Endoglin Upregulation During Experimental Renal Interstitial Fibrosis in Mice

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Abstract—The goal of the present study was to evaluate the role of endoglin, a transforming growth factor-ß1 (TGF-ß1) accessory receptor, in the pathogenesis of renal fibrosis. This was achieved by testing a model of tubulo-interstitial fibrosis induced by unilateral ureteral obstruction in endoglin heterozygous (Eng+/−) mice. Northern and Western blot analysis revealed that endoglin expression in kidneys of these mice was significantly reduced compared with Eng+/+ littermates. Pronounced interstitial fibrosis induced by ureteral obstruction was confirmed histologically by Masson’s trichromic staining and by increased immunostaining for fibronectin and laminin without significant differences between Eng+/+ and Eng+/− mice. Ureteral obstruction induced significant increases in α2(I) and α1(IV) collagen, fibronectin, and TGF-ß1 mRNA levels, as well as in total kidney collagen but changes were similar in Eng+/- and Eng+/+ mouse kidneys. Ureteral obstruction also induced a 2-fold increase in endoglin mRNA levels in both Eng+/− mice and Eng+/+ mice, which was confirmed by Western blot analysis. Thus, the present study provides clear evidence that endoglin is upregulated in the kidneys of mice with interstitial fibrosis induced by unilateral ureteral ligation. However, Eng+/- mice do not show any changes in the severity of renal disease induced in this model when compared with normal mice, suggesting that the absolute level of endoglin is not critical for the effects of TGF-ß1 in the renal fibrosis process. (Hypertension. 2002;40:713-720.)

Key Words: collagen ■ renal disease ■ fibrosis ■ transforming growth factors

End-stage renal disease is one of the most prevalent complications of hypertension, diabetes, and intrinsic renal diseases.1 It is characterized by the progressive accumulation of extracellular matrix (ECM) in the glomeruli (glomerulosclerosis) and between tubules (tubulo-interstitial fibrosis). Many studies suggest that it is the severity of tubulo-interstitial fibrosis that best correlates with the degree of renal impairment and the risk for renal failure progression.2 There is much accumulated evidence about the role of transforming growth factor-ß1 (TGF-ß1) in promoting renal fibrosis. The upregulation of TGF-ß1 and its receptors (types I and II) has been reported in several models of experimental renal fibrosis.3 Renal fibrosis was also diminished by administration of antibodies or antisense oligonucleotides to TGF-ß1 and by transfection with decorin, a molecule that can bind and inactivate TGF-ß1.4,5

Endoglin (CD105) is a 180-kD homodimeric membrane glycoprotein expressed by human endothelial cells,7 macrophages,8 vascular smooth muscle cells,9 mesangial cells,10,11 and fibroblasts.12 Endoglin binds TGF-ß1 and TGF-ß3 in human endothelial cells,13 in association with the type II receptor.14 The gene encoding endoglin is mutated in the autosomal dominant vascular disorder known as hereditary hemorrhagic telangiectasia (HHT) type 1.15 Endoglin heterozygous (Eng+/−) mice can develop clinical signs of HHT, particularly the 129/Ola strain, confirming the haploinsufficiency model for the disorder.16,17 Mice deficient in endoglin (Eng−/−) die at mid-gestation with vascular and cardiovascular defects.16,18,19 as do mice deficient in Tgfbr1,20 Tgfbr2,21 or Tgfbr1,22 suggesting an important role for endoglin in mediating effects of TGF-ß1.23

Endoglin has been detected in normal kidneys.10 It has been shown by immunohistochemistry to be overexpressed in biopsies from patients with chronic progressive renal disease.23 We have also reported that endoglin is upregulated in a model of hypertension and renal fibrosis induced by 5/6 renal mass reduction in rats.24 However, in spite of the association between renal fibrosis and upregulation of endoglin, there is no evidence for the role of this glycoprotein in renal fibrosis.

Unilateral ureteral obstruction (UUO) is an intervention that leads to tubulo-interstitial fibrosis of the obstructed

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kidney, likely via TGF-β1 overexpression. The goal of the present study was to evaluate the effect of UUO on endoglin expression and on renal fibrosis in asymptomatic C57BL/6-Eng+/− mice.

