TGF-β1 Is an Autocrine Mediator of Renal Tubular Epithelial Cell Growth and Collagen IV Production

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Recent studies in cultured cells have provided evidence that a variety of pathobiologic stimuli, including high glucose, angiotensin II, and thromboxane A_2, trigger a signaling pathway leading to autocrine induction of TGF-β1. TGF-β1 production through this pathway may profoundly affect cell growth, matrix synthesis, and response to injury. This study examines the role of autocrine versus exogenously added TGF-β1 in cellular proliferation and collagen IV production, critical targets of TGF-β1 signaling, using renal cells derived from TGF-β1 knockout (KO) animals or wild-type (WT) controls. Growth of WT and KO cells was assessed by cell counting and [^{3}H]thymidine uptake. Basal and TGF-β1-stimulated collagen production was assessed by Northern and Western blotting; transcriptional activity of the α1(IV) collagen gene was assessed by transient transfection analysis. KO cells grew at a faster rate than WT cells carefully matched for plating density and passage number. This increased growth rate was paralleled by increases in [^{3}H]thymidine uptake. KO cells expressed lower levels of the cell cycle inhibitors p21 and p27 than WT cells. KO cells failed to express TGF-β1, as expected. Basal TGF-β3 mRNA levels were higher in KO cells than in WT cells. WT cells expressed higher basal levels of TGF-β2 mRNA than KO cells. Basal α1(IV) and α2(IV) collagen mRNA and protein expression were significantly lower in KO cells than WT cells. Administration of exogenous TGF-β1 induced collagen IV production in both KO and WT cells. Although basal transcriptional activity of an α1(IV) collagen-CAT construct was lower in KO cells than WT cells, administration of exogenous TGF-β1 was associated with significant increases in transcriptional activity of this construct in both KO and WT cells. These studies provide evidence that autocrine production of TGF-β1 may play a critical role in regulation of growth and basal collagen IV production by renal tubular epithelial cells.

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Transforming growth factor-β1 (TGF-β1) has a wide variety of effects on cell growth, development, and tissue remodeling following injury (1, 2). TGF-β1 potently inhibits the growth of a variety of cells, including those derived from epithelium, endothelium, the glomerular mesangium, and the lymphoid system (3–6). Abnormalities in TGF-β signaling have been identified in a variety of proliferative disorders, including glomerulonephritis, cancer, and atherosclerosis (6–9). TGF-β1 stimulates the accumulation of extracellular matrix by enhancing the rate of biosynthesis of various extracellular matrix macromolecules and by inhibiting synthesis of enzymes capable of degrading the extracellular matrix (1, 10). Excessive production of TGF-β1 following injury has been implicated in progressive organ dysfunction, culminating in fibrosis (11, 12).

TGF-β1 signaling may occur by autocrine, paracrine, or endocrine pathways. Circulating cells, including monocytes (13) and platelets (14, 15), are abundant sources of TGF-β1, and are capable of delivering TGF-β1 to sites of tissue injury. Parenchymal cells of many organs also have a high capacity for TGF-β1 production (1, 16–19). Recent studies in cultured cells have demonstrated that a variety of pathobiologic stimuli, including high glucose, angiotensin II, reactive oxygen species, lipids, and thromboxane A_2, are capable of inducing TGF-β1 synthesis, with subsequent alterations in cell growth and matrix production (20–25). Because TGF-β1 is a potent stimulus for its own production (26), autocrine synthesis of TGF-β1 may play an important role in the maintenance of tissue homeostasis and the cellular response to injury.

The use of TGF-β1 knockout animals for *in vivo* studies to address these issues is complicated by the TGF-β1 knockout phenotype, which is characterized by a high embryonic lethality and the development of a systemic inflammatory wasting syndrome in liveborn animals within 1 week, leading to death after 3–4 weeks of age (9, 27–30). Although many studies have underscored the importance of TGF-β1 in progressive tissue injury, the role of autocrine TGF-β1 production in regulation of cell proliferation and matrix synthesis has not been adequately explored. Cells derived from TGF-β1 null mice provide an excellent model system to address these issues. The purpose of this study is to define the role of autocrine (endogenous) or paracrine (exogenous) TGF-β1 signaling on proliferation and on collagen IV production utilizing renal cells isolated from experimental animals bearing a targeted disruption of the TGF-β1 gene (knockout cells). Our data suggest that TGF-β1 is an autocrine modulator of both cell growth and collagen IV biosynthesis in murine renal tubular epithelial cells. The use of TGF-β1 knockout cells may provide an ideal model system for elucidation of the role of TGF-β1 in the cellular response to pathobiologic stimuli.

Materials and Methods

Cell Culture. Primary cultures of renal epithelial cells were prepared from homogenates of renal cortex obtained from mice bearing a targeted disruption of the TGF-β1 gene (knockout, KO) (29). Renal tubular epithelial cells harvested from wild-type animals (WT) served as controls. Epithelial cells were selected by using appropriate growth media, as described below. To characterize the cells, monolayer cultures grown on Lab Tech slides were stained with a battery of immunohistochemical markers using the VECTASTAIN kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Both the WT and KO cells are cytokeratin positive, smooth muscle actin negative, desmin negative, and vimentin negative, consistent with an epithelial cell phenotype. For the studies described here, four separately prepared renal tubular epithelial cell cultures were derived from two KO mice, and two renal tubular epithelial cell cultures were obtained from one WT mouse. When the primary cultures became confluent, cell stocks were frozen in liquid nitrogen. In all experiments, WT and KO cells were thawed at the same time and were matched for passage number and for plating density. Passages 10–27 for both WT and KO cells were used in this study.

