the activities of mitochondrial enzymes in viable cells. At the termination of the experiment, all media were replaced with serum-free medium containing 1 mg/mL MTT for 4 hours at 37°C. After aspirating the MTT solution, the purple formazan reaction product was extracted from the cells with acidified isopropanol for 10 minutes by shaking, and then the optical density was read on a ThermoMax microplate scanning spectrophotometer (Molecular Devices Corporation, Menlo Park, CA, USA) at 562 nm with background subtraction at 650 nm.

Collagen assay

Collagen synthesis was measured by the incorporation of [^{3}H]-proline into collagen. Renal fibroblasts were cultured and treated with TGF-β isoforms as described above. 1 μCi/mL L-[2,3,4,5-^{3}H]-proline (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 50 μg/mL ascorbic acid was added in the last 24 hours. Cells were solubilized with 2 mL 0.5 N NaOH and collected with medium into 4 mL 0.5 mol Tris-HCl, pH 7.4, containing 0.11 mol NaCl, 1 mmol unlabeled proline, and 1 mg/mL albumin. Proteins were precipitated for 30 minutes with 8 mL ice-cold 20% trichloroacetic acid (TCA), and the precipitates were collected after centrifugation for 15 minutes at 3000*g*. The supernatant was removed, and the precipitate was dissolved and washed twice in 10 mL of ice-cold 10% TCA. The resultant precipitate was dissolved in 0.5 mL 0.2 N NaOH. Duplicate 0.2 mL aliquots were neutralized by 0.16 mL 0.15 mol HCl and 0.1 mL 1 mol HEPES/Tris buffer, pH 7.3, and added with 10 μL 25 mmol $CaCl_2$, 20 μL 62.5 mmol N-ethylmaleimide and 20 μL elution buffer (50 mmol Tris-HCl, pH 7.4, 5 mmol $CaCl_2$) or 20 μL elution buffer containing purified collagenase (600 U/mL). After a 120 minute incubation period at 37°C with or without collagenase, the samples were placed on ice and precipitated with 0.5 mL ice-cold 10% TCA plus 0.5% tannic acid. Acid-insoluble material (noncollagen protein) was pelleted by centrifugation. The supernatants were counted for β emission. The amount of radioactivity solubilized by collagenase was subtracted from the radioactivity of tubes without collagenase (nonspecific digest) as a measure of [^{3}H]-proline incorporated into collagen. All counts were corrected for cell number [15–17].

Measurement of proteoglycan production by metabolic labeling of cells

This assay was performed as previously described [18]. Briefly, rat mesangial cells were cultured and treated with TGF-β isoforms as described above. [^{35}S]-sulfate (200 μCi/mL) (NEN, Boston, MA, USA) was added in the last 18 hours. At the end of experiments, supernatants were removed and analyzed by electrophoresis on 10% Tris-Glycine gels (Invitrogen, Carlsbad, CA, USA) under reducing conditions. ^{14}C protein molecular weight marker was obtained from NEN. Gels were dried and exposed to x-ray films for 72 to 96 hours. Autoradio-

graphs were scanned on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to compare and quantitate the relative intensities of proteoglycan bands.

Measurement of fibronectin, TGF-β1, TGF-β2, and TGF-β3 in culture supernatants

Fibronectin synthesis was measured with modified enzyme-linked immunosorbent assay (ELISA) according to published methods [19]. For TGF-β isoform ELISAs, all supernatant samples were acid-activated. TGF-β1 was measured with a commercially available sandwich ELISA kit (DuoSet®, R&D Systems, Inc.) according to the manufacturer's instructions. TGF-β2 sandwich ELISA system was set up using MAB612 (R&D Systems, Inc.) as capture antibody and BAF302 (R&D Systems, Inc.) as detection antibody. Similarly, the TGF-β3 ELISA used MAB643 (R&D Systems, Inc.) as capture antibody and BAF243 (R&D Systems, Inc.) as detection antibody.

Measurement of extracellular matrix degradation by cultured renal fibroblast

Production of radioactive matrices. Renal fibroblasts were seeded into 6-well plates at 5×10^4 cells/well. One day later the culture medium was changed to DMEM supplemented with 5% FBS, 50 μg/mL ascorbic acid, and 1 μCi/mL L-[2,3,4,5-^{3}H]-proline and changed daily thereafter. One week after seeding of the cells the cultures were washed with phosphate-buffered saline (PBS). Cells were lysed by the addition of 2 mL 25 mmol NH_4OH to each well for 30 minutes at 37°C. The matrices left in each well were washed carefully with PBS twice and kept covered with sterile water at 4°C until further use.

Degradation of the matrices by renal fibroblasts. Renal fibroblasts were seeded onto labeled matrices at 5×10^4 cells/well. When cells grew to confluence, the medium was carefully removed and the cells were washed with serum-free DMEM medium and then incubated in 2 mL DMEM with 5% FBS and different doses of TGF-β isoforms for 48 hours. At the end of the experiment, supernatants were collected. To release undigested radioactivity from the matrix, 2 mL 2N NaOH were added to each well and plates were incubated at 37°C for 18 hours. Aliquots (50 μL) from both samples were counted and results were expressed as the percentage of radioactivity released into supernatants. Background values obtained with medium placed on radiolabeled matrix in the absence of cells were subtracted from these values [20].

PAI-1 Western blot

Growth-arrested tubular epithelial cells were incubated in serum-free medium with 0.1 mmol H_2O_2 and different TGF-β antibodies (AF-101-NA 1 μg/mL; AF-302-NA 1 μg/mL; AF-243-NA 1 μg/mL; and 1D11 10 μg/mL). The antibody dose was selected based on the neutralization dose$_{50}$ for each isoform and was sufficient to block the corresponding isoform. Twenty-four hours later, supernatants were collected for Western blot and cells were used for MTT assays. Supernatants (40 μL) were separated by 10% Tris-glycine gel electrophoresis and transferred to a 0.45 μm nitrocellulose membrane (Millipore, Bedford, MA, USA). Nonspecific binding was blocked by 10% non-fat milk powder in Tris-buffered saline (TBS) for 1 hour at room temperature, followed by 4°C overnight incubation with primary antibody [stock solution: 250 μg/mL, rabbit-anti-rat plasminogen activator inhibitor type 1 (PAI-1) immunoglobulin G (IgG) (American Diagnostica, Inc., Greenwich, CT, USA) diluted 1:200 in 5% BSA in TBS/0.1%Tween-20 with 0.02% NaN_3]. The blot was washed three times for 10 minutes in TBS/0.1%Tween-20. The second antibody, goat anti-rabbit horseradish peroxidase (stock solution: 400 μg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), was incubated at a dilution of 1:5000 for an additional hour at room temperature, followed by three washes as described above. Bound antibodies were detected by developing the blot in enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech) for 1 minute. Quantitation of the bands on autoradiograms was performed using a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Inc.).