Methods

Animals and Disease Model

Eng+/− mice were generated by homologous recombination using embryonic stem cells of 129/Ola (129) origin, as previously described, and by backcrossing onto the C57BL/6 (B6) background. B6-Eng+/− mice of N4 generation were used in the present studies, as well as their B6-Eng+/+ littermate controls. These mice do not show clinical signs of HHT. Genotypes were determined by PCR analysis using 2 sets of primers amplifying a 470-bp product in the mutated allele and a 300-bp product in the wild-type allele.

Mice were kept in ventilated rooms in a germ-free facility under controlled conditions of light, temperature, humidity, food, and water (Unidad de Experimentación Animal, Edificio Experimental, University of Salamanca, Spain) and fed on a standard chow for laboratory mice (Panlab) and water ad libitum. Studies were performed in males, 4 to 6 months old and weighing 20 to 25 g. Studies were designed in paired form with the Eng+/− and Eng+/+ mice. The left ureter of each Eng+/− and Eng+/+ mouse (13/group) was ligated with 7.0 silk suture at 2 separate locations under general anesthesia induced by intraperitoneal injection of ketamine chlorohydrate/diazepam/atropine (1.56/12.0/0.3 mg/kg body weight). Preliminary findings showed that maximal changes in renal fibrosis occur at 15 days post-UUO in rats. Studies were thus performed using renal tissue at 15 days post-UUO with these experimental groups: right nonligated ureter of Eng+/− (NL Eng+/−) and Eng+/+ (NL Eng+/+) mice and left ligated ureter of Eng+/− (L. Eng+/−) and Eng+/+ (L. Eng+/+) mice. Data obtained from mice with manipulated but nonligated ureters of the nonligated kidney in UUO mice were indistinguishable.

In all procedures, animals were treated in accordance with the international and national institution’s guidelines for the care and use of laboratory animals: Conseil de l’Europe (published in the Official Daily N. L358/1-358/6, 18 December 1986), Spanish Government (published in Boletín Oficial del Estado N. 67, pp 8509–8512, 18 March 1988, and Boletín Oficial del Estado N. 256, pp 31349–31362, 28 October 1990), and the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23).

Kidney Tissue Preparation

The L. and NL (contralateral) kidneys of each animal were obtained under ether anesthesia and decapsulated. Kidneys (n=4 per group), recovered for protein and total RNA extraction, were frozen in liquid nitrogen, weighed, individually ground into a fine powder, and stored at −80°C until use. Intact kidneys (n=5 per group) were frozen and stored at −80°C for total collagen measurement. For histological studies, animals were perfused with heparinized saline solution (0.9% sodium chloride, 37°C) and 4% buffered formalin. Kidneys (n=5 per group) were removed, cut in halves, fixed for 24 hours in 4% buffered formalin, processed, and embedded in paraffin.

Total Kidney Collagen Content

Frozen powdered kidney was homogenized in distilled water to obtain a weight:volume ratio of 10% and hydrolyzed in 12 N HCl at 115°C for 24 hours. The hydroxyproline content was measured by a previously described spectrophotometric method. Total renal collagen was calculated assuming that collagen contains 12.7% hydroxyproline by weight. Final results were expressed as micrograms per milligram of total protein.