Cells were cultured in renal epithelial cell growth medium (REGM) containing 0.5% fetal bovine serum (FBS), supplemented with epidermal growth factor (EGF), insulin, hydrocortisone, epinephrine, T_4, transferrin, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Clonetics, San Diego, CA) at 37°C in a humidified 5% CO_2 incubator. In selected experiments, cultures were treated with porcine TGF-β1 (R&D Systems, Minneapolis, MN).

Determination of Cell Numbers. WT and KO cells were seeded in triplicate in 6-well plates at 1.2×10^5 cells/well in complete REGM. To assess the effect of exogenously administered TGF-β1, selected cultures were treated with porcine TGF-β1 (10 ng/ml) for 72 hr. At 24-hr intervals, cultures were washed with PBS and adherent cells were harvested by trypsinization. The number of viable cells in each sample was determined using a Coulter counter (Coulter Electronics, Hialeah, FL).

[^{3}H]Thymidine Incorporation. WT and KO cells were plated in 24-well plates at 2.5×10^4 cells/well in complete REGM. After 24 hr, cells were treated with TGF-β1 (10 ng/ml) or vehicle. Twenty hours later, 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) was added to each well and was incubated at 37°C for an additional 4 hr. Cells were washed three times with 10% trichloroacetic acid and one time with diH_2O, followed by lysis in 0.2 N sodium hydroxide with salmon sperm DNA (40 μg/ml) as a carrier. Radioactivity in the total lysates was counted in a liquid scintillation counter.

Northern Blot Analysis. WT and KO cells were plated in 10-cm dishes at 2×10^6 cells/dish in complete REGM. Basal collagen IV and TGF-β expression in WT and KO cells was assessed 24 hr after plating. TGF-β1-mediated induction of collagen IV was assessed in cells withdrawn from serum 24 hr prior to addition of TGF-β1 (10 ng/ml, 6 hr). Total cellular RNA was isolated using the RNeasy Total RNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA (10 μg/lane) was electrophoresed through a 1% agarose, 2.2 *M* formaldehyde denaturing gel and was transferred to nylon membranes (Schleicher & Schuell, Keene, NH), as previously described (31, 32). Ethidium bromide was added to each lane to allow visualization of the RNA with UV light. Complete transfer of the RNA from the gel to the membrane was documented by examining the gel under UV light.

cDNA probes for collagen IV were prepared by PCR amplification of the sequence corresponding to the triple helical domain of the murine α1(IV) and the 3′-untranslated region of the murine α2(IV) collagen genes, as previously described (32). cDNA probes for TGF-β1–3 were obtained from the American Type Culture Collection (ATCC nos. 63197, 63198, and 63199 for TGF-β1–3, respectively; Rockville, MD). Probes (25 ng) were labeled with α-^{32}P dCTP by the random primer method (33). Membranes were hybridized at 65°C in a 0.5 *M* sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 7% SDS, and 1% bovine serum albumin (BSA), as described by Church and Gilbert (34). Autoradiograms were quantitated by computer-assisted video densitometry. Data for 18S RNA were obtained from a negative image of the ethidium bromide-stained nylon membrane according to the method of Correa-Rotter *et al.* (35). As an additional control, blots were reprobed for the housekeeping gene GAPDH, as previously described (32).

Bioassay for TGF-β Activity. Bioassay for TGF-β was performed as previously described (36, 37). Mink lung epithelial cells (ATCC no. CCL-64), which are strongly growth inhibited by TGF-β, were trypsinized, resuspended in 10% FBS, and pelleted at 500*g* for 5 min. Cells were washed once with 10 ml of assay buffer (Dulbecco's modified Eagle's medium [DMEM]; Life Technologies, Rockville, MD; supplemented with 0.2% FBS) and were seeded at 60,000 cells/0.2 ml of assay buffer in 96-well Costar dishes. After 1 hr, conditioned media was added; control cultures received TGF-β1. Twenty-two hours later, the cells were pulsed with 1 μCi [^{3}H]thymidine and were incubated at 37°C for an additional 2 hr. [^{3}H]thymidine incorporation into DNA was assessed as described above. Data presented are representative of two experiments, each performed in duplicate.

Preparation of Conditioned Medium. Conditioned media were prepared from KO and WT cells grown in DMEM supplemented with 0.2% FBS. Conditioned media were centrifuged and immediately added to mink lung epithelial cells as described above. Conditioned media was transiently acidified to activate latent TGF-β as previously described (37).

Western Blot Analysis. WT and KO cells were plated at 5 × 10^6 cells/15-cm dish in complete REGM, followed by incubation with or without TGF-β1 (10 ng/ml) for 18 hr. Analysis of p21 and p27 was performed on cell lysates. Cells were washed with cold PBS, lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml aprotinin, and 200 μ*M* sodium orthovanadate), sonicated, and centrifuged at 2000*g* for 5 min at 4°C. Cell homogenates were denatured for 3 min at 95°C in loadin gbuffer according to Laemmli *et al.* (38). SDS-PAGE was performed using gels cast in the Ready Gel system (Bio-Rad, Hercules, CA). Equal amounts of protein were added to each well, as assessed by the method of Lowry *et al.* (39) with BSA as a standard. Western blot analysis for collagen IV was performed on aliquots of culture media (~100 μg of protein) as previously described (31). Electrophoresis was performed at a constant voltage (200 V), and protein was transferred to PVDF or nitrocellulose membranes. Blots were incubated with one of the following primary antibodies: mouse anti-rat p21 antibody (catalog no. 556430; PharMingen, San Diego, CA); rabbit anti-rat p27 (C-19) antibody (catalog no. sc528; Santa Cruz Biotechnology, Santa Cruz, CA); β-actin antibody as a control for loading (catalog no. A5441; Sigma, St. Louis, MO); or rabbit anti-human antibody collagen IV (catalog no. 681241; ICN Biomedicals, Costa Mesa, CA). The blots were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature and were visualized by exposure to x-ray film using an enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ).

