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Endoglin Expression in Human and Rat Mesangial Cells and Its Upregulation by TGF-β1

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Endoglin is a component of the TGF-β receptor complex present in the kidney at the human glomerular mesangium. Since the cellular origin of the glomerular endoglin is unknown, in the present study we investigated the expression of endoglin in mesangial cells in culture, as well as their response to TGF-β1. Western and Northern blot analysis identified the expression of endoglin protein and mRNA transcript in both human and rat mesangial cells. Flow cytometry and immunocytochemistry analyses revealed that endoglin is present on the cell membrane. Exogenous TGF-β1 stimulated not only the expression of collagen α1 (I) I and TGF-β1, but also that of endoglin. These data provide the first evidence for the expression of endoglin in mesangial cells, as well as its upregulation by TGF-β1, thus suggesting that endoglin may have a role in modulating the effects of TGF-β1 on the glomerular mesangium.

Key Words: **mesangial cells; endoglin; transforming growth factor beta (TGF-β); TGF-β receptor system; extracellular matrix (ECM); collagen.**

The ability of mesangial cells (MCs) to modulate their phenotype, proliferate, and secrete extracellular matrix underlies their crucial role in the development and progression of glomerular diseases to chronic glomerular sclerosis (1). These aspects of MC behavior can be modified by transforming growth factor beta 1 (TGF-β1), an autocrine growth factor thought to play a significant role in the pathogenesis of chronic renal fibrosis (2). Overexpression of this cytokine has been observed in experimental and clinical glomerulosclerosis (3–5). Treatment with anti-TGF-β antiserum (6), decorin (a natural inhibitor of TGF-β) (7, 8), or oligodeoxynucleotides antisense to TGF-β (9), reduced the excessive ECM deposition in the acute anti-Thy-1 glomerulonephritis model. Conversely, TGF-β enhances glomerular mesangial cell production of collagen and fibronectin (10–12). These data support the hypothesis that TGF-β is a central mediator in the sclerosis processes of diseased glomeruli.

Endoglin is a membrane-bound glycoprotein, which shows structural homology with the type III TGF-β receptor, also known as betaglycan. Human endoglin consists of two 90 kDa disulfide-linked subunits containing N- and O-linked oligosaccharides. Its primary sequence contains a 561 amino acid extracellular domain, a single transmembrane region, and a short cytoplasmic tail of 47 amino acid residues (13, 14). Endoglin binds TGF-β1, TGF-β3, activin, BMP-2 and BMP-7 with high affinity, and associates with both the type I and the type II receptors (15–18). Human endoglin was originally identified in human vascular endothelial cells and was shown to be highly expressed on umbilical vein endothelial cells (19) and in vascular smooth muscle cells in culture (20). It was subsequently found to be expressed in murine ovary, uterus, heart, and skeletal muscle, and at low levels in placenta and spleen. Endoglin tissue distribution is remarkably similar to that of TGF-β1, particularly in the heart and uterus, suggesting that it may be involved in TGF-β1 signaling in stromal fibroblast-like cells (21).

Roy-Chaudhury *et al.* (22) have described in biopsies from kidneys without renal disease and from patients with glomerulonephritis, that endoglin was present within the glomerular mesangium and interstitium in normal kidneys. In diseased biopsies, there was a weak but significant correlation between staining for endoglin in the interstitium and the extent of chronic histological damage. One of the areas overexpressing endoglin was the glomerular mesangium, and there was also a positive correlation between mesangial and intersti-

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tial endoglin expression, thus suggesting that endoglin was upregulated in chronic renal disease. Moreover, we have recently demonstrated that both endoglin protein and mRNA were upregulated in the kidney of rats with glomerular sclerosis and hypertension induced by 5/6th nephrectomy (23).

Since endoglin modulates TGF-β1 signaling and given the involvement of this growth factor and MCs in chronic glomerular fibrosis, it was of interest to determine whether endoglin mRNA and protein are present in MCs. In the present study we have investigated the expression of endoglin in rat and human mesangial cells in culture, as well as their response to TGF-β1. We provide the first evidence for the existence of both endoglin mRNA and protein in rat and human MCs, as well as its upregulation by TGF-β1, thus suggesting that endoglin may have a role in regulating the effects of TGF-β1 on the glomerular mesangium.