Northern Blot Analysis

Total kidney RNA was isolated from each animal using the guanidine thiocyanate-phenol-chloroform method, size-fractionated by electrophoresis (20 μg/lane) through a denaturing 1% agarose–2.2 mol/L formaldehyde gel and transferred to a nylon membrane. The following cDNA probes were used: a 938 bp BamHI/AccI fragment of mouse endoglin inserted into pBluescript M13+ plasmid, 2 ECORI fragments of 451 and 408 bp corresponding to mouse TGF-β1 and fibronectin, respectively, and inserted into the pGEMT plasmid (Jean Pierre Girolami, U388 INSERM, Toulouse, France), a 600-bp PstI fragment of rat collagen α1 (I) and a 1600-bp ECORI/HindIII fragment of mouse collagen α1 (IV) inserted into pUC18 plasmid (D. Rodriguez-Puyol, University of Alcalá de Henares, Madrid, Spain), and a 1500-bp ECORI fragment of human ribosomal subunit 18S in pBluescript SK+. cDNA probes (30 ng) were radiolabeled with γ-32P dCTP (3000 Ci/mmol) by random priming. Results were expressed as mRNA/18S relative optical density ratios to correct for any RNA-loading inequality. We have assessed that 18S RNA control is not saturated and that we work in the lineal range or the relation intensity of the band/mRNA amount.

Western Blot Analysis

Renal cell membranes were obtained by differential centrifugation of homogenized powdered tissue. Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (0.45 μm, Bio-Rad). The membrane was incubated for 1 hour with the rat anti-mouse endoglin monoclonal antibody M1718, kindly provided by Dr Butcher, Stanford University Medical School, Calif. The membrane was then incubated for 30 minutes in standard buffer containing horseradish peroxidase–labeled goat anti-rat IgG (Santa Cruz Biotechnology) diluted 1:10000. The presence of mouse endoglin monomers, with a characteristic band of 90 to 95 kDa, was detected using a chemiluminescence assay (ECL detection reagents, Amersham) and recorded on a x-ray film.

Histological Analysis

Sections 3-μm thick were cut, mounted on glass slides, and stained either with hematoxylin–eosin or Masson’s trichrome for light microscopy analysis.

Immunohistochemical Studies

Immunohistochemistry was performed on buffered formalin fixed, paraffin-embedded tissues. Briefly, 3-μm sections were deparaffinized in xylene and rehydrated in graded ethanol before staining with the peroxidase-antiperoxidase method. An antigen retrieval process was carried out on slides with citrate solution (BioGenex) in a microwave oven for 3 minutes. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Primary antibodies were monoclonal anti-human fibronectin, clone 568 (1:50 dilution; Biogenex), rabbit anti-laminin (prediluted; Biogenex), rabbit anti-human TGF-β1 (1:50 dilution; Sta. Cruz Biotechnology), and monoclonal anti-human CD105 endoglin, clone SN6h (1:400 dilution; DAKO). Following washes in PBS, the sections were sequentially incubated with the DAKO LSAB+HRP 2 system, and f, 3,3′-diaminobenzidine (DAB) was used as chromogen. Negative controls were prepared without primary antibody.

Morphometric Studies

Renal sections 5-μm thick were stained with Sirius red to evaluate the area occupied by collagen fibrils. From each kidney, a total of 25 interstitial random cortical fields were captured at 20-fold magnification using a green optical filter (IF 550) and a high-resolution videocamera (SONY CCD-iris) connected to a light microscope (LEITZ Laborlux S). The area occupied by collagen was measured using a computerized image analysis system (Fibrosis HR®, Master Diagnostica) as previously described. The values obtained for tubulointerstitial fibrosed tissue were expressed in square micrometers.
The tubulo-interstitial space occupied by extracellular matrix was markedly increased after UUO, but to a similar extent in nonligated kidneys from Eng−/+ mice (Figure 3).

Immunostaining of fibronectin and laminin showed a normal localization in tissues from non-UUO kidney groups. For instance, fibronectin expression was circumscribed to mesangium, with low levels seen around tubules (Figures 4a and 4b). A marked increase in glomerular and interstitial fibronectin was seen in tissues from obstructed kidneys, but no significant differences were observed between Eng+/+ and Eng−/+ mice (Figures 4c and 4d). Laminin was well detected in glomerular capillaries and mesangium and along the tubular basal membrane and interstitial capillaries (Figures 4e and 4f). It was also significantly increased in the obstructed kidneys (Figures 4g and 4h).

**Results**

**Severe Renal Fibrosis After Unilateral Ureteral Ligation**

The total collagen content was similar in nonligated kidneys from Eng+/+ and Eng−/+ mice. It was, however, significantly increased in both groups of mice in the ligated kidneys, 15 days after UUO. No difference in collagen content was found between Eng+/+ and Eng−/+ mice following UUO (Figure 1).

