Up-regulation of endoglin, a TGF-β-binding protein, in rats with experimental renal fibrosis induced by renal mass reduction

Ana Rodríguez-Peña, Marta Prieto, Annette Duwel, Juan V. Rivas, Nélida Eleno, Fernando Pérez-Barriocanal, Miguel Arévalo, Joshua D. Smith, Calvin P. H. Vary, Carmelo Bernabeu and José M. López-Novoa

Instituto ‘Reina Sofía’ de Investigación Nefrológica, Departamento de Fisiología y Farmacología, Departamento de Anatomía e Histología Humanas, Universidad de Salamanca, Salamanca, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain and 2The Center for Molecular Medicine, Maine Medical Center Research Institute, South Portland, Maine, USA

Abstract

Background. The central process in chronic renal failure is the progressive accumulation of extracellular matrix in the glomeruli and in the tubulo-interstitial space, resulting in renal fibrosis. Transforming growth factor-β1 (TGF-β1) up-regulation plays a major role in the genesis of renal fibrosis. Endoglin is a membrane glycoprotein that binds TGF-β1 and TGF-β3 with high affinity. An increased level of endoglin immunostaining has been demonstrated previously in biopsies from patients with chronic progressive renal disease. We have assessed the expression of endoglin in the rat 5/6th renal mass reduction (RMR) model.

Methods. One, 3 and 5 months after RMR, mean arterial pressure and renal function were measured, animals were sacrificed, renal fibrosis was evaluated quantitatively and the expression of endoglin was assessed by western blot, northern blot and immunohistochemistry.

Results. RMR induced a progressive increase in mean arterial pressure and urinary protein excretion. Renal corpuscular area, and mesangial and interstitial fibrosis increased with time after RMR. Immunohistochemical staining for endoglin demonstrated its expression mainly on the endothelial surface of major vessels. In kidneys 1 and 3 months after RMR, the expression of endoglin in renal corpuscles was limited to Bowman’s parietal epithelium. In rats 5 months after RMR, the immunoeexpression in glomerular endothelium was more marked. Northern blot analysis revealed that rats with RMR showed an increase in the expression of mRNA for endoglin, only at 5 months after RMR. Western blot analysis gave a different time course: a marked increase in the first month, a decrease in the 3rd month and a further increase in the 5th month after RMR.

Conclusions. The present study demonstrates increased endoglin expression in rats with severe hypertension and renal damage. This increased endoglin expression coincides with the period of higher renal damage and renal dysfunction.

Keywords: chronic renal failure; endoglin; glomerulosclerosis; renal mass reduction; transforming growth factor-β; tubulo-interstitial fibrosis

Introduction

The central process in chronic renal failure is the progressive accumulation of extracellular matrix in the glomeruli and in the tubulo-interstitial space. Extracellular matrix is subjected to continued remodelling, the rate of synthesis and degradation being similar under normal conditions. Accumulation of extracellular matrix occurs as a result of increased synthesis, decreased degradation or some combination of both processes. Transforming growth factor-β (TGF-β) is a member of a large family of proteins that has many biological effects including regulation of cellular proliferation, differentiation and migration, extracellular matrix formation, and modulation of the immune response [1,2]. TGF-β seems to play a major role in the genesis of renal fibrosis. First, the up-regulation of TGF-β expression in several models of experimental renal fibrosis, as well as the expression of both type I and type II receptor mRNA has been reported [3]. In addition, renal fibrosis has been improved by the administration of antibodies against TGF-β, by the administration of antisense oligonucleotides or by transfection with decorin, a
Endoglin and renal fibrosis

soluble molecule that can bind and inactivate TGF-β1 [4–6]. Endoglin is a 180 kDa homodimeric membrane glycoprotein expressed by human endothelial cells [7], macrophages [8], vascular smooth muscle cells and other cell types [9]. The gene encoding endoglin has been identified as the target gene for the autosomal dominant vascular disorder known as hereditary haemorrhagic telangiectasia type 1 [10]. Endoglin binds TGF-β1 and TGF-β3 with high affinity in human endothelial cells [11]. Recently it has been demonstrated that endoglin is up-regulated in biopsies from patients with chronic progressive renal disease [12]. However, in that study, endoglin was only detected by immunohistochemistry, and no detailed study of protein and mRNA expression or the time course of up-regulation was performed. The purpose of the present study was to assess the expression of endoglin by western blot, northern blot and immunohistochemistry in a model of renal fibrosis associated with hypertension and renal mass reduction (RMR), the rat 5/6th nephrectomy model.