RNA preparation and Northern hybridization

For Northern blots, renal fibroblasts and rat mesangial cells were treated with TGF-β isoforms for 8 hours and tubular epithelial cells for 48 hours. Total RNA was extracted by a guanidinium isothiocyanate method using Trizol® reagent according to the manufacturer's instructions. For Northern analysis, RNA was denatured and fractionated by electrophoresis through a 1.0% agarose gel (20 μg/lane) and transferred to a nylon membrane (BrightStar™ Plus, Ambion, Inc., Austin, TX, USA). Nucleic acids were immobilized by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). Membranes were prehybridized with ULTRAhyb™ buffer (Ambion, Inc.) and hybridized with DNA probes labeled with ^{32}P-dATP by random oligonucleotide priming (Strip-EZ DNA™, Ambion, Inc.). The blots were washed once for 10 minutes in 2 X SSC, 0.1% SDS at room temperature and twice for 15 minutes in 0.1 X standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 42°C. DNA probes used were rat GAPDH cDNA (kindly provided by M.B. Sporn) [21]; rat procollagen α_1 cDNA (kindly provided by Dr. D. Rowe) [22]; fibronectin-EDA cDNA (kindly provided by Dr. R.O. Hynes) [23]; TGF-β1 and -β2 cDNA (kindly provided by Dr. H.L. Moses) [24, 25]; and TGF-β3 (ATCC). Three blots per probe were performed. Autoradiographic signals obtained with the

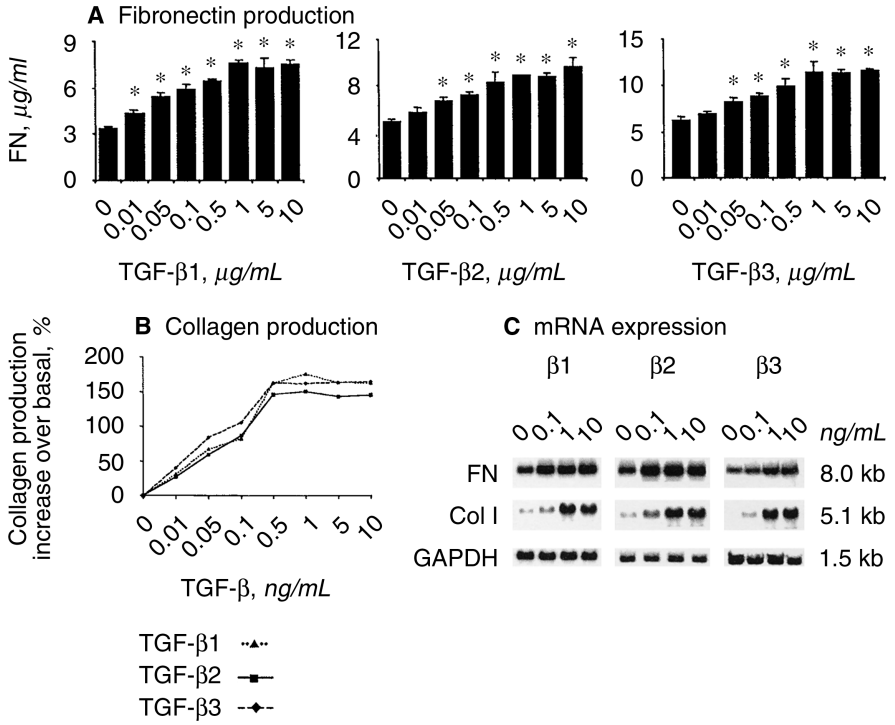

Fig. 1. Effects of transforming growth factor (TGF)-β1, -β2, and -β3 on production of extracellular matrix (ECM) proteins by renal fibroblasts. (*A*) Production of fibronectin, (*B*) collagen, and (*C*) mRNA expression. $^*P <$ 0.05 vs. control.

GAPDH cDNA probe served as control for equal loading of the gel. Autoradiographs were scanned on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Inc.). Changes in mRNA levels were determined by first correcting for the densitometric intensity of GAPDH for each sample. For comparison, this ratio was set at unity for normal control samples and other lanes on the same gel were expressed as fold-increase over this value.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis of differences between the groups was performed by analysis of variance (ANOVA) and subsequent Student-Newman-Keuls or Dunnett testing for multiple comparisons. Comparisons with a P value <0.05 were considered significantly different. Triplicate wells were analyzed for each experiment, and each experiment was performed independently a minimum of three times.

RESULTS

TGF-β isoforms -β1, -β2, and -β3 stimulate extracellular matrix protein production equally

Renal fibroblasts. Treatment of renal fibroblasts with increasing concentrations of TGF-β did not significantly alter the number of living cells (data not shown). All isoforms increased production of the matrix proteins fibronectin (Fig. 1A) and collagen I (Fig. 1B) up to 200% in a dose-dependent manner. The ED_{50} of this profibrotic effect is approximately 0.05 ng/mL, and doses higher than 1 ng/mL did not further increase matrix protein production. These protein changes were consistent with mRNA expression (Fig. 1C). No differences were found among the three isoforms.

Tubular epithelial cells. TGF-β isoforms did not alter cell number (data not shown). Compared with fibroblasts, which responded to TGF-β isoforms within 24 hours, the epithelial cell response was slow, showing no obvious increase in fibronectin production until 72 hours of incubation (data not shown). Measured at 72 hours, each isoform increased fibronectin production in a dose-dependent manner (Fig. 2A). At the mRNA level, all TGF-β isoforms increased collagen I expression to about the same extent as fibronectin expression (Fig. 2B).

Rat mesangial cells. Because the collagen and fibronectin response of mesangial cells to TGF-β was small (data not shown), we measured changes in proteoglycan synthesis. Proteoglycan production by mesangial cells was significantly increased at the low dose (0.1 ng/mL) of each TGF-β isoform, reaching a maximum 3-fold increase at 0.1 or 1.0 ng/mL (Fig. 3). Treatment of mesangial cells with each isoform at 10 ng/mL caused a decrease in proteoglycan production compared to that seen at lower TGF-β concentrations (Fig. 3).

TGF-β isoforms -β1, -β2, and -β3 similarly reduce extracellular matrix protein degradation. Renal fibroblasts cultured on renal fibroblast-produced radiolabeled ma-

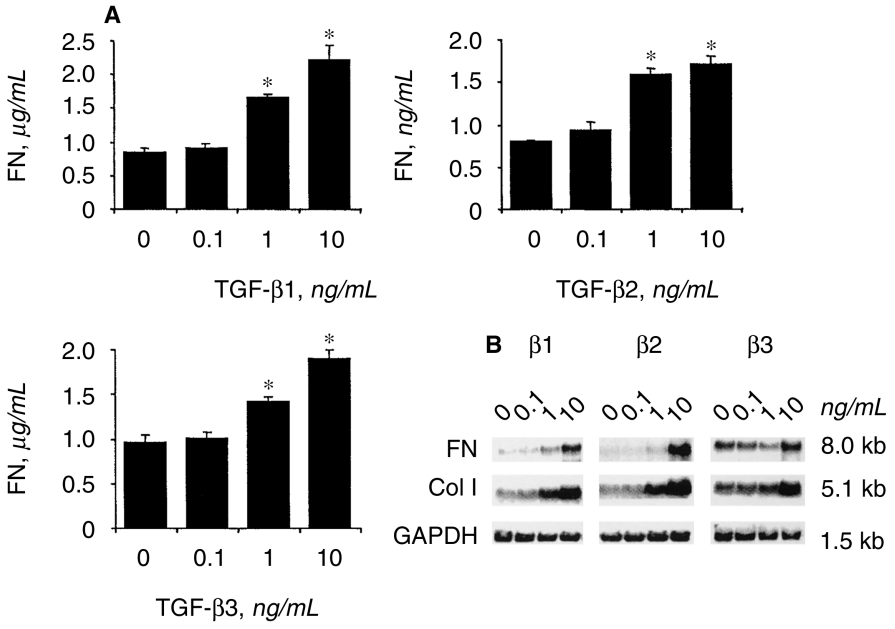

Fig. 2. Effects of transforming growth factor (TGF)-β1, -β2, and -β3 on production of extracellular matrix (ECM) proteins by tubular epithelial cells. (*A*) Production of fibronectin and (*B*) mRNA expression. *$P < 0.05$ vs. control.