MATERIALS AND METHODS

Mesangial cell culture. Primary cultures of rat mesangial cells were obtained from rat glomeruli isolated by successive mechanical sieving (24), following the regulations for animal handling of the Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18th December 1986). Primary cultures of human mesangial cells were obtained from glomeruli prepared from the cortex of the intact pole of kidneys removed for circumscribed tumor. Histological examination of these samples revealed no renal pathology. Glomerular cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, glutamine, penicillin/streptomycin, insulin, transferrin, selenium and amphotericin at 37°C in the presence of 5% CO_2 as previously described (25). Cells were confirmed as mesangial by standard morphological and functional criteria (24, 26). Experiments were performed on mesangial cells from passages 2–4. For TGF-β experiments, cultures were expanded to 70–80% confluence and serum was withdrawn from the culture media for 24 h. Cells were treated with human TGF-β1 (10 ng/ml, R&D Systems) for 24 h in the absence of serum. Cell extracts were then prepared as outlined below. All experiments described were, at least, performed twice.

Western blot analysis. Cells were washed twice in ice-cold PBS before being lysed in lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% dodecyl sulfate (SDS)] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin]. Cell lysates were centrifuged at 15,000*g* for 20 min at 4°C, and the supernatant was collected. The protein content was determined by a commercially available variant of the Lowry method (Bio-Rad) using BSA as standard. Fresh cell lysates were analyzed by SDS–polyacrylamide gel (8% acrylamide gel) electrophoresis. Samples were prepared in the Laemmli nonreducing buffer (final concentration: 50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 1% bromophenol blue) and equal amount of protein were loaded. Gels were blotted onto nitro-cellulose membranes (Bio-Rad). Membranes were blocked with 5% dried milk in Tris-buffered saline (TBS)-Tween (0.1%) for one hour at room temperature followed by incubation with the mouse anti-human endoglin monoclonal antibody P4A4 (27) for 2 h at room temperature. Blots were then washed in TBS-Tween, followed by incubation with the secondary antibody, HRP-conjugated goat anti-mouse IgG (Bio-Rad). Blots were developed by chemiluminescence using the ECL Western blotting system (Amersham-Pharmacia-Biotech).

Flow cytometry. Cells (5×10^5) were incubated with the rabbit polyclonal antibody VV-endo (28), which detects rat endoglin, for 30 min at 4°C, washed twice in PBS, and incubated for 30 min at 4°C with Cy3-labeled anti-rabbit IgG conjugate (DAKO Diagnosticos). Cells were washed twice in PBS and their fluorescence was estimated with an EPICs-CS (Coulter Cientifica), using logarithmic amplifiers.

Inmunocytochemistry. Mesangial cells were seeded onto glass cover-slides and left for 24 h in RPMI growth medium to adhere and flatten. Cells were washed in PBS and fixed in 4% formaldehyde/PBS. After two more washes in PBS, nonspecific binding sites were blocked by incubation in 2% BSA/PBS for 15 min at room temperature. Human mesangial cells were incubated with the mouse mAb P3D1 (anti-human endoglin) (27), and rat mesangial cells with the rabbit polyclonal antibody VV-endo (anti-endoglin) (28), followed by the corresponding Cy3-conjugated secondary antibody (goat anti-mouse or goat anti-rabbit) (Amersham-Pharmacia Biotech). Slides incubated only with the secondary antibody were used to control for nonspecific binding of the anti-endoglin antibody. Cells were washed in 0.2% BSA/PBS, rinsed briefly in 2 μM Hoechst (Sigma) to stain the nuclei, and mounted with mowiol (Hoechst). Immunofluorescence was studied using a Leica DM RxA fluorescence microscope.