Transfection Studies. WT and KO cells were plated at 3 × 10^5 cells/60-mm plate in complete REGM. Twenty-four hours after plating, cells were transfected with a chimeric collagen IV promoter-CAT construct containing 479 bp of the bidirectional collagen IV promoter (COL4A1/COL4A2, bases 2932–3410 from Genbank sequence RNU85606) oriented in the α1(IV) direction (40). Transfections were performed using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions. Five hours after transfection, cells were treated with TGF-β1 (10 ng/ml); control cells received vehicle only. Forty-eight hours after transfection, CAT activity was assessed relative to β-galactosidase activity using standard methods.

Statistical Analysis. Data presented are representative of at least three independent experiments. Statistical analysis was performed using InStat (GraphPad, San Diego, CA). Pairwise comparisons were evaluated by the student's *t* test when the standard deviations were similar, and the alternate Welch *t* test when the standard deviations were different. For the thymidine uptake studies, multiple comparisons between control and TGF-β1-treated WT and KO cells were performed by one-way analysis of variance (ANOVA) following reciprocal transformation of data (to normalize differences in standard deviations between groups); the Tukey-Kramer multiple comparisons *post hoc* test was employed. For the transfection studies, multiple comparisons between control and TGF-β1-treated WT and KO cells were performed by one-way ANOVA using the Student-Neuman-Keuls multiple comparisons *post hoc* test.

Results

Morphologic appearance of the WT and KO cells was similar as assessed by phase contrast microscopy. Both WT and KO cells grew in cohesive clusters of cells. Immunohistochemical phenotype of WT and KO cells was identical—keratin positive, smooth muscle actin negative, desmin negative, and vimentin negative—consistent with a tubular epithelial cell phenotype. In all subsequent experiments, KO and WT cells were matched for passage number, and were plated at identical densities.

KO Cells Grow Faster than WT Cells. TGF-β1, alone and in concert with other growth factors/cytokines, has been shown to regulate growth of many cell types, including tubular epithelial cells. To assess cell growth in the presence of growth factors/cytokines that may act in concert with TGF-β1, WT and KO cell growth was assessed in complete REGM, a heavily supplemented medium containing a variety of growth factors and FBS. Counts of WT and KO cells were similar 24 h after plating in complete REGM (Fig. 1). After 48 hr, the cell counts of KO cells were consistently greater than those of WT cells. TGF-β1 (10 ng/ml) exogenously administered during the 72-hr growth period reduced cell numbers by 67% for WT cells and 90% for KO cells (Fig. 1). These experiments were

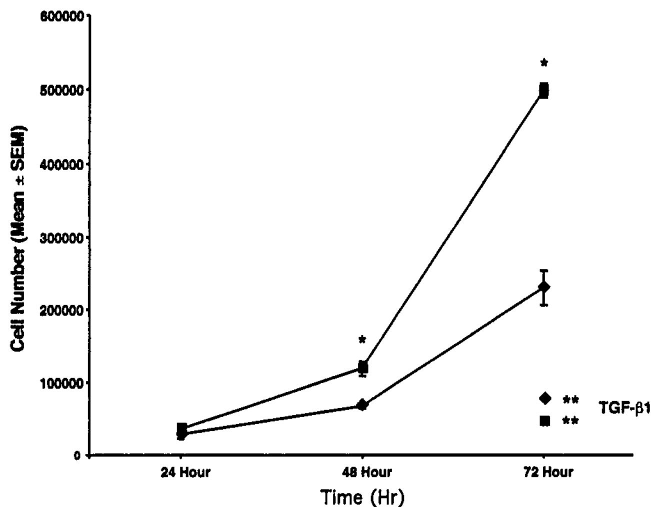

Figure 1. KO grow at a higher rate than WT cells. WT and KO cells were plated at identical densities, and cell counts were obtained at 24, 48, and 72 hr. ◆, WT cells; ■, KO cells; An asterisk designates a significant difference in number of WT versus KO cells ($P < 0.05$). WT and KO cell counts following a 72-hr incubation in the presence of TGF-β1 (10 ng/ml) are shown in the lower right of the figure (TGF-β1). A double asterisk designates significant growth inhibition compared with vehicle-treated cells ($P < 0.05$). Each bar designates mean ± standard error. Data are representative of four independent experiments.

repeated using different vials of frozen isolates and seeded at various initial plating densities with similar results: cell counts of subconfluent KO cells were significantly greater than those of WT cells plated under identical conditions. These studies indicate that TGF-β1 acts as an autocrine negative growth regulator of WT cells. Exogenously administered TGF-β1 markedly suppressed growth of both WT and KO cells. The growth inhibitory response of KO cells to TGF-β1 indicates that these cells retain functional TGF-β receptors and intracellular signaling pathways.

[^{3}H]Thymidine Uptake Is Greater in KO Cells than WT Cells. After 48 hr of growth in REGM, [^{3}H]thymidine uptake in KO cells was 1.6-fold higher than that of WT cells (Fig. 2). Exogenous administration of TGF-β1 (10 ng/ml) during the growth period reduced [^{3}H]thymidine uptake by 24% in WT cells and by 60% in KO cells.