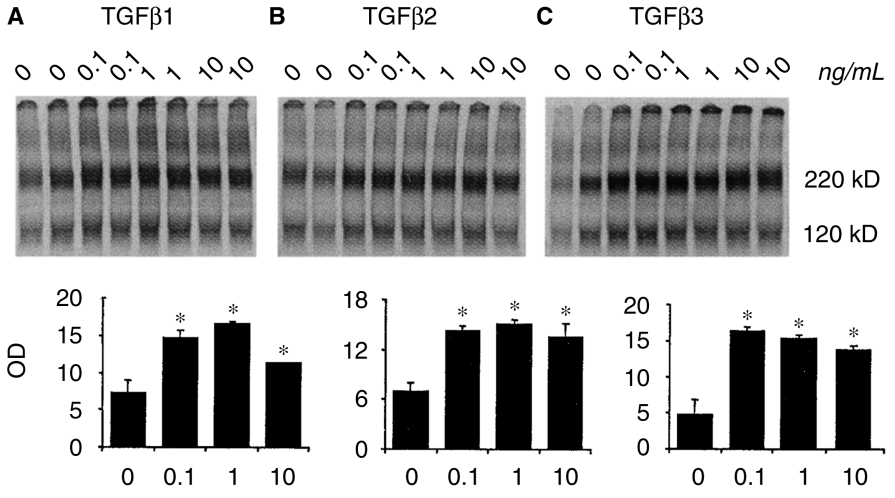

Fig. 3. Effects of transforming growth factor (TGF)-β1, -β2, and -β3 on proteoglycan production by renal mesangial cells. (*A*) Effects of TGF-β1, (*B*) TGF-β2, and (*C*) TGF-β3. Proteoglycan production by mesangial cells was increased dose dependently up to 3-fold the control level. *$P < 0.05$ vs. control.

trix degraded the preexisting matrix proteins and released ^{3}H into supernatant. Exogenously added TGF-β dose dependently inhibited extracellular matrix (ECM) degradation. As shown in Figure 4, TGF-β1 and -β3 reduced matrix degradation approximately 75% at 10 ng/mL TGF-β, while TGF-β2 reduced matrix degradation less, reaching approximately 60% reduction at 10 ng/mL TGF-β2.

Combination of TGF-β isoforms shows additivity in their profibrotic action. We next asked whether we could detect any antifibrotic effect of TGF-β3 on renal cells as has been reported in dermal scarring and in dermal fibroblasts [11, 12]. We compared collagen production by renal fibroblasts stimulated with TGF-β1 or -β3 alone with collagen production stimulated with a mixture of

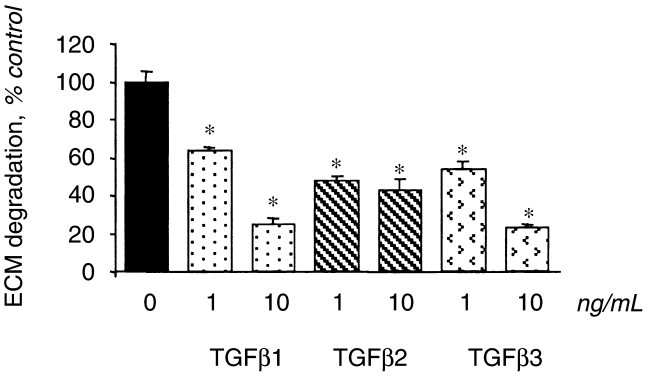

Fig. 4. Effects of transforming growth factor (TGF)-β1, -β2, and -β3 on matrix degradation. * $P < 0.05$ vs. control.

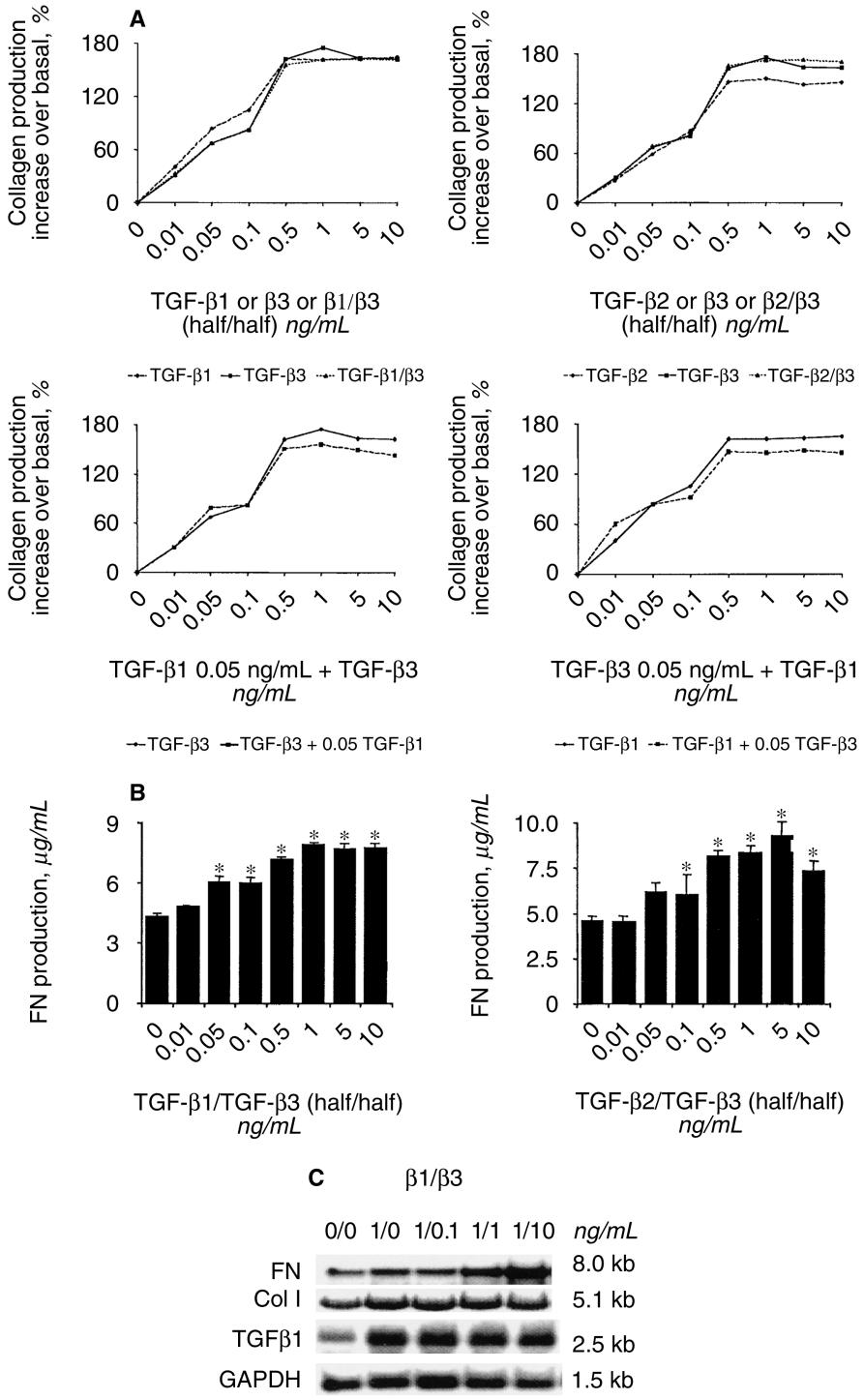

Fig. 5. Additive effects of combinations of transforming growth factor-β (TGF-β) isoforms on matrix protein production by renal fibroblasts. (*A*) Collagen production in response to 50%/50% TGF-β1 and -β3 (top left), 50%/50% TGF-β2 and -β3 (top right), 0.05 ng/mL TGF-β1 combined with different amounts of TGF-β3 (bottom left) and 0.05 ng/mL TGF-β3 combined with different amounts of TGF-β1 (bottom Right). (*B*) Fibronectin production in response to 50%/50% TGF-β1 and -β3 (left) and 50%/50% TGF-β2 and -β3 (right). (*C*) TGF-β1 (1 ng/mL) combined with different doses of TGF-β3 was added to fibroblasts for 6 hours.

half -β1 and half -β3. Similarly, collagen production in response to TGF-β2 or TGF-β3 added alone or in 1:1 combination was measured. The results, shown in the top left and right panels of Figure 5A, indicate that the collagen response of renal fibroblasts depends on the total TGF-β concentration added, and not on which isoform is added. This result is seen over a wide range of total TGF-β concentrations from 0.01 to 10 ng/mL. Similarly, if a constant amount of TGF-β1 or TGF-β3 (0.05 ng/mL) is added with varying amounts of TGF-β3 or TGF-β1 (0 to 10 ng/mL), the collagen produced again is extremely similar over a wide range of TGF-β concentrations, as shown in the bottom panels of Figure 5A. Figure 5B shows the results of fibronectin production by renal fi-