Northern blot. Total RNA was isolated from mesangial cells with the guanidinium thiocyanate-phenol–chloroform method (29), size-fractionated by electrophoresis (20 μg/lane) through denaturing 1% agarose–1.1% formaldehyde gels, transferred to Hybond membranes (Amersham-Pharmacia Biotech), and UV cross-linked. Hybridization was performed at 60°C for 12–16 h, and membranes were then washed at final stringency conditions of 60°C, 2× SSC, 1% SDS, two times for 30 min each, and at 20°C, 0.2× SSC, 0.1% SDS for 1 h. Filters were exposed on XAR Kodak film, using an intensifying screen. The probe used for endoglin was a 360-bp *Sac*I/*Sac*II fragment of rat endoglin cDNA in pGEM-T, kindly provided by Dr. Calvin Vary (Maine). The probe for rat collagen Iα2 (0.6-kb fragment) was obtained by digestion of the corresponding cDNA inserted in plasmid PUC18, and was kindly provided by Dr. Diego Rodríguez Puyol (University of Alcalá de Henares, Madrid, Spain). The probe of mouse TGF-β1, that hybridizes with rat TGF-β1 mRNA, is a fragment of 451 bp obtained as a PCR product kindly gifted by Jean Pierre Girolami and Jean Loup Bascand (U388 INSERM, Toulouse, France). An 18S ribosomal subunit probe (1.5 kb) was used as an internal loading control.

Collagen synthesis and accumulation. The collagen content in the medium was quantified by measuring the incorporation of [^{3}H]-Proline into collagen proteins, as described elsewhere (30). In brief, cells were cultured in flat-bottomed 24-well plates. The culture medium was changed 24 h before the start of the radiolabeling period to DMEM serum free medium lacking proline. Radiolabeling was carried out by incubation for 24 h in fresh DMEM serum-free medium containing 0.15 mM β-aminopropionitrile, 210 μM ascorbic acid and 183 μM proline, 1 μCi/well [^{3}H]-Proline (Specific activity 24 Ci/mmol). Following completion of metabolic radiolabeling, an aliquot of medium was mixed with a proteinase inhibitor solution (3 mM PMSF, 0.1 M EDTA, 40 mM *N*-ethylmaleimide). Proteins were precipitated in ice-cold 10% trichloroacetic acid, and the pellet was extensively washed and resuspended in 0.1 N NaOH. [^{3}H]Proline incorporated to collagen proteins was measured in a β scintillation counter. The protein content of the wells was determined using a commercial kit (Bio-Rad).

RESULTS

Endoglin is expressed in human and rat mesangial cells. Using specific antibodies against endoglin, Western blot and flow cytometry analysis revealed that both human and rat mesangial cells expressed endoglin (Figs. 1A and 1B) respectively. In addition, immu-