Expression of the Cell Cycle Inhibitors p21 and p27 by WT and KO Cells. Previous studies have suggested that TGF-β1 inhibits G1-S phase transition at least in part through upregulation of the cell cycle inhibitors p27 and/or p21. We found that KO cells expressed lower basal levels of p21 and p27 than WT cells (Fig. 3). These observations suggest that autocrine TGF-β1 produced by WT cells limits cell growth through induction of p21 and p27 levels.

Expression of Collagen IV and TGF-β1–3 mRNA by WT and KO Cells. TGF-β1 has been recognized as a predominant mediator of extracellular matrix synthesis. Therefore, we examined the expression of collagen α1(IV) and collagen α2(IV) mRNA in WT and KO cells to determine the effects of autocrine TGF-β1 on the production of these matrix components. Basal expression of TGF-β1–3 was also assessed in WT and KO cells. Total RNA was isolated from WT and KO cells 24 hr after plating, was analyzed by Northern hybridization, and was quantified by densitometric scanning. Both collagen α1(IV) (Fig. 4) and α2(IV) (Fig. 5) mRNA expression were four to five times greater in WT cells than KO cells. The KO cells did not express TGF-β1 mRNA, as expected (Fig. 4). TGF-β2 mRNA expression was over four times greater in WT cells than in KO cells (Fig. 5). However, TGF-β3 mRNA expression was not detectable in WT cells, whereas a strong signal corresponding to TGF-β3 was identified in KO cells (Fig. 6). Exogenous administration of TGF-β1 to the WT and KO cells induced collagen α1(IV) mRNA expression

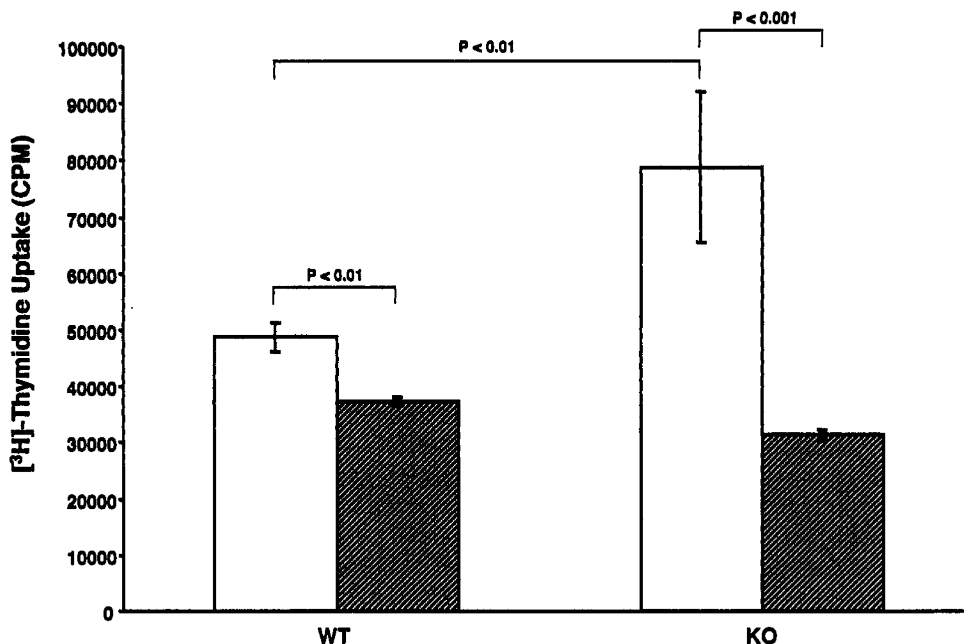

Figure 2. [^{3}H]thymidine uptake is greater in KO cells than WT cells. [^{3}H]Thymidine uptake was assessed in WT and KO cells treated with TGF-β1 (10 ng/ml) or vehicle, as described in "Materials and Methods." □, vehicle-treated WT or KO cells; ▨, TGF-β1-treated WT or KO cells. Each bar designates mean ± standard error. Data are representative of four independent experiments.

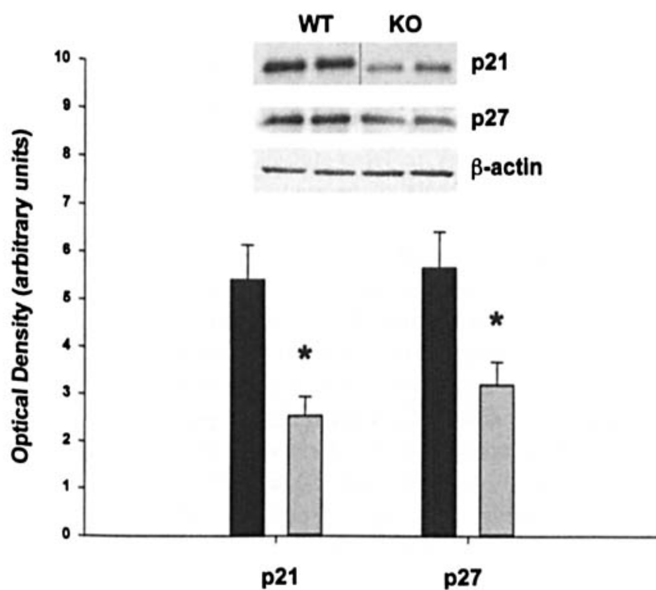

Figure 3. Expression of the cell cycle inhibitors p21 and p27 is higher in WT cells than in KO cells. Basal p21 and p27 levels were assessed by Western blot analysis of WT and KO cell homogenates, as described in "Materials and Methods." Inset shows Western blot analysis of a representative experiment. The blots were probed for β-actin to control for loading. The bar graph represents densitometric analysis of eight independent experiments, each performed in duplicate (mean ± standard error). ■, WT cells; ▩, KO cells. An asterisk designates a significant difference in p21 or p27 expression between WT and KO cells ($P < 0.05$).