Light microscopy examination of the hematoxylin (not shown) or Masson trichrome–stained sections showed that nonligated kidneys from Eng−/+ and Eng−/+ mice had a normal histological appearance and showed no fibrotic lesions (Figures 2a and 2b). On the contrary, obstructed kidneys from both Eng+/+ and Eng−/+ mice exhibited a wide range of alterations caused by the unilateral ureteral ligation. The total thickness of the cortex and medulla was diminished. Tubules showed changes ranging from a dilated lumen with a flattened epithelium to necrosis of varying degrees. In most cases, an interstitial inflammatory infiltrate was observed, mainly in the perivascular areas. Masson trichrome revealed peritubular interstitial fibrosis irregularly distributed and more pronounced around blood vessels (Figures 2c and 2d). No evident alterations, but a slight increase in mesangial tissue, were seen in renal corpuscles. No noticeable differences were observed between Eng+/+ and Eng−/+ mice.

Similar findings were observed when the extent of tubulo-interstitial fibrosis was evaluated by staining with Sirius red. The tubulo-interstitial space occupied by extracellular matrix was markedly increased after UUO, but to a similar extent in Eng+/+ and Eng−/+ mice (Figure 3).

**TGF-β1 and Matrix Protein Gene Expression After Unilateral Ureteral Ligation**

Northern blot analysis revealed no differences between Eng+/+ and Eng−/+ mice with respect to the levels of mRNA...
for α2(I) and α1(IV) collagens, fibronectin, and TGF-β1 in the nonligated kidneys of Eng<sup>+/+</sup> and Eng<sup>−/−</sup> mice. UUO induced a significant increase in α2(I) and α1(IV) collagen, fibronectin, and TGF-β1 mRNA in Eng<sup>+/+</sup> mouse kidneys. Similar changes occurred in the ligated kidneys from Eng<sup>−/−</sup> mice (Figure 5).

TGF-β1 expression was not detected in glomeruli or arterial blood vessels from non-UUO Eng<sup>−/−</sup> kidneys (Figures 6a and 6b). Similarly, kidney tissues from non-UUO Eng<sup>+/+</sup> showed no TGF-β1 glomerular expression, except for low levels in cortical arterial walls (Figures 6e and 6f). However, intense anti-TGF-β1 reactivity was detected in glomeruli, arterial walls, and interstitial fibrotic tissues from both obstructed groups, but no differences were observed between Eng<sup>+/+</sup> and Eng<sup>−/−</sup> mice (Figures 6c, 6d, 6g, and 6h).

Figure 4. Light micrographs of fibronectin (a through d) and laminin (e through h) immunostaining. Fibronectin is almost restricted to mesangial matrix in nonligated kidneys from Eng<sup>−/−</sup> (a) and Eng<sup>+/+</sup> mice (b). However, markedly increased glomerular and interstitial fibronectin expression is seen after unilateral ureteral obstruction in both Eng<sup>+/+</sup> (c) and Eng<sup>−/−</sup> mice (d). Laminin immunostaining is observed in mesangium and basal membranes surrounding tubules and in interstitial capillaries in nonobstructed kidneys from Eng<sup>+/+</sup> (e) and Eng<sup>−/−</sup> mice (f), whereas a strong staining is seen in obstructed kidneys from Eng<sup>−/−</sup> (g) and Eng<sup>+/+</sup> animals (h). Bar is 100 μm.