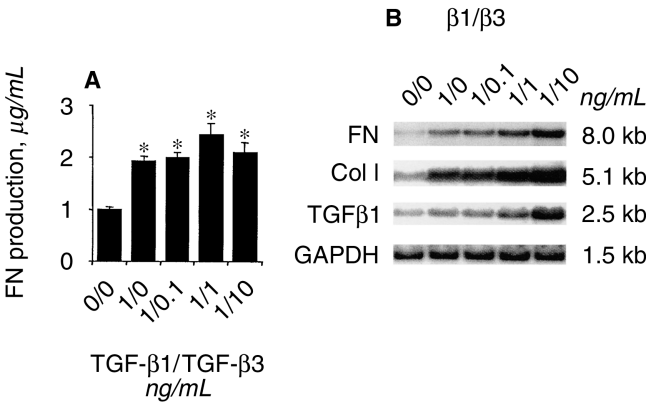

Fig. 6. Additive effects of combinations of transforming growth factor-β (TGF-β) isoforms on matrix protein production by tubular cells. TGF-β1 (1 ng/mL) was combined with different doses of TGF-β3 and added to renal tubular epithelial cells. (*A*) Fibronectin production during 72-hour treatment. (*B*) TGF-β and matrix protein mRNA expression after 48-hour treatment.

broblasts in response to total TGF-β concentrations from 0 to 10 ng/mL added as 1:1 TGF-β1 and TGF-β3 (Fig. 5B, left) or 1:1 TGF-β2 and TGF-β3 (Fig. 5B, right). One can readily see, when comparing Figures 1A and Figure 5B, that fibronectin production is very similar, whether the total TGF-β is composed of one or two isoforms. Figure 5C shows the renal fibroblast mRNA levels in response to constant -β1 levels and increasing -β3 levels. Similar data are shown for fibronectin production by renal epithelial cells in response to constant TGF-β1 levels and increasing TGF-β3 levels (Fig. 6A). Levels of mRNA are shown in Figure 6B. When 1 ng/mL TGF-β1 is added, fibronectin production and mRNA levels increase (Figure 6A, second bar; Fig. 6B, second lane). As shown in the third and fourth bars of Figure 6A and the third and fourth lanes of Figure 6B, when 0.1 or 1.0 ng/mL TGF-β3 is added to this 1.0 ng/mL TGF-β1, both fibronectin protein and mRNA levels continue to increase as the total dose of TGF-β increases. Also, rat mesangial cells in response to addition of 1 ng/mL TGF-β1, -β2, or -β3 show a marked increase in proteoglycan production (Figure 7 A, B, and C, respectively, 2 left bars). When TGF-β3 is added to either -β1 (Fig. 7A) or -β2 (Fig. 7B), or when TGF-β1 is added to -β3 (Fig. 7C), a further increase in proteoglycan production is seen. Thus, for all three of the renal cell types shown in Figures 5, 6, and 7, no evidence was found suggesting that addition of TGF-β3 in the presence of another isoform leads to decreased matrix protein production.

The final investigation of the possible antifibrotic effect of TGF-β3 on renal cells involved addition of TGF-β1 to renal fibroblasts for 40 hours. During the last 24 hours, different doses of TGF-β3 from 0 to 5 ng/mL were added in an attempt to mimic the in vivo situation of early expression of TGF-β1 and later up-regulation of TGF-β3. When renal fibroblasts were pretreated with TGF-β1, collagen production was increased dose dependently (Fig. 8). Later addition of TGF-β3 resulted in an obvious further dose-dependent increase in collagen production when the preincubation concentration of TGF-β1 was low (0.05 ng/mL). No further increase and no decrease were seen when TGF-β1 (5 ng/mL) pretreatment had already maximized collagen synthesis (Fig. 8).

Interactions among TGF-β isoforms in renal cells. We next studied the unstimulated production of TGF-β isoforms in the three cell types and the ability of one isoform to increase the production of the other two. As a first step we determined the specificity of the TGF-β isoform ELISAs. The results, shown in Table 1, indicate very limited cross reactivity that was seen only at the highest concentration. The unstimulated, endogenous production of TGF-β1, TGF-β2, and TGF-β3 in the three cell types is shown in Table 2. In renal fibroblasts and tubular epithelial cells, TGF-β1 predominated, while rat mesangial cells produced each isoform at a similar level. Tubular epithelial cells produced a large amount of TGF-β1 in 72 hours compared with that produced by renal fibroblasts and mesangial cells in 24 hours (Table 2). TGF-β2 was moderately expressed in tubular epithelial cells and mesangial cells and was barely detectable in renal fibroblasts (Table 2). TGF-β3 was not detected in renal fibroblasts and was moderately produced in both epithelial cells and in mesangial cells (Table 2).

Production of TGF-β1, TGF-β2, or TGF-β3 in response to stimulation by the other two isoforms is shown in rows 2-4 of Table 2, respectively. Corresponding Northern blots are shown in Figure 9. TGF-β2 and -β3 stimulated TGF-β1 production two-fold in renal fibroblasts. Tubular epithelial cells, with high endogenous production of TGF-β1, were less responsive to exogenous TGF-β. Addition of 1 ng/mL TGF-β2 or -β3 resulted in a small increase in TGF-β1 protein and no detectable increase in mRNA. At the highest dose of -β2 or -β3, TGF-β1 production was increased 25% at the protein level and considerably more at the mRNA level. Mesangial cells showed two-fold, dose-dependent increases in TGF-β1 mRNA and protein in response to TGF-β2 and TGF-β3 (Table 2 and Fig. 9).

Table 2 shows that TGF-β2 production in fibroblasts was stimulated almost three-fold by both TGF-β1 and -β3, although -β2 levels remained low. TGF-β3 remained undetectable in renal fibroblasts (Table 2). In addition, stimulation of epithelial cells or mesangial cells with TGF-β1 or TGF-β3 had little effect on production of either TGF-β2 or TGF-β3. Similarly, stimulation of epithelial cells or mesangial cells with TGF-β3 or TGF-β2 had no effect on production of TGF-β2 or TGF-β3, respectively (Table 2).

We next investigated the ability of antibodies to TGF-β1

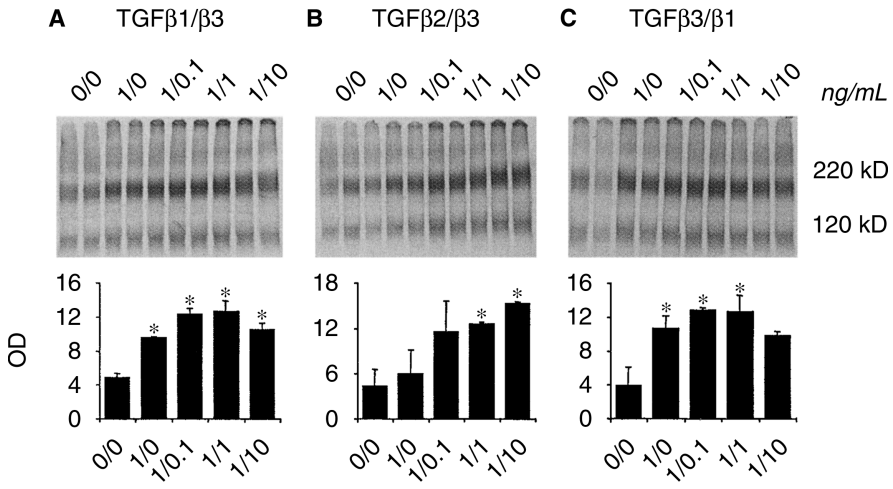

Fig. 7. Additive effects of combinations of transforming growth factor-β (TGF-β) isoforms on matrix protein production by mesangial cells. (*A*) Mesangial cells were treated with 1 ng/mL TGF-β1 combined with different doses of TGF-β3 for 24 hours, (*B*) TGF-β2 (1 ng/mL) combined with different doses of TGF-β3, (*C*) TGF-β3 (1 ng/mL) combined with different doses of TGF-β1. $*P < 0.05$ vs. control.