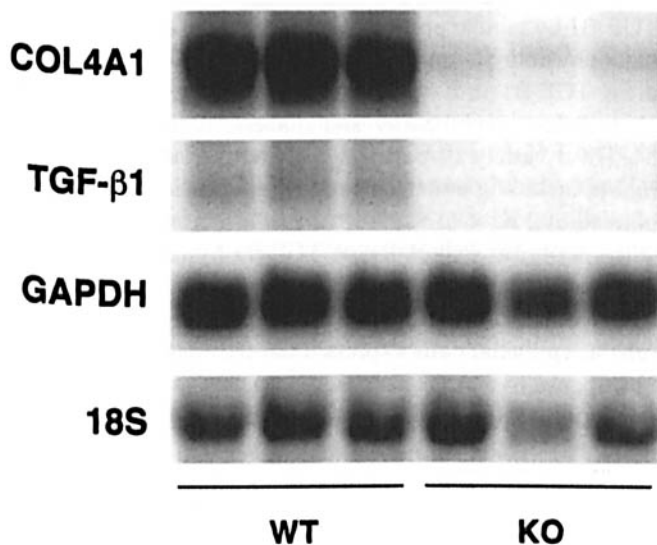

Figure 4. Basal collagen α1(IV) chain expression is higher in WT cells than KO cells. Total RNA was isolated from WT and KO cells, as described in "Materials and Methods." Northern blots were probed for the collagen α1(IV) chain, TGF-β1, and the housekeeping gene, GAPDH. Following densitometric analysis, data were normalized to 18S signal, as described in "Materials and Methods." Data are representative of three independent experiments.

(1.3- and 1.6-fold, respectively). These studies demonstrate that the matrix signaling pathway in response to exogenously added TGF-β1 is intact in the KO cells (Fig. 7). Secretion of collagen IV into the medium was assessed by Western blot analysis. Two bands corresponding to the 185-kDa α1(IV) collagen chain and the 170-kDa α2(IV) collagen chain were observed (32, 41). Basal collagen IV production was 5.5-fold higher in WT cells than in KO cells (Fig. 8). Collagen IV production was induced by TGF-β1 (by 1.5-fold for WT cells and 2.4-fold for KO cells; Fig. 8), indicating that the differences in collagen IV mRNA expression are paralleled by differences in collagen IV production.

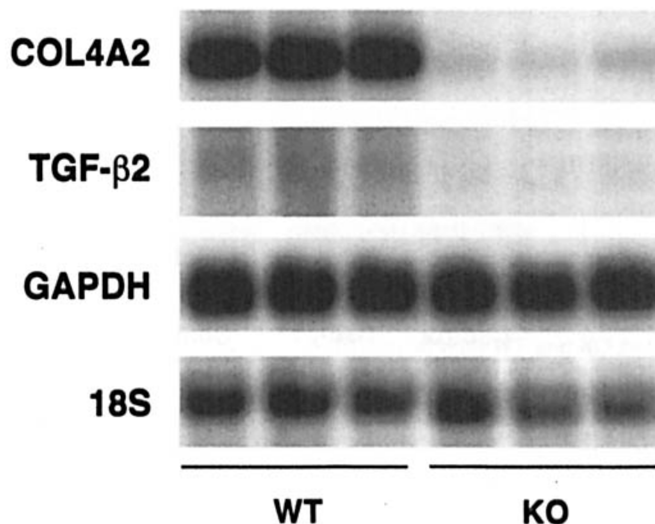

Figure 5. Basal expression of collagen α2(IV) and TGF-β2 is higher in WT cells than KO cells. Northern blots prepared from WT and KO cells were probed with cDNAs for collagen α2(IV) chain and for TGF-β2, as described in "Materials and Methods." Data are representative of three independent experiments.

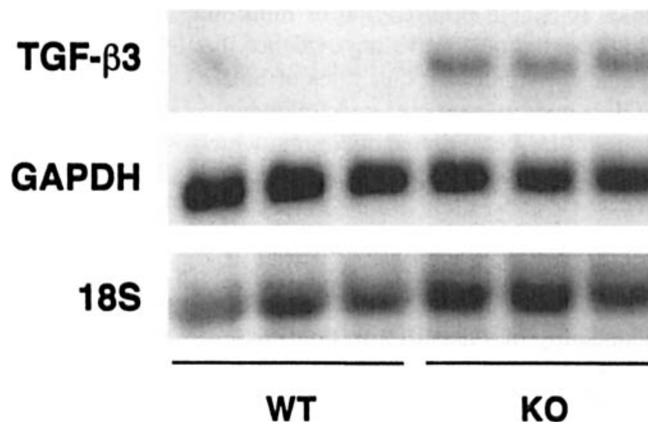

Figure 6. Basal TGF-β3 expression is higher in KO cells than WT cells. Northern blots obtained from WT and KO cells were probed with a cDNA for TGF-β3, as described in "Materials and Methods." Data are representative of three independent experiments.