Materials and methods

Disease model and experimental design

Experiments were performed in male Wistar rats weighing ~250 g. Rats were housed in standard cages in a daylight- (12 h), temperature- (20°C) and humidity- (60%) controlled room. Rats were allowed free access to food (standard rat chow containing 20% protein by weight) and water.

Rats were anaesthetized with 2-2.5% tribromoethanol and subjected to 5/6th RMR by ligation of two or three branches of the left renal artery and right uninephrectomy, as previously reported [13]. After 2 days, the survival rate was 60%. Verification of RMR was confirmed by elevated blood pressure, increased values for urinary protein excretion and visual inspection of the remnant kidney at the end of the study. Sham-operated animals were used as controls.

Each month after surgery, systolic and diastolic arterial pressure were measured in awake animals with a tail-cuff method ( Electro-sphygmomanometer, Letica LE 5000, Letica Barcelona, Spain). Then, body weight was measured and rats were placed into metabolic cages with free access to food and water during four equilibration days. On the two following days, urine was collected free of food and faeces. A sample of blood (0.15 ml) was also obtained from a cut in the tail tip collected into heparinized capillaries.

Plasma and urine creatinine levels were measured by a colorimetric method based on the Jaffé reaction. Endogenous creatinine clearance was used to estimate the glomerular filtration rate (GFR). Urinary protein excretion was determined by Bradford’s method.

Surviving RMR animals were distributed randomly into three groups: group 1, sacrificed 1 month after surgery; group 2, sacrificed 3 months after surgery; and group 3, sacrificed 5 months after surgery.

Morphological and morphometric studies

For this purpose, animals were anaesthetized with ether and the kidney was perfused with heparinized isotonic saline to clear the kidney of blood. A piece of the kidney, including cortex and medulla, was trimmed and fixed by immersion in 4% buffered formalin for 24 h. The blocks thus obtained were dehydrated in a graded series of ethanol washes and embedded in paraffin. Sections 3 µm thick were cut, mounted on glass slides and counterstained with either haematoxylin-eosin or Masson’s trichrome for light microscopy analysis.

For quantitative determination of glomerular and interstitial fibrosis, 5 µm sections were stained with sirius red. Images were captured using a high-resolution videocamera (SONY ccd-iris) connected to a light microscope (Leitz Laborlux S) using the 20 × objective and a green optical filter (IF 550). The evaluation and image analysis procedures were performed with specific software (Fibrosis HR® Master Diagnostica, Granada, Spain) as previously reported [13]. To increase the precision of this method further, the same person captured the images in a blind manner.

A total of 25 glomerular and 10 interstitial images for each slide were captured and processed, in six rats per group. For each image, the values, obtained in μm² were: (i) glomerular fibrosis area; (ii) glomerular section area; and (iii) tubulo-interstitial fibrosis area. To assess the variability of the technique, a single glomerular image was captured and processed 20 times. Coefficients of variation for the parameters analysed were: glomerular area, 0.19%; intra-glomerular fibrosis, 1.52%; and tubulo-interstitial fibrosis, 0.78%.

Renal endoglin expression was detected in 3 μm coated sections using a rabbit polyclonal anti-endoglin antibody [14], followed by a standard avidin–biotin complex method (Santa Cruz Biotechnology, CA), as previously described [15].