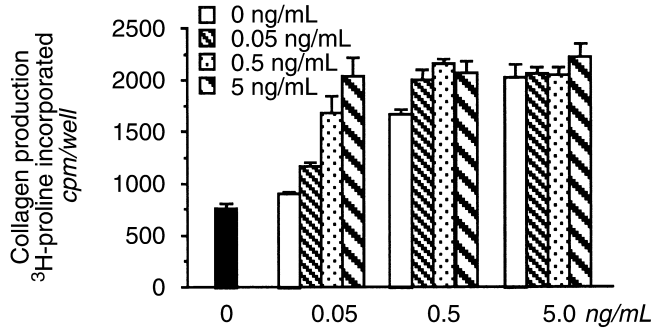

Fig. 8. Effects of addition of transforming growth factor (TGF)-β3 16 hours after addition of TGF-β1 on collagen production by renal fibroblasts. Open bars show a dose-dependent increase in collagen as preincubation TGF-β1 increases. Taking each set of bars, the data show that at low TGF-β1 concentration (0.05 ng/mL), later addition of TGF-β3 resulted in a dose-dependent increase in collagen synthesis. As the preincubation concentration of TGF-β1 increased, later addition of TGF-β3 resulted in smaller or no increase in collagen production.

Table 1. Cross-reactivity of TGF-β isoform-specific enzyme-linked immunosorbent assays (ELISAs)

Isoform added	Concentration tested *ng/mL*	Observed value *pg/mL*	Cross-reactivity %
A TGF-β2	1.0	0	0
	10.0	21.1	0.21
TGF-β3	1.0	0	0
	10.0	43.1	0.43
B TGF-β1	1.0	0	0
	10.0	32.1	0.32
TGF-β3	1.0	0	0
	10.0	12.7	0.13
C TGF-β1	1.0	0	0
	10.0	13.2	0.13
TGF-β2	1.0	0	0
	10.0	0	0

Row A shows TGF-β1 ELISA values with TGF-β2 and TGF-β3; row B shows TGF-β2 ELISA values with TGF-β1 and TGF-β3; row C shows TGF-β3 ELISA values with TGF-β1 and TGF-β2.

or TGF-β3 to reduce the actions of these isoforms to increase fibronectin production. The specificities of the TGF-β isoform antibodies used in this study were tested by R&D Systems, Inc. Cross-reactivity is less than 2% for anti-TGF-β1 and anti-TGF-β2, and less than 10% for anti-TGF-β3. The results are shown in Figure 10. The first two bars of each graph in figure 10 show that addition of either anti-TGF-β1 or anti-TGF-β3 had no effect on endogenous fibronectin production by either renal fibroblasts (Fig. 10 A and C) or tubular epithelial cells (Fig. 10 B and D). This suggests that baseline fibronectin production in these cells is not dependent upon exogenous TGF-β1 or -β3.

Comparing the first and third bars of each graph in Figure 10, one can see the TGF-β3- and TGF-β1–induced increases in fibronectin production. As expected, increasing doses of the antibody corresponding to the isoform added block the increase in fibronectin production (Fig. 10, third through fifth bars of each graph). The last two bars of each graph show a different story. If fibronectin production is increased by addition of TGF-β1, anti-TGF-β3 cannot block this effect in fibroblasts. Since TGF-β3 was not detected in renal fibroblasts this is expected (Fig. 10A, right two bars). However, anti-TGF-β3 also did not reduce fibronectin production by epithelial cells (Fig. 10B, right two bars). This suggests that the TGF-β1–induced increase in fibronectin in epithelial cells is not mediated at all by production of TGF-β3. (It also suggests that the limited reactivity between TGF-β1 and this anti-β3 antibody can be ignored in this experiment.) In contrast, if fibronectin production is induced with TGF-β3, addition of anti-TGF-β1 blocks the observed increase in fibronectin production 77% and 86%, respectively, in fibroblasts and epithelial cells (Fig. 10 C and D, right two bars). This strongly suggests that a large component of the action of TGF-β3 to increase fibronectin synthesis is mediated by production of TGF-β1.

Table 2. Effects of TGF-β isoforms -β1, -β2 and -β3 on production of TGF-β isoforms by renal cells

A	TGF-β1 (*pg/mL*) produced in response to TGF-β2 or TGF-β3					
Dose	TGF-β2			TGF-β3		
ng/mL	NRK-49F	NRK-52E	RMC	NRK-49F	NRK-52E	RMC
0	130 ± 3.7	1307 ± 37	54.8 ± 3.1	157 ± 3.7	1387 ± 29	54.8 ± 5.4
0.1	193 ± 2.0[a]	1302 ± 43	93.0 ± 9.5[a]	230 ± 2.2[a]	1343 ± 27	99.2 ± 4.6[a]
1	245 ± 4.4[a]	1520 ± 12[a]	106 ± 5.5[a]	269 ± 15[a]	1526 ± 17[a]	114 ± 2.0[a]
10	248 ± 10[a]	1729 ± 50[a]	118 ± 2.5[a]	270 ± 6.4[a]	1733 ± 68[a]	129 ± 5.4[a]
B	TGF-β2 (*pg/mL*) produced in response to TGF-β1 or TGF-β3					
Dose	TGF-β1			TGF-β3		
ng/mL	NRK-49F	NRK-52E	RMC	NRK-49F	NRK-52E	RMC
0	24.0 ± 9.5	170 ± 3.7	113 ± 4.4	22.4 ± 3.3	179 ± 7.3	113 ± 4.4
0.1	47.1 ± 5.4	188 ± 17	119 ± 8.3	24.2 ± 1.6	177 ± 6.1	115 ± 8.3
1	62.5 ± 0.6[a]	188 ± 12	112 ± 7.7	64.3 ± 6.1[a]	209 ± 7.0[a]	113 ± 2.5
10	64.4 ± 6.3[a]	218 ± 6.0[a]	116 ± 4.6	63.0 ± 7.2[a]	242 ± 2.3[a]	110 ± 3.0
C	TGF-β3 (*pg/mL*) produced in response to TGF-β1 or TGF-β2					
Dose	TGF-β1			TGF-β2		
ng/mL	NRK-49F	NRK-52E	RMC	NRK-49F	NRK-52E	RMC
0	0	140 ± 5.2	174 ± 23	0	126 ± 10	174 ± 23
0.1	0	136 ± 5.3	202 ± 6.2	0	133 ± 4.7	189 ± 12
1	0	122 ± 10	195 ± 15	0	111 ± 5.3	186 ± 15
10	0	139 ± 4.4	190 ± 8.4	0	118 ± 11	190 ± 16

[a] $P < 0.05$ vs. control

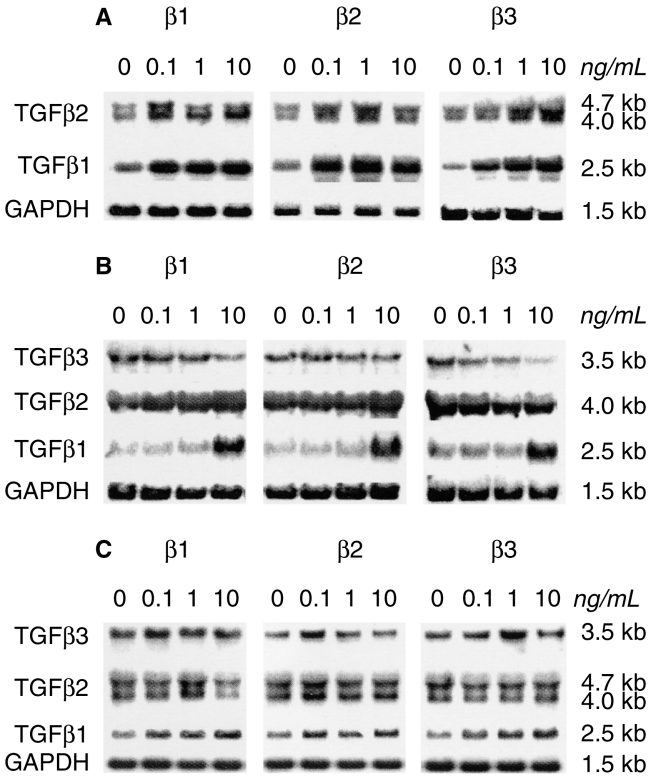

Fig. 9. Effects of transforming growth factor-β (TGF)-β isoforms -β1, -β2, and -β3 on isoform mRNA expression. (*A*) Renal fibroblasts, (*B*) tubular epithelial cells, (*C*) mesangial cells.