WT Cells Produce More Active and Total TGF-β than KO Cells. It is generally recognized that TGF-β1, TGF-β2, and TGF-β3 signal through the same receptor complex. Although KO cells express high levels of TGF-β3 mRNA (Fig. 6), the higher growth rate and lower basal collagen IV production suggests that total TGF-β production by these cells is less than that of WT cells. To test this hypothesis, a bioassay for active and total TGF-β activity was performed using mink lung epithelial cells, which are strongly growth-inhibited by TGF-β (37, 42). As expected, conditioned media from WT cells inhibited proliferation of

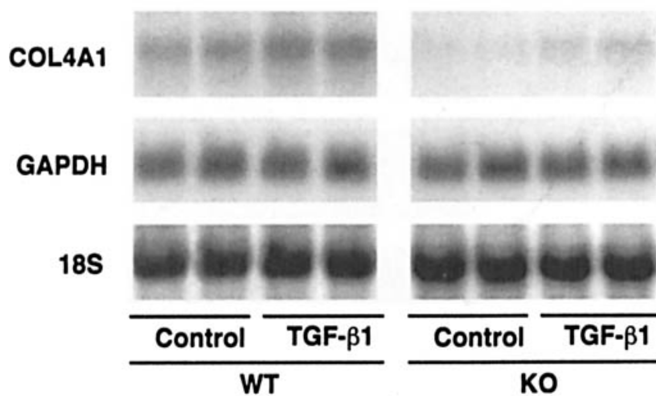

Figure 7. TGF-β1 induces collagen α1(IV) mRNA expression by KO and WT cells. Cells were withdrawn and treated with TGF-β1 (10 ng/ml) or vehicle for 6 hr prior to isolation of total RNA, as described in "Materials and Methods." Northern blots were probed with a cDNA for collagen α1(IV) mRNA, as described in "Materials and Methods." Data are representative of three independent experiments.

mink lung epithelial cells by 36.8%, indicating the presence of bioactive TGF-β. Conditioned media from KO cells failed to inhibit proliferation of mink lung epithelial cells (2.3% inhibition), providing evidence that KO cells do not synthesize significant quantities of bioactive TGF-β. Total TGF-β activity was obtained following transient acidification of the conditioned medium, which activates latent TGF-β produced by the WT and KO cells (37, 42). Following this treatment, conditioned media from WT cells inhibited proliferation of mink lung epithelial cells by 96.6% and inhibited proliferation of KO cells by 75.8%. These studies indicate that both WT and KO cells produce TGF-β, predominantly in a latent form. Presumably, the latent TGF-β activity observed in the KO cells is due to the production of TGF-β3.

Based on standard curves obtained by adding known quantities of porcine TGF-β1, concentrations of active and total TGF-β in conditioned medium from WT and KO cells were estimated. The total TGF-β concentration in conditioned media obtained from WT cells was approximately 2.6 ng/ml; the concentration in conditioned media obtained from KO cells was 0.2 ng/ml. Active TGF-β concentration in conditioned media was <1 ng/ml in WT cells and was undetectable in KO cells.

Transcriptional Activity of a Chimeric Collagen IV Promoter-CAT Construct Is Higher in WT Cells than KO Cells. We, and others, have previously shown that induction of collagen IV mRNA and protein is in large part directed by increased transcriptional activity of the collagen IV genes (32). Therefore, we sought to determine the effects of autocrine or exogenously added TGF-β1 on transcriptional activity of a chimeric collagen IV promoter-CAT construct transfected into WT and KO cells. In accordance with differences in basal collagen IV mRNA expression, basal transcriptional activity of the collagen IV promoter-CAT construct was 2.3-fold greater in WT cells than KO cells (Fig. 9). Exogenously administered TGF-β1 increased transcriptional activity of the collagen IV promoter-CAT construct 2-fold in WT cells and 6-fold in KO cells (Fig. 9).

Discussion

Although many *in vitro* studies have underscored the critical regulatory role of exogenously added TGF-β1 on cell growth and matrix production, a potential role for autocrine production of TGF-β1 by parenchymal cells following tissue injury has received little attention. *In vivo* studies to address this issue using TGF-β KO mice are complicated by a high intrauterine mortality and a phenotype characterized by progressive wasting syndrome with multifocal inflammatory disease (27, 43–45). Using primary cultures of renal tubular epithelial cells derived from TGF-β1 KO animals, carefully matched for passage number and growth conditions with renal tubular epithelial cells derived from WT animals, we found that the KO cells grow more rapidly than WT cells, but synthesize less collagen IV than WT cells. These differences in cell growth and collagen IV production between WT and KO cells could be abolished through exogenous administration of TGF-β1. Based on these considerations, we propose that autocrine production of TGF-β1 regulates growth and collagen IV production by renal tubular epithelial cells.

Since severity of chronic tubulointerstitial disease is the strongest predictor of outcome in patients with a wide variety of renal diseases (46–50), autocrine production of TGF-β1 by tubular epithelial cells may play a critical role in pathophysiologic states. Renal tubular epithelial cells express TGF-β1 in a variety of human kidney diseases, including IgA nephropathy and diabetic renal disease (51–54). In a variety of experimental models of chronic renal injury, including unilateral ureteric obstruction, subtotal nephrectomy, diabetic nephropathy, and chronic tubulointerstitial nephritis, expression of TGF-β1 by renal tubular epithelial cells appears to correlate with the extent of interstitial fibrosis and tubular atrophy (53, 55–60). *In vitro*, tubular epithelial cells express TGF-β1 mRNA and protein in response to a variety of stimuli, including angiotensin II, cyclosporin A, and high glucose (61–66). From these studies, it may be concluded that renal tubular epithelial cells are capable of TGF-β1 production *in vivo* and may be stimulated to produce TGF-β1 in response to pathobiologic stimuli.