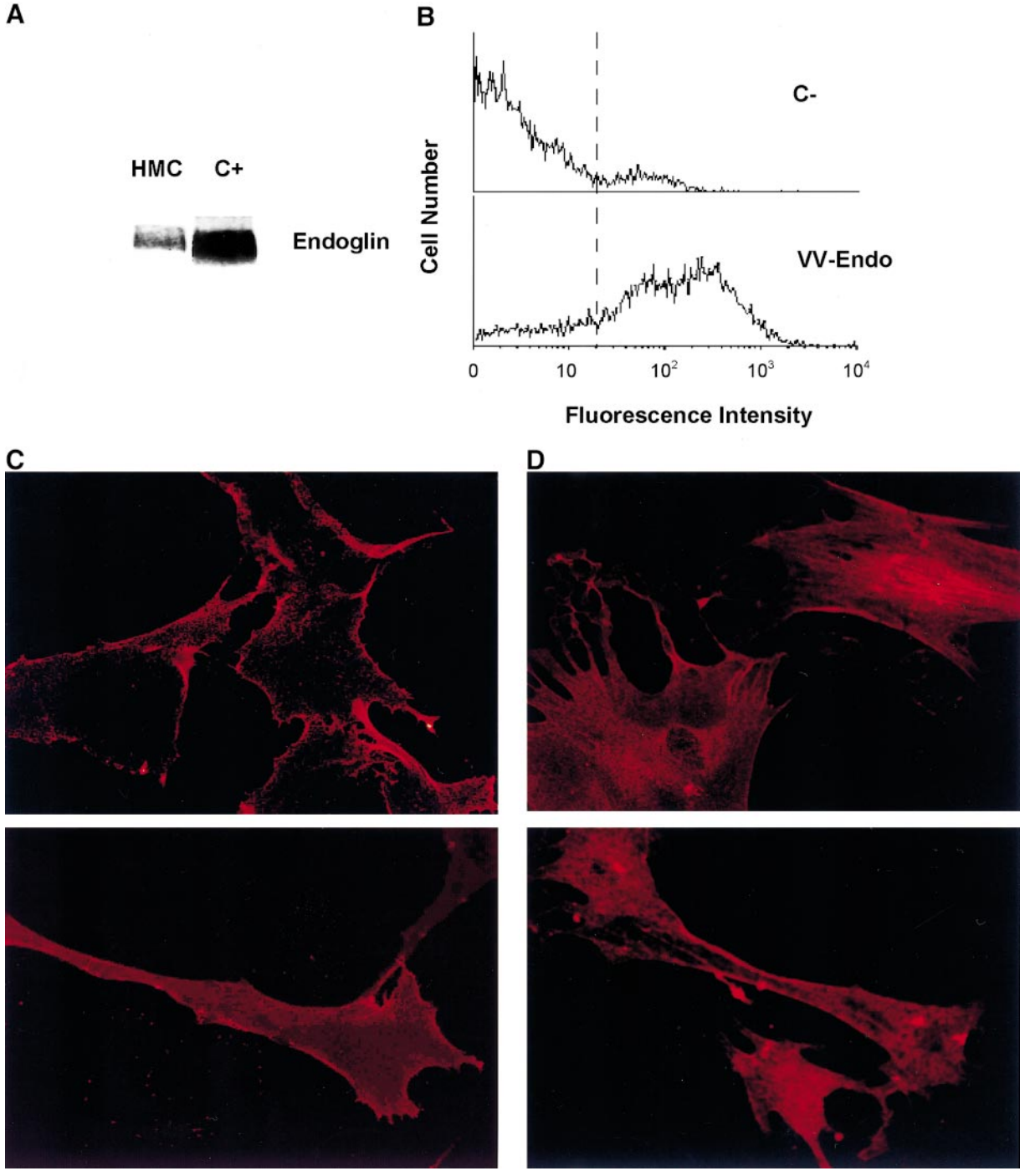

FIG. 1. Endoglin protein expression in cultured mesangial cells. (A) Western blot analysis. Extracts from human mesangial cells (HMC) were electrophoresed and transferred to a nitrocellulose membrane. The presence of endoglin was revealed with the anti-endoglin mAb P4A4 using a chemiluminescence assay. Rat myoblast transfectants expressing human endoglin were used as a positive control (C+). (B) Expression of endoglin in rat mesangial cells was analyzed by flow cytometry for surface expression of endoglin. After trypsinization, cells were stained by indirect immunofluorescence with the rabbit polyclonal antibody VV-endo (anti-endoglin). Control staining with nonimmune rabbit IgG was used to delineate the limit of the specific fluorescence and it is represented by the broken vertical line. Immunocytochemistry was performed on freshly plated mesangial cells. Human mesangial cells (C) were incubated with the mouse mAb P3D1 (anti-human endoglin). Rat mesangial cells (D) were incubated with the rabbit polyclonal antibody VV-endo (anti-endoglin). The presence of endoglin was revealed by incubation with the corresponding Cy3-conjugated secondary antibody, followed by immunocytochemical analysis. Endoglin was found to localize homogenously on the plasma membrane of the mesangial cells.