Blockade of TGF-β isoforms in "fibrotic" tubular epithelial cells. Finally, we attempted to mimic an in vivo fibrotic situation using oxidative stress and isoform-specific antibodies to determine the effectiveness of blockade of TGF-β isoforms on production of the marker of fibrosis, PAI-1. As seen in Figure 11 A and B, the addition of hydrogen peroxide (0.1 mmol) increased TGF-β1 and TGF-β2 production. The 24-hour treatment period yielded TGF-β3 levels that were too low to be detected by ELISA (Fig. 11). Based on absolute levels of TGF-β produced by peroxide stimulation, TGF-β1 appears to be the main isoform involved. The first two bars from the left in Figure 11C show the very large, 22-fold increase in PAI-1 protein in response to peroxide. Large concentrations of isoform-specific antibodies were then added to peroxide-treated cells. As seen in the fourth to sixth bars of Figure 11C, all three isoform-specific antibodies yielded reductions in PAI-1 production. The reduction reached significance only for anti-TGF-β1. The last bar of Figure 11C shows that a combination of all three isoform-specific antibodies was more effective than any isoform-specific antibody alone. The pan-specific mouse monoclonal antibody 1D11, shown in the third bar of Figure 11C, was most effective at reducing the peroxide-induced increases in PAI-1 production. If one uses the difference in PAI-1 production between control and peroxide-stimulated cells as 100%, PAI-1 production was reduced 29% by anti-β1, 13% by anti-β2, 16% by anti-β3, 36% by a combination of the three isoform-specific antibodies, and 44% by the pan-specific antibody, 1D11. These results indicate that blockade of all three isoforms is most effec-

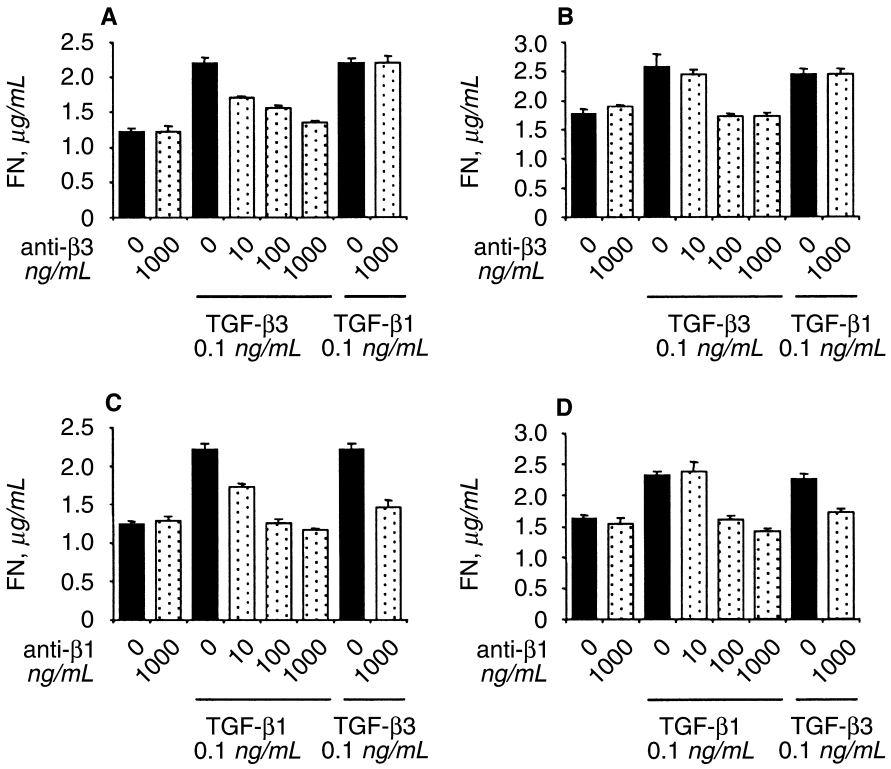

Fig. 10. Effects of anti-transforming growth factor-β (TGF)-β1 and anti-TGF-β3 on fibronectin production. (*A* and *C*) Renal fibroblasts. (*B* and *D*) Epithelial cells. TGF-β3 antibody dose dependently neutralized TGF-β3 effect. Blockade of TGF-β3 had no effect on TGF-β1's effect (A, B). TGF-β1 antibody dose dependently neutralized TGF-β1's effect. Blockade of TGF-β1 reduced TGF-β3's effect on fibronectin production by 76.7% in fibroblasts (C) and 85.6% in epithelial cells (D).

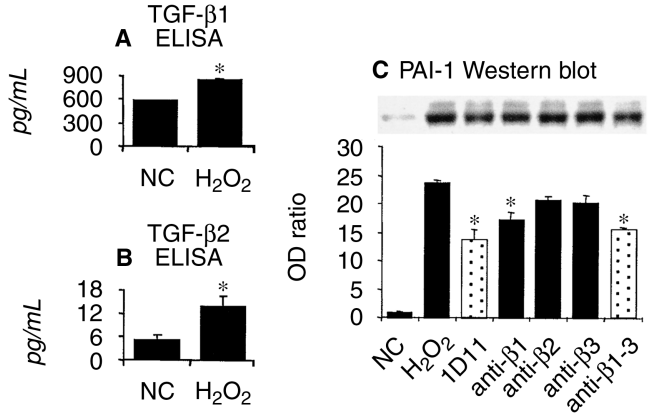

Fig. 11. Effects of transforming growth factor-β (TGF-β) blockade on plasminogen activator inhibitor type 1 (PAI-1) production after treatment of epithelial cells with H_2O_2. Stimulation with H_2O_2 caused marked increases in (*A*) TGF-β1, (*B*) TGF-β2, and (*C*) PAI-1 production. Blockade of all TGF-βs significantly reduced PAI-1 production (C). $^*P < 0.05$ vs. H_2O_2. TGF-β3 levels were too low to be measured (data not shown).

tive in reducing peroxide-induced increases in PAI-1 production. Interestingly, the data also show that 56% of the peroxide-induced up-regulation of PAI-1 is TGF-β–independent.

DISCUSSION

The results of this study clearly show that addition of TGF-β1, -β2, or -β3 to three types of renal cells produced very similar effects on matrix protein metabolism. Although there were differences in the magnitude of response among the cell types, all TGF-β isoforms dose dependently increased matrix protein synthesis and reduced matrix degradation (Figs. 1–4).