It is well recognized that prior to secretion, TGF-β non-covalently associates with its latency-associated peptide to produce an inactive TGF-β complex (67, 68). The biological activity of TGF-β in the kidney depends upon the relative abundance of the active or mature form of TGF-β (69). Latent TGF-β may be activated by a variety of agents, including heat, pH extremes, thrombospondin-1, plasmin, and deglycosylation (70–75). We found that conditioned medium from WT cells inhibited proliferation of mink lung epithelial cells in a well-characterized bioassay for TGF-β activity (76). Conditioned medium from KO cells had no effect on proliferation of mink lung epithelial cells, indicat-

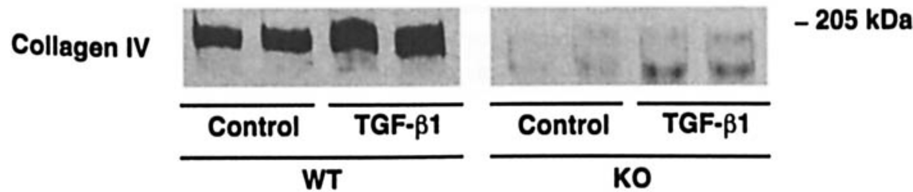

Figure 8. Collagen IV protein production is greater in WT than KO cells. Collagen IV secretion into the culture medium was assessed by Western blot analysis. Cells were withdrawn and treated with TGF-β1 (10 ng/ml) or vehicle for 18 hr prior to harvest, as described in Materials and Methods. Data are representative of four independent experiments.

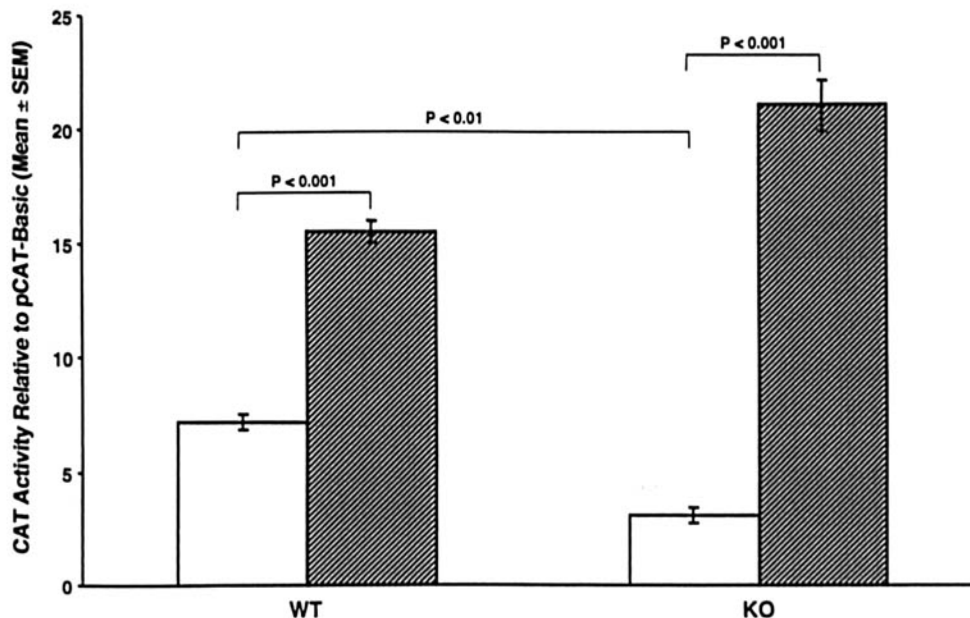

Figure 9. Transcriptional activity of a chimeric collagen α1(IV) promoter-CAT construct is induced by TGF-β1 in both WT and KO cells. WT and KO cells were transfected with a chimeric collagen IV promoter-CAT construct, oriented in the α1(IV) direction, as described in "Materials and Methods." Five hours after transfection, cells were treated with TGF-β1 (10 ng/ml; ▨) or vehicle (□). Forty-eight hours after transfection, cells were harvested for assessment of CAT and β-galactosidase activity. Data are expressed as CAT activity/β-galactosidase activity of WT or KO cells transfected with the collagen IV promoter-CAT construct, relative to activity of cells transfected with pCAT-Basic, a promoterless, enhancerless construct. Mean pCAT-Basic activity in WT cells was 299.5, and in KO cells was 109.0. Each bar designates mean ± standard error. Data are representative of three independent experiments.

ing that these cells do not produce measurable quantities of active TGF-β. Production of active TGF-β by WT cells, but not by KO cells, is likely responsible for the observed differences in cell growth and basal collagen IV production in this study. These studies demonstrate that WT cells possess a capacity for activation of TGF-β produced in an autocrine fashion. Further studies are required to define the mechanism by which renal tubular epithelial cells activate latent TGF-β, both *in vivo* and *in vitro*.

Immunohistochemical studies have shown that rat proximal tubules stain positively for TGF-β2 and TGF-β3, in addition to TGF-β1 (77). Although KO cells express high levels of TGF-β3 mRNA, they do not produce measurable quantities of active TGF-β, as assessed by the mink lung epithelial cell bioassay. Following transient acidification to activate TGF-β produced in a latent form, conditioned medium from KO cells inhibited proliferation of mink lung epithelial cells. This TGF-β bioactivity is likely due to production of TGF-β3 by the KO cells. However, production of TGF-β by KO cells is more than 10-fold less than production of TGF-β by WT cells. Furthermore, KO cells do not produce detectable quantities of active TGF-β. Therefore, the observed differences in cell growth and collagen IV production in this study are most likely related to significant quantitative differences in TGF-β production by the WT and KO cells.