Northern and western blot

Renal endoglin expression was assessed by northern blot analysis. Total RNA was isolated from rat homogenates using the guanidinium thiocyanate–phenol–chloroform method. The probe used was a 360 bp SacI–SacII fragment of rat endoglin cDNA in pGEM-T. A probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Endoglin expression was also measured by western blot in renal plasma membranes obtained by differential centrifugation. The crude membrane preparation was resuspended in lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 20 mM Tris, pH 8.0 containing 100 U/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 60 μg of soy bean trypsin inhibitor and 1% NP-40). A sample containing 150 μg of protein was mixed with the same volume of 2× sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue). Proteins were separated on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (0.45 mm pore) at 400 mA for 2 h in a buffer containing 20 mM Tris and 190 mM glycine, pH 8.3. Non-specific binding blockade was performed overnight in blocking buffer (20 mM Tris containing 150 mM NaCl and 0.1% Tween-20) to which 8% dry milk was added. The membrane was then rinsed with blocking buffer, and incubated for 1 h in standard solution containing rabbit anti-endoglin polyclonal antiserum [14] at 1:1000 dilution. The membrane was washed four times for 10 min in blocking buffer and incubated for 30 min in standard buffer containing horseradish peroxidase-labelled goat anti-rabbit IgG (1:10000 dilution). The membrane was washed a further four times for 10 min with the standard incubation buffer.
The detection of rat endoglin was performed with the ECL chemiluminescence western blotting system from Amersham (Buckinghamshire, UK). For quantification, films were scanned and relative optical densities of each lane were obtained from the digitalized image using an image analysis program (MacBAS, version 2.2, Fuji, Japan).

Statistical methods

The Kolmogorov–Smirnov test was used to assess normality of the data distribution. One-way analysis of variance was used to compare differences between groups. Scheffé’s t-test for parametric data and the Kruskal–Wallis multiple comparison Z-value test for non-parametric data were used. A P-value < 0.05 was considered statistically significant. All the data are expressed as means ± SEM.

Results

RMR induced a progressive increase in mean arterial pressure and urinary protein excretion, as shown in Table 1. Light microscopy analysis of haematoxylin–eosin- and Masson’s trichrome-stained slides showed that most glomeruli in group 1 had a preserved architecture with only a slight mesangial matrix increment observed (Figure 1a). No alterations were detected in tubule interstitial area in these animals. Renal tissues from group 2 showed a moderate increment in mesangial matrix. In addition, inflammatory infiltration and low-grade interstitial fibrosis were observed in the interstitial tissue (Figure 1b). Group 3 showed evident glomerulosclerosis in a large number of renal corpuscles, with tuft adhesions to Bowman’s capsule. Inflammatory infiltration and focal tubular atrophy and dilatation with PAS-positive casts and fibrosis were observed in the interstitium (Figure 1c).

As shown in Table 2, computer-assisted quantitative detection of fibrous tissue revealed that renal corpuscular area, and mesangial and interstitial fibrosis were increased with the time of evolution.

Immunohistochemical staining for endoglin demonstrated expression mainly on the endothelial surface of large vessels in all groups of the study. The immunoreactivity in renal corpuscles was limited to Bowman’s parietal epithelium in group 1, while glomerulus showed very slight staining in this group and in group 2 (Figure 1d and e). The immunoreactivity in conserved glomerular endothelium from group 3 was more evident (Figure 1f).

Northern blot experiments revealed that rats with RMR showed an increase in the expression of mRNA for endoglin, only at 5 months after RMR (Figure 2A). Western blot analysis (Figure 2B) produced a different time course: a marked increase in the first month, a decrease in the 3rd month and a further increase in the 5th month after RMR.

Discussion

The present study demonstrates increased expression of endoglin in rats with severe hypertension and renal damage. The up-regulation of endoglin has been demonstrated by northern blot, western blot and immunohistochemistry, and this increased endoglin expression coincides with the major renal damage and renal dysfunction.

The increase in endoglin expression in the kidneys after RMR can be explained by the increase in TGF-β1 expression in remnant rat kidneys after subtotal nephrectomy [16,17]. TGF-β1 itself can stimulate the expression of endoglin in cultured human monocytes and in the U-937 monocytic line [18], mouse fibroblasts (Eleno et al., unpublished data) and human arterial smooth muscle cells [19] in vitro.