The three mammalian TGF-β isoforms are encoded by distinct genes. They are synthesized as large preproproteins, of which the biologically active TGF-β isoforms reside in the C-terminus. Although their N-terminal precursor sequences are dissimilar, the C-terminal 112 amino acids of the mature TGF-β peptides exhibit 60% to 80% conservation and 100% conservation of the nine cysteine residues [26]. In most biological aspects they are interchangeable. The clear difference among them is tissue-specific and developmentally regulated expression. TGF-β1 is expressed in endothelial, hematopoietic, and connective tissue cells; TGF-β2 in epithelial and neuronal cells; and TGF-β3 primarily in mesenchymal cells. In normal kidney, TGF-β1 is expressed in glomeruli and tubules, predominantly in distal tubules. TGF-β1 mRNA has been found in tubular epithelial cells, mesangial cells, glomerular epithelial cells, as well as interstitial fibroblast like cells [27]; TGF-β2 mRNA and protein was found in glomeruli and tubular cells. Potassium depletion or water deprivation induced TGF-β2 in renal arteries and the juxtaglomerular apparatus [28–30]; TGF-β3 expression was located at tubular cells and to a lesser extent in glomeruli [29, 30]. During development, TGF-β1 and -β3 are expressed early in structures under-

going morphogenesis, and TGF-β2 is expressed later in mature and differentiating epithelium [31, 32]. In a tissue where all three isoforms are expressed it is not clear whether they act synergistically.

At the level of signal transduction, TGF-β2 is different from the other two isoforms. TGF-β1 and -β3 directly combine with the type II TGF-β receptor (TβRII). The complex activates the type I TGF-β receptor (TβRI) and signaling proceeds. In contrast, TGF-β2 needs the type III TGF-β receptor (TβRIII, betaglycan) in order to combine with TβRII [33]. Thus, it is possible that some cells respond differently to TGF-β2, as has been shown in some studies [34, 35]. However, most studies have shown similar profibrogenic effects of TGF-β2, probably because TβRIII is widely expressed [9, 10, 36]. In the present study, addition of exogenous TGF-β2 to renal cells produced increases in matrix production that were interchangeable with those seen with addition of TGF-β1.

There has been a great deal of controversy over the reported anti-fibrotic effect of TGF-β3. A number of in vitro studies have failed to show antifibrotic effects of TGF-β3 [9, 10, 36, 37]. On the other hand, Murata et al [12] observed that addition of TGF-β3 to dermal fibroblasts increased collagen production when added alone, but when both TGF-β1 and TGF-β3 were added together, α1(I) procollagen and TGF-β1 mRNA levels decreased. These data may result from the well-known phenomenon that low levels of TGF-β increase matrix protein production while very high doses decrease matrix production in some cell types. Thus, it was not surprising that when 1 or 5 ng/mL TGF-β3 was added to 1 ng/mL TGF-β1, mRNA expression decreased. In the present study we first determined dose response curves for all isoforms. Mesangial cell proteoglycan production showed decreases at high doses, while renal fibroblast and tubular epithelial cell matrix protein synthesis leveled off at high doses. Because of the sensitivity and reproducibility of the ^{3}H-proline incorporation assay, we used collagen production as a read-out for isoform effects in most of the studies. The data indicate that all three isoforms increase matrix protein production and they are equipotent. Whether we added isoforms together simultaneously, whether different ratios of isoforms were used, or whether TGF-β3 was added at a later time than TGF-β1, we could find no anti-fibrotic effects of TGF-β3 in these cells.

We also investigated the possibility that TGF-β isoforms differentially reduce matrix protein degradation that could result in different levels of pathologic matrix accumulation. While increased PAI-1 production is generally thought to result in decreased matrix degradation via decreased plasmin generation, the only direct evidence has been provided in studies by Baricos et al [38]. PAI-1 was shown to mediate human mesangial cell degradation of radiolabeled matrigel and TGF-β1 was later shown to mediate the decreased degradation seen with increased PAI-1 production [39]. We found TGF-β2 and -β3 also increased PAI-1 expression on these renal cells (data not shown). The data presented here show that renal fibroblasts cultured on their own matrix degrade the pre-existing matrix proteins as they produce new ones and that all TGF-β isoforms reduce this matrix degradation similarly. The question of whether this decrease is entirely mediated by PAI-1 is, as yet, unknown.

While the in vitro data presented here suggest that TGF-β3 has no anti-fibrotic actions, it is possible that the in vivo and in vitro situations differ. The first study suggesting TGF-β3 had anti-fibrotic effects came from a rat model of wound repair. Shah et al [11] reported that exogenous addition of the TGF-β3 to rat dermal wounds reduced monocyte/macrophage infiltration, as well as fibronectin, collagen I, and collagen III deposition in the early stages of wound healing. The architecture of the neodermis was improved and scarring was reduced. However, the rat incisional model has been questioned by other researchers because rat wounds usually heal without an obvious scar [40]. In addition, in Shah's study, TGF-β isoforms and antibodies were injected into different wounds on the same rat, introducing the question of whether systemic effects might explain their results. In fact, when Tyrone et al [41] did similar experiments on rabbits they found the opposite results. Exogenous TGF-β3 significantly increased the wound-breaking strength and collagen deposition.

Interpretation of our in vitro studies may also be limited because of the multiple roles of TGF-β in vivo. While TGF-β is chemotactic for monocytes and neutrophils, which would enhance inflammation, TGF-β may also suppress activated inflammatory cells, thereby decreasing inflammation [42, 43]. Only empirical in vivo data on TGF-β isoforms will truly shed light on whether inflammation is an important factor in many fibrotic diseases. For example, a study on nephrotoxic nephritis with TGF-β3 infusion showed profound reduction of macrophage infiltration, but no decrease in glomerular matrix accumulation [30].

Perhaps the most interesting and clinically relevant finding in this study is that while TGF-β1 had a small and inconsistent action on TGF-β2 and -β3 synthesis, TGF-β2 and -β3 up-regulated TGF-β1 expression in all three cell types. This led us to hypothesize that the profibrotic actions of TGF-β2 and TGF-β3 may be mediated by TGF-β1. Use of isoform-specific antibody to TGF-β3 revealed that nearly 80% of the profibrotic effect of TGF-β3 in tubular epithelial cells and renal fibroblasts was dependent on induction of TGF-β1 synthesis by TGF-β3. In contrast, the profibrotic actions of TGF-β1 were not mediated by TGF-β3. This is consistent with Murata's work on dermal fibroblasts, in which more than 50% of the stimulatory effect of TGF-β3 on α1(I) procol-

lagen mRNA expression could be inhibited by TGF-β1 anti-sense oligonucleotide [12]. When one combines these findings with the fact that TGF-β1 production by these renal cells is quantitatively greater than production of TGF-β2 and TGF-β3, it appears likely that TGF-β1 is the major mediator of fibrogenesis in renal cells.

The interactive relationship between isoforms was further demonstrated in an in vitro system. Oxidative stress causes renal injury and promotes renal fibrosis [44, 45]. In this study, exposure of tubular epithelial cells to hydrogen peroxide produced dramatic increases in PAI-1 protein production (Fig. 11C). This was accompanied by up-regulation of TGF-β1 and -β2 (Fig. 11 A and B). Blockade of TGF-β1, TGF-β2, or TGF-β3 reduced PAI-1 by 29%, 13%, and 16%, respectively. A further reduction to 44% was achieved by blocking all three isoforms together, suggesting that pan-specific blockade of all TGF-β isoforms may be the best strategy for therapeutic reduction of fibrosis. Consistent with this idea is the fact that expression of all three TGF-β isoforms is increased in most human renal fibrotic diseases [46, 47]. Interestingly, the PAI-1 increase in response to peroxide treatment could only be blocked by 44%, indicating that TGF-β–independent pathways are responsible for part of this increase.

CONCLUSION

The study presented here demonstrates a clear profibrotic effect of all three TGF-β isoforms. It suggests that increases in any of the TGF isoforms can contribute to pathologic matrix accumulation in renal fibrosis. Although TGF-β1 may be the main mediator, blockade of all isoforms together may yield the best therapeutic effect.