Figure 5. a, Northern blot analysis of renal mRNA expression following 15 days of UUO in Eng<sup>−/−</sup> and Eng<sup>+/+</sup> mice. b, Graphs illustrating the average ratio mRNA/18S densitometric values for collagen α2(I), collagen α1(IV), fibronectin, TGF-β1, and endoglin from at least 3 different Northern blots performed in similar conditions. #, *Statistically significant differences (P<0.05 and P<0.01, respectively) versus experimental groups lacking significance symbols.
Increased Endoglin Expression in a Murine Model of Kidney Fibrosis

Northern blot analysis of total kidney mRNA revealed that endoglin expression in kidneys of Eng<sup>−/−</sup> mice was about half that in kidneys from Eng<sup>+/+</sup> littermates, as expected (Figure 5). UUO induced a significant (2.1-fold) increase in endoglin mRNA levels in Eng<sup>+/+</sup> animals. The UUO-mediated increase was similar (1.9-fold) in Eng<sup>−/−</sup> mice. Western blot analysis of kidney plasma membrane extracts also revealed a lower amount of endoglin in Eng<sup>−/−</sup> mice than in their littermate controls (Figure 7). UUO induced an increase in this glycoprotein in kidneys of both Eng<sup>+/+</sup> (2.4-fold) and Eng<sup>−/−</sup> (2.3-fold) animals (Figure 7).

Endoglin expression was not detected by immunohistochemistry in tissue from contralateral, nonligated kidneys from Eng<sup>−/−</sup> mice (Figures 8a and 8b). This lack of staining is probably due to the low amount of endoglin present in these vessels, as demonstrated by Western and Northern blot analyses, and to the low sensitivity of the antibody used (anti-human endoglin) in formalin-fixed murine tissues. Only a slight expression of endoglin was seen in glomerular capillaries from obstructed Eng<sup>−/−</sup> mouse kidneys (Figure 8c),
with almost no expression in arterial endothelium (Figure 8d). Kidneys from Eng+/−/− mice showed clear endoglin expression in glomerular and interstitial capillaries (Figure 8e) and in interlobar arterial endothelium (Figure 8f). In kidneys from Eng+/−/− mice with ureteral obstruction, endoglin localization was similar, but the level of expression was higher than in the unobstructed kidney (Figures 8g and 8h).

**Discussion**

Experimental unilateral ureteral obstruction (UUO) serves as an excellent model of renal tubulo-interstitial inflammation and fibrosis. It is characterized by a slight and transient uremia and the absence of severe hypertension. From a histological point of view, it is associated with an increase in mononuclear cell infiltrate and proliferation of interstitial cells within the first few days following ureteral ligation. Within 7 to 14 days, the obstructed kidney develops interstitial fibrosis as demonstrated by increased synthesis of several extracellular matrix proteins such as collagens type I, III, and IV, fibronectin, and heparan sulfate proteoglycans. Our results demonstrate that UUO can induce tubulo-interstitial fibrosis in C57/BL6 mice. It was characterized by increased extracellular matrix deposition in the interstitial space, as demonstrated by increased collagen content, more intense extracellular matrix staining by the Masson’s technique, and increased laminin immunostaining. It was also accompanied by a higher expression of type I and IV collagen and fibronectin mRNA in the obstructed compared with the nonobstructed kidney.

This study also detected a marked increase in renal endoglin mRNA and protein levels after unilateral ureteral obstruction in mice. The mechanism responsible for the increase in endoglin expression is not clear but could be related to overexpression of TGF-β1 and the renin-angiotensin system in the ligated kidney. In this study and others, it has been demonstrated that UUO induces an increase in TGF-β1 expression. Additionally, TGF-β type I and type II receptors were also overexpressed during chronic UUO.
TGF-β1–induced endoglin expression was also shown in cultured human monocytes, in the U-937 monocyte line,\textsuperscript{34} and in cultured mesangial cells.\textsuperscript{11} Thus, increased endoglin expression in kidneys with UUO may occur as a consequence of the increase in TGF-β1. Recently, it has been reported that angiotensin II induces endoglin overexpression in cultured endothelial cells.\textsuperscript{35} Previous studies have demonstrated that angiotensin II increases TGF-β1 synthesis in fibroblasts, smooth muscle cells, and renal mesangial cells.\textsuperscript{36,37} Angiotensin II also induced the expression of TGF-β type I and type II receptors.\textsuperscript{38} Previous studies reported increased renin mRNA and protein in the UUO kidney and decreased expression in the contralateral kidney.\textsuperscript{32} Angiotensin-converting enzyme activity was also increased in UUO, but not in contralateral kidney. This is associated with a transient increase in plasma renin activity. However, the present study and others\textsuperscript{31,32} demonstrated that TGF-β1 mRNA and protein (as detected by immunostaining) are increased in the obstructed, but not in the contralateral, kidney. In addition, treatment with enalapril, an inhibitor of angiotensin II synthesis, or losartan, an angiotensin II type I receptor, decreases TGF-β1 mRNA in UUO, but not in contralateral kidneys.\textsuperscript{31,32} This suggests that the locally formed angiotensin II, but not the circulating one, is involved in the stimulation of TGF-β1 mRNA expression in an autocrine-paracrine effect. Thus, we can suggest that UUO induces a local activation of the renin-angiotensin system in the ligated kidney that leads to an increase in TGF-β1 synthesis in the UUO kidney, which, in turn, causes increased renal endoglin expression. This could explain the lack of endoglin overexpression in the contralateral kidney.