There are several potential targets whereby autocrine TGF-β1 may downregulate cell growth. These include binding of growth factor ligand to receptor, inhibition of mitogenic signaling pathways, or inhibition of the cell cycle. Increases in TGF-β1 receptor expression have been described in glomeruli of rats with experimental membranous nephropathy (78), adriamycin nephropathy (79), and in hypertensive diabetic rats (80). Although we have not studied TGF-β receptor expression by WT versus KO cells in detail, we found that short-term treatment of KO cells with TGF-β1 significantly inhibits cell growth and [^{3}H]thymidine uptake, indicating that the KO cells possess a functionally intact TGF-β signaling system. In a previous study of embryonic fibroblasts derived from TGF-β1 KO mice, no differences in expression of TGF-β receptor types I, II, or III were identified compared with cells isolated from WT controls (81). Furthermore, there were no differences between WT and KO cells in binding of TGF-β to receptor complexes (81).

It is unlikely that the growth inhibitory effects of TGF-β1 are due to downregulation of mitogenic signaling cascades. Recent studies have shown that TGF-β1 can directly activate the mitogen-activated protein kinase (MAPK) intracellular signaling pathways, including extracellular signal-related kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) (82, 83). TGF-β-mediated activation of ERK has

been shown to be necessary for several TGF-β responses, including collagen synthesis (84) and expression of p21, a cell cycle regulatory protein (85).

Based on these considerations, TGF-β most likely inhibits cell growth through negative regulation of the cell cycle. When a cell is stimulated to proliferate, cyclin D associates with the cyclin-dependent kinases cdk4 and cdk6, whereas cyclin E associates with cdk2. In several cell types, including mesangial cells isolated from rat kidney (86), TGF-β prevents cell cycle progression by inhibiting cyclin E-dependent kinase activity (87, 88) without significantly changing cyclin D-cdk4, 6, or cyclin E-cdk2 levels (86).

We observed that KO cells expressed significantly lower levels of the cdk inhibitors p21 and p27 than WT cells. p21 and p27 belong to the KIP family of cell cycle inhibitory proteins, which are capable of blocking the activity of a variety of cyclin-cdk complexes, including cyclin E-cdk2 (89, 90). We hypothesize that autocrine production of TGF-β1 by WT cells limits cell growth through upregulation of p21 and/or p27 levels.

p27 was originally described as a TGF-β-inducible protein that arrests growth of mink lung epithelial cells in G1 phase of the cell cycle by inhibiting the activity of the cyclin E-cdk2 complex (91). Platelet-derived growth factor (PDGF)-stimulated mitogenesis of mesangial cells is associated with reduction of p27 levels (92). This reduction of p27 is prevented by TGF-β1 during inhibition of PDGF-induced mesangial cell proliferation. However, TGF-β1 still inhibits proliferation of growth factor-stimulated mesangial cells treated with p27 antisense oligonucleotides (92). Mice with homozygous deletion of the p27 gene develop hyperplasia of multiple organs in association with elevation of cdk2 activity (93). However, TGF-β1 is capable of inducing cell cycle arrest in T cells isolated from p27 KO animals (93). These studies suggest that p27 is not essential for the growth inhibitory effects of TGF-β, at least in some cell types.

TGF-β1 may also inhibit cell growth through induction of p21 in some cell types (87, 94, 95). The p21 promoter contains TGF-β-responsive elements (96). In experimental diabetes, renal hypertrophy is associated with increased TGF-β1 production and increased p21 expression (97, 98). When diabetes is induced in animals with homozygous deletion of the p21 gene (p21 KO), the kidneys do not develop hypertrophy despite increased TGF-β levels (99). Interestingly, p21 KO animals do not develop progressive renal failure following subtotal nephrectomy compared with WT controls (100). These studies suggest that TGF-β1-mediated hypertrophy occurs through induction of p21 and may be a maladaptive response that contributes to the progression of renal injury.

We found that basal collagen IV mRNA expression and protein production were significantly lower in KO cells than WT cells. Many studies linking TGF-β1 production to progressive injury have focused on collagen I, or fibrillar collagen deposition and tissue fibrosis. However, progressive human renal disease is associated with excessive deposition of collagen IV, or basement membrane collagen (101–104). Within the renal interstitium, collagen IV deposition contributes more to the development of interstitial expansion and renal failure than deposition of collagens I and III, the fibrillar collagens associated with tissue fibrosis (105). Increased deposition of collagen IV within the interstitium is a well-recognized feature of experimental models of progressive renal disease such as ureteric obstruction, purine amino nucleoside nephrosis, tubulointerstitial nephritis, anti-glomerular basement membrane nephritis, and diabetic nephropathy (53, 55, 106, 107). Based on previous observations that TGF-β1 is a predominant mediator of collagen IV production (31) and that TGF-β1 primarily acts through increasing transcription of the collagen IV genes (32), we propose that autocrine TGF-β1 production is a critical determinant of basal collagen IV production by renal tubular epithelial cells.

Using chimeric collagen IV promoter-CAT constructs, we found that basal transcriptional activity is significantly higher in WT cells than KO cells. Transcriptional activity of the collagen IV promoter-CAT construct is significantly augmented following administration of exogenous TGF-β1 in both WT and KO cells. Future studies are needed to define mechanisms underlying the transcriptional response of renal tubular epithelial cells to TGF-β1.

Based on these observations, we propose that autocrine production of TGF-β1 limits proliferation of renal tubular epithelial cells, and that basal transcription of the collagen IV genes is regulated by autocrine TGF-β1 production. It is of interest that the growth inhibition in collagen IV induction by exogenously added TGF-β1 was greater in KO cells than WT cells. This increase in response may be due to compensatory induction of TGF-β-signaling molecules due to the absence of TGF-β1. Alternatively, the relatively high level of TGF-β1 signaling in the WT cells may render them less able to respond to exogenous TGF-β1 than KO cells. In either case, the TGF-β1 KO cells, with limited basal production of signaling molecules responsible for growth regulation and collagen IV production, may be useful tools for dissecting mechanisms whereby TGF-β1 regulates these processes.

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