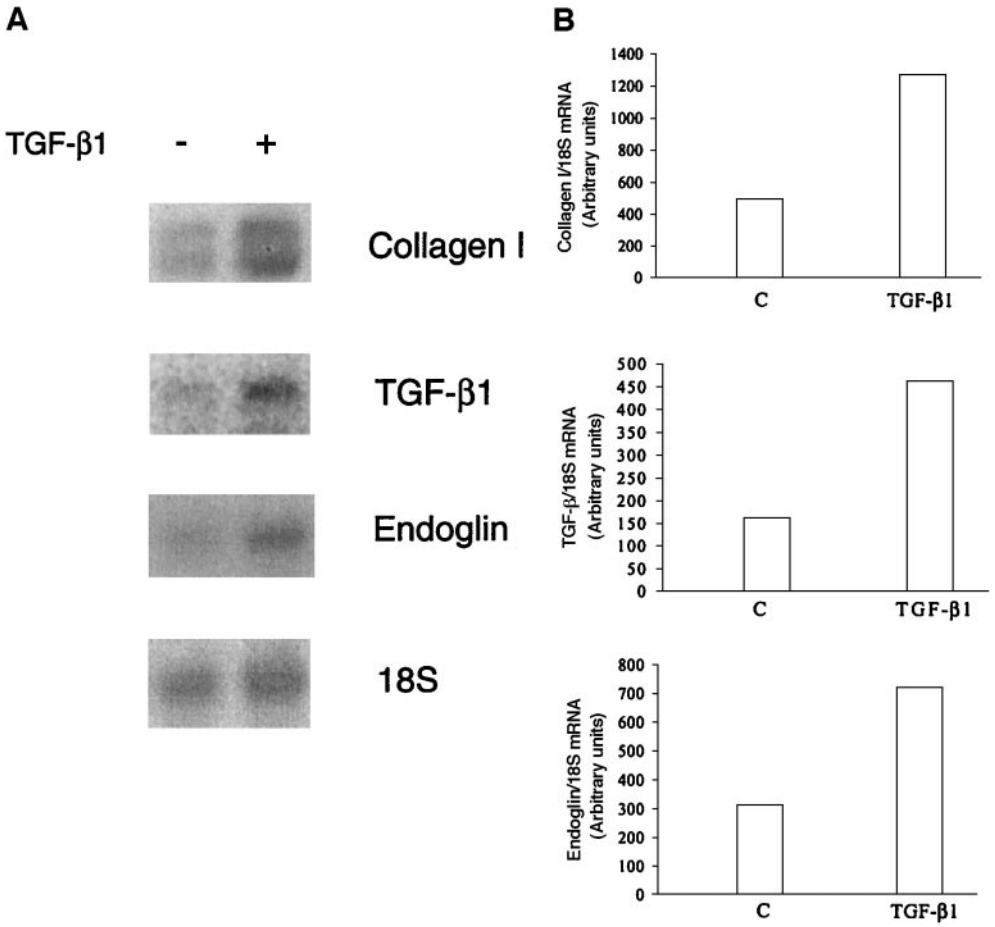

FIG. 2. Effects of TGF-β1 on expression of collagen I, TGF-β1, and endoglin mRNA in rat mesangial cells. (A) Cells were incubated with and without 10 ng/ml TGF-β1 for 24 h and the levels of specific transcripts of collagen, TGF-β1, and endoglin mRNA were detected by Northern blot analysis. RNA blots were stained with ethidium bromide to visualize the integrity of the 28S and 18S ribosomal subunits. The levels of collagen I, TGF-β1, and endoglin mRNA were quantitated by densitometric analysis using the imageQuant software and are illustrated in B.

nocytochemistry studies revealed that endoglin is present on the cell membrane of both human and rat mesangial cells, in a diffuse pattern (Figs. 1C and 1D) respectively. Expression of endoglin was also substantiated by the fact that Northern blot analysis identified the endoglin transcripts in rat mesangial cells (Fig. 2).

Effect of exogenous TGF-β1 on collagen, TGF-β1 and endoglin expression in mesangial cells. To examine the effect of exogenous TGF-β1 on endoglin expression, quiescent rat mesangial cells were incubated with 10 ng/ml TGF-β1 for 24 h. This incubation stimulated the production of collagen by mesangial cells, as demonstrated by the increase in collagen I mRNA, assessed by Northern blot (Fig. 2) and by the accumulation of collagen in the incubation medium (Fig. 3A). In addition, TGF-β1 also induced the expression of TGF-β1 mRNA (Fig. 2). Under these conditions, TGF-β1 induced an increase in endoglin expression, as shown by Western and Northern blots (Figs. 3B and 1) respectively.

DISCUSSION

The present study is the first to demonstrate that endoglin, a membrane glycoprotein which plays a role in modulating TGF-β signaling and vasculogenesis, is expressed at both mRNA and protein level in human and rat cultured mesangial cells. In addition, endoglin expression in rat mesangial cells is up-regulated by incubation with TGF-β1. TGF-β1-induced endoglin expression has also been demonstrated in cultured human monocytes (17). Roy-Chaudhury *et al.* (22) have described in biopsies from kidneys without renal disease that endoglin was present within the glomerular mesangium and interstitium in normal kidneys, whereas endoglin was found in significantly higher levels in the diseased kidneys from patients with glomerulonephritis. We have recently reported that endoglin expression is up-regulated in the kidneys of rats with renal mass reduction (23) and in the ligated kidney of rats with unilateral ureteral ligation (31). The