The biological significance of the increased endoglin expression in the interstitium of diseased kidneys remains to be elucidated. Endoglin is a component of the TGF-β receptor system that functions as a modulator of the interaction between TGF-β and its signaling receptors.\textsuperscript{39} The promatrix effects of TGF-β are well recognized as a key factor in the glomerulosclerosis and interstitial fibrosis characteristic of chronic progressive renal disease. Furthermore, transgenic mice overexpressing TGF-β1 develop severe glomerulosclerosis.\textsuperscript{40} 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.\textsuperscript{51,42} TGF-β1 induces collagen IV gene expression in both NIH-3T3 cells and NRK-49F normal rat kidney fibroblasts.\textsuperscript{43} Previous studies have obtained preliminary evidence that endoglin overexpression can diminish rather than enhance the effects of TGF-β on extracellular matrix synthesis and cell proliferation.\textsuperscript{44–46} Thus, one might have expected that the increased expression of endoglin by renal cells would contribute to a reduction in the amount of TGF-β1–induced ECM production. Consequently, we expected that endoglin haploinsufficiency would increase the severity of UUO-induced renal damage.

However, our results demonstrate clearly that Eng haploinsufficiency does not modify the severity of UUO-induced renal fibrosis. The most likely explanation is that, even in Eng-haploinsufficient mice, endoglin is present in quantities sufficient to partially counteract the profibrotic effects of TGF-β1. Alternatively, the negative results might be explained by endoglin and TGF-β1 protein and receptors not being coexpressed in the same cell type. In the present study, we have observed that after UUO, endoglin is overexpressed in glomerular and interstitial capillaries and in endothelium of large arteries. However, in this study and others,\textsuperscript{31,32} TGF-β1 overexpression was observed around blood vessels, mainly in the adventitia, and in connective fibrotic tissues of UUO kidneys. TGF-β receptors I and II were detected exclusively in renal tubules and were increased in both the obstructed and contralateral kidneys, relative to sham operated animals.\textsuperscript{33} Thus the lack of coexpression of endoglin, TGF-β1, and the receptors I and II might explain the similar extent of renal fibrosis in Eng-\textsuperscript{−/−} and Eng-\textsuperscript{+/−} mice.

**Perspectives**

The present study provides evidence that endoglin, a TGF-β–binding protein, until now associated with the pathogenesis of hereditary hemorrhagic telangiectasia type I, is upregulated in the kidneys of mice with interstitial fibrosis induced by unilateral ureteral ligation. However, mice haploinsufficient for endoglin do not show any change in the severity of UUO-induced renal disease when compared with their normal littermates, probably because endoglin, TGF-β1, and its receptors are overexpressed in different cell types. Because endoglin can counteract the fibrogenic effects of TGF-β1 in several in vitro systems, the precise mechanism of endoglin upregulation and the elucidation of its pathological or physiologic role in renal fibrosis could lead to further insights into this disease. It could also help to develop treatments aimed at prevention or even reversal of renal tubulo-interstitial fibrosis associated with chronic kidney diseases, diabetes, or hypertension.

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