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REFERENCES

1. 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
2. Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
3. Yu L, Noble N, Border W: Therapeutic strategies to halt renal fibrosis. *Curr Opin Pharmacol* 2:177–181, 2002
4. Hill C, Flyvbjerg A, Gronbaek H, *et al*: The renal expression of transforming growth factor-beta isoforms and their receptors in acute and chronic experimental diabetes in rats. *Endocrinology* 141:1196–1208, 2000
5. Hill C, Flyvbjerg A, Rasch R, *et al*: Transforming growth factor-beta2 antibody attenuates fibrosis in the experimental diabetic rat kidney. *J Endocrinol* 170:647–651, 2001
6. Pasquale LR, Dorman-Pease ME, Lutty GA, *et al*: Immunolocalization of TGF-beta 1, TGF-beta 2, and TGF-beta 3 in the anterior segment of the human eye. *Invest Ophthalmol Vis Sci* 34:23–30, 1993
7. Lutty GA, Merges C, Threlkeld AB, *et al*: Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid. *Invest Ophthalmol Vis Sci* 34:477–487, 1993
8. Cordeiro MF, Gay JA, Khaw PT: Human anti-transforming growth factor-beta2 antibody: A new glaucoma anti-scarring agent. *Invest Ophthalmol Vis Sci* 40:2225–2234, 1999
9. Coker RK, Laurent GJ, Shahzeidi S, *et al*: Transforming growth factors-beta 1, -beta 2, and -beta 3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. *Am J Pathol* 150:981–991, 1997
10. Kinbara T, Shirasaki F, Kawara S, *et al*: Transforming growth factor-beta isoforms differently stimulate proalpha2 (I) collagen gene expression during wound healing process in transgenic mice. *J Cell Physiol* 190:375–381, 2002
11. Shah M, Foreman DM, Ferguson MWJ: Neutralisation of TGF-β1 and TGF-β2 or exogenous addition of TGF-β3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 108:985–1002, 1995
12. Murata H, Zhou L, Ochoa S: TGF-β3 stimulates and regulates collagen synthesis through TGF-β1-dependent and independent mechanisms. *J Invest Dermatol* 108:258–262, 1997
13. Kagami S, Border WA, Miller DE, Noble NA: Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 93:2431–2437, 1994
14. Okuda S, Languino LR, Ruoslahti E, Border WA: Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. *J Clin Invest* 86:453–462, 1990
15. Kidwell WR: Assays of growth factor stimulation of extracellular matrix biosynthesis. *Methods Enzymol* 147:407–414, 1987
16. Trevisan R, Yip J, Sarika L, *et al*: Enhanced collagen synthesis in cultured skin fibroblasts from insulin-dependent diabetic patients with nephropathy. *J Am Soc Nephrol* 8:1133–1139, 1997
17. Orth SR, Ritz E, Suter-Crazzolara C: Glial cell line-derived neurotrophic factor (GDNF) is expressed in the human kidney and is a growth factor for human mesangial cells. *Nephrol Dial Transplant* 15:589–595, 2000
18. Border WA, Okuda S, Languino LR, Ruoslahti E: Transforming growth factor-beta regulates production of proteoglycans by mesangial cells. *Kidney Int* 37:689–695, 1990
19. Rennard SI, Berg R, Martin GR, *et al*: Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal Biochem* 104:205–214, 1980
20. Laug WE, Weinblatt ME, Jones PA: Endothelial cells degrade extracellular matrix proteins produced in vitro. *Thromb Haemost* 54:498–502, 1985
21. Fort P, Marty L, Piechaczyk M, *et al*: Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res* 13:1431–1442, 1985
22. Genovese C, Rowe D, Kream B: Construction of DNA sequences complementary to rat alpha 1 and alpha 2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. *Biochem.* 23:6210–6216, 1984
23. Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO: Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell* 35:421–431, 1983
24. Derynck R, Jarrett A, Chen EY, Goeddel DV: The murine transforming growth factor-β precursor. *J Biol Chem* 261:4377–4379, 1986
25. Miller DA, Lee A, Pelton RW, *et al*: Murine transforming growth factor-beta 2 cDNA sequence and expression in adult tissues and embryos. *Mol Endocrinol* 3:1108–1114, 1989
26. Roberts AB, Sporn MB: The transforming growth factor-betas, in *Peptide Growth Factors and their Receptors 1*, edited by Sporn MB, Roberts AB, 1st edition, Berlin, Heidelberg, Germany, Springer-Verlag, 1990, pp 419–472
27. Fukuda K, Yoshitomi K, Yanagida T, *et al*: Quantification of

TGF-beta1 mRNA along rat nephron in obstructive nephropathy. *Am J Physiol Renal Physiol* 281:F513–521, 2001
28. Horikoshi S, McCune BK, Ray PE, *et al*: Water deprivation stimulates transforming growth factor-beta 2 accumulation in the juxtaglomerular apparatus of mouse kidney. *J Clin Invest* 88:2117–2122, 1991
29. Ray PE, McCune BK, Gomez RA, *et al*: Renal vascular induction of TGF-β2 and renin by potassium depletion. *Kidney Int* 44:1006–1013, 1993
30. Wilson HM, Minto AWM, Brown PAJ, *et al*: Transforming growth factor-β isoforms and glomerular injury in nephrotoxic nephritis. *Kidney Int* 57:2434–2444, 2000
31. Pelton RW, Saxena B, Jones M, *et al*: Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: Expression patterns suggest multiple roles during embryonic development. *JCB* 115:1091–1105, 1991
32. Roberts AB, Sporn MB: Differential expression of the TGF-β isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol Repro Dev* 32:91–98, 1992
33. Lopez-Casillas F, Wrana JL, Massague J: Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 73:1435–1444, 1993
34. Merwin JR, Newman W, Beall LD, *et al*: Vascular cells respond differentially to transforming growth factors beta 1 and beta 2 in vitro. *Am J Pathol* 138:37–51, 1991
35. Salzman SA, Mazza JJ, Burmester JK: Regulation of colony-stimulating factor-induced human myelopoiesis by transforming growth factor-beta isoforms. *Cytokines Cell Mol Ther* 7:31–36, 2001
36. Klein MB, Yalamanchi N, Pham H, *et al*: Flexor tendon healing in vitro: Effects of TGF-beta on tendon cell collagen production. *J Hand Surg [Am]* 27:615–620, 2002
37. Eickelberg O, Kohler E, Reichenberger F, *et al*: Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *Am J Physiol* 276:L814–824, 1999
38. Baricos WH, Cortez SL, El-Dahr SS, Schnaper HW: ECM degradation by cultured human mesangial cells is mediated by a PA/plasmin/MMP-2 cascade. *Kidney Int* 47:1039–1047, 1995
39. Baricos WH, Cortez SL, Deboisblanc M, Xin S: Transforming growth factor-β is a potent inhibitor of extracellular matrix degradation by cultured human mesangial cells. *J Am Soc Nephrol* 10:790–795, 1999
40. Wu L, Siddiqui A, Morris DE, *et al*: Transforming growth factor beta 3 (TGF beta 3) accelerates wound healing without alteration of scar prominence. Histologic and competitive reverse-transcription-polymerase chain reaction studies. *Arch Surg* 132:753–760, 1997
41. Tyrone JW, Marcus JR, Bonomo SR, *et al*: Transforming growth factor beta3 promotes fascial wound healing in a new animal model. *Arch Surg* 135:1154–1159, 2000
42. Letterio JJ, Roberts AB: Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 16:137–161, 1998
43. Pawluczyk IZ, Harris KP: Transforming growth factor-beta suppresses macrophage-induced mesangial cell fibronectin expression. *Kidney Int* 60:533–542, 2001
44. Shah SV: Role of reactive oxygen metabolites in experimental glomerular disease. *Kidney Int* 35:1093–1106, 1989
45. Iglesias-De La Cruz MC, Ruiz-Torres P: Alcami J, *et al*: Hydrogen peroxide increases extracellular matrix mRNA through TGF-beta in human mesangial cells. *Kidney Int* 59:87–95, 2001
46. Yamamoto T, Noble NA, Cohen AH, *et al*: Expression of transforming growth factor-β isoforms in human glomerular diseases. *Kidney Int* 49:461–469, 1996
47. Yamamoto T, Noble NA, Miller DE, *et al*: Increased levels of transforming growth factor-β in HIV-associated nephropathy. *Kidney Int* 55:579–592, 1999