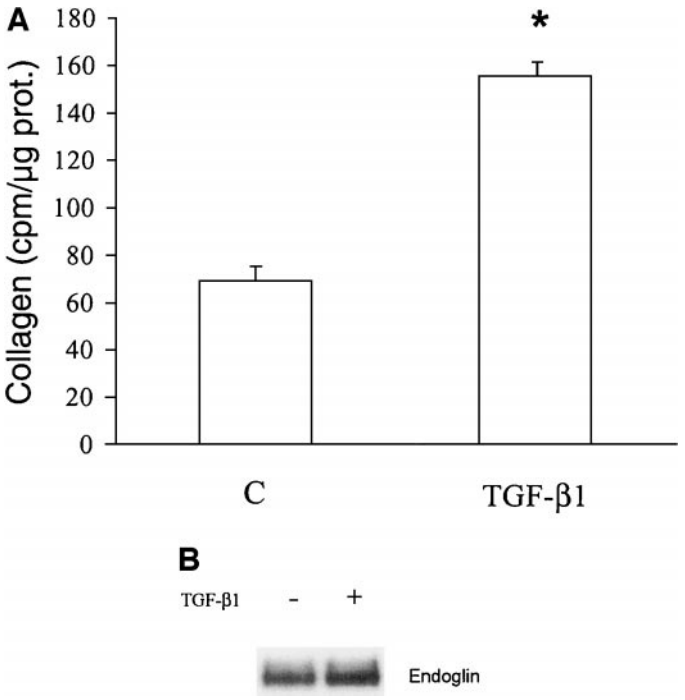

FIG. 3. Effects of TGF-β1 on mesangial cell collagen accumulation and endoglin expression. Quiescent rat mesangial cells were incubated with and without 10 ng/ml TGF-β1 for 24 h. (A) Metabolic radiolabeling with [^{3}H]proline was carried out during 24 h. Collagen accumulation was measured according to the amount of radioactivity incorporated into the protein fraction of the medium. Each value represents the mean ± SEM of six samples. *$P < 0.001$ versus control (C). (B) Western blot analysis of human endoglin. Human mesangial cells were lysed and total extracts were transferred to nitrocellulose. Specific immunodetection was performed by incubation with the mAb P4A4 (anti-human endoglin) followed by peroxidase-conjugated goat anti-mouse IgG. Endoglin was detected by using a chemiluminescence assay.

biological significance of the increased endoglin expression in the diseased kidneys remains to be determined. Endoglin is a component of the TGF-β receptor system and probably functions as a modulator of the interaction between TFG-β and its signaling receptors (15–18). The pro-matrix effects of TGF-β are well recognized to be a key factor in the glomerulosclerosis and interstitial fibrosis that characterizes chronic progressive renal disease. Furthermore, transgenic mice overexpressing TGF-β1 develop severe glomerulosclerosis (32). TGF-β1 causes ECM accumulation by enhancing glomerular mesangial cell production of collagen and fibronectin, suppressing the expression of ECM degrading proteases and increasing the synthesis of ECM protease inhibitors (9–11, 33). In addition, we have reported that endoglin is overexpressed in experimental models of renal fibrosis (23, 31). Thus, it is tempting to speculate that the increased expression of endoglin by renal cells during renal disease contributes to regulate the stimulation of ECM production by TGF-β1.

The cellular TGF-β responses modulated by endoglin include the synthesis of extracellular matrix components such as collagen, fibronectin and plasminogen activator inhibitor type 1 (18). We have already obtained some preliminary evidence showing that endoglin overexpression can diminish, rather than enhance, the effect of TGF-β on extracellular matrix synthesis and cell proliferation (17, 18). Thus, it can be suggested that the presence of endoglin as a part of the TGF-β receptor system could be important in modulating the extent of ECM protein production by renal cells following the binding of TGF-β1.

In summary, the finding that endoglin is expressed in human and rat mesangial cells, and that it is upregulated by TGF-β1 suggests that it may play an important role in regulating the effects of TGF-β1 in chronic renal diseases associated with glomerulosclerosis.

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