The biological meaning of the increased endoglin expression in glomeruli and interstitium of diseased kidneys remains to be determined. Since endoglin is a component of the TGF-β receptor system and the pro-matrix effects of TGF-β1 are recognized to be a key factor in the glomerulosclerosis and interstitial fibrosis that characterize chronic progressive renal disease [3,20], it is tempting to speculate that the increased expression of endoglin by interstitial fibroblasts or glomerular mesangial cells modulates the stimulation of extracellular matrix production. Endoglin is an accessory protein that requires the presence of the signalling receptor type II for ligand binding [21,22]. The association of endoglin with type I and II receptors, as well as the modulation of cellular TGF-β responses by endoglin overexpression have been demonstrated [18,21]. Among the cellular TGF-β responses

<table>
<thead>
<tr>
<th>Table 1. Effect of renal mass reduction on renal function in rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Sham</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Mean arterial pressure (mmHg)</strong></td>
</tr>
<tr>
<td><strong>Creatinine clearance (ml/min)</strong></td>
</tr>
<tr>
<td><strong>Urinary protein excretion (mg/24 h)</strong></td>
</tr>
<tr>
<td><strong>Months after RMR</strong></td>
</tr>
<tr>
<td><strong>1</strong></td>
</tr>
<tr>
<td><strong>2</strong></td>
</tr>
<tr>
<td><strong>3</strong></td>
</tr>
<tr>
<td><strong>4</strong></td>
</tr>
<tr>
<td><strong>5</strong></td>
</tr>
<tr>
<td><strong>Data are the mean ± SEM of nine animals per group.</strong></td>
</tr>
</tbody>
</table>
| *Significant difference (P < 0.05) vs sham-operated rat values.**
modulated by endoglin is the synthesis of the extracellular matrix components fibronectin and plasminogen activator inhibitor type 1. Thus, the presence of endoglin as a part of the TGF-β receptor system could be important in determining the extent of extracellular matrix protein production by renal cells following the binding of TGF-β1. It is unclear whether TGF-β1 binding to endoglin in the kidney would lead to increased or decreased TGF-β signalling. In this regard, 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 [18,21, Eleno et al., unpublished results, Smith et al., unpublished results]. Thus, endoglin hyperexpression could protect the kidney against the continuous profibrogenic effect of TGF-β1, thus fitting into the concept of ‘glomerular self-defence’ developed by...
Table 2. Quantitative morphometric study of renal fibrosis in rats with renal mass reduction

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Months after RMR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Corpuscular section area</td>
<td>10.558 ± 205</td>
<td>11.349 ± 310</td>
</tr>
<tr>
<td>Glomerular fibrosis</td>
<td>874 ± 64</td>
<td>959 ± 82</td>
</tr>
<tr>
<td>Tubulo-interstitial fibrosis</td>
<td>3225 ± 197</td>
<td>3811 ± 293</td>
</tr>
</tbody>
</table>

Data are shown in µm² as median ± SEM of six animals per group. No statistical differences were found between sham-operated and 1 month after RMR in the parameters observed.

*Statistically significant difference (P < 0.01; Kruskal-Wallis test) vs sham-operated or 1 month after RMR.

#Statistically significant difference (P < 0.01; Kruskal-Wallis test) vs 3 months after RMR.

Fig. 2. (A) Representative northern blot analysis for endoglin (upper blot) and GAPDH (lower blot) using kidney extracts from rats 1 month (lanes 1–3), 3 months (lanes 4–7) and 5 months (lanes 8–11) after RMR and sham-operated rats (lanes 12–15). This is a representative northern blot of three different experiments performed in the same conditions. The average endoglin/GAPDH signal (phosphorimager; n = 9 animals per group) is 1.44 ± 0.03 for sham-operated rats, 1.49 ± 0.11 for 1 month RMR, 1.60 ± 0.18 for 3 months RMR and 1.99 ± 0.05 for 5 months RMR (P < 0.05 vs sham-operated rats and 1 month RMR). (B) Representative western blot for endoglin using kidney extracts from rats 1 month (lanes 1 and 2), 3 months (lanes 3 and 4) and 5 months after RMR (lanes 5 and 6) and sham-operated rats (lanes 7 and 8). The figure shows one of three different but equally performed experiments. The average signal (densitometry; n = 6 animals per group) is 9.6 ± 1.8 for sham-operated rats, 15.5 ± 0.9 for 1 month RMR (P < 0.05 vs sham-operated rats), 11.5 ± 1.1 for 3 months RMR and 14.4 ± 1.1 for 5 months RMR (P < 0.05 vs sham operated rats and 1 month RMR).

Kitamura and Fine [23]. In support of this hypothesis is the finding that the time course of endoglin induction is slightly delayed as compared with that of the extracellular matrix deposition in the kidney upon RMR.

Endoglin also contains the RGD sequence in an exposed region of the extracellular domain [9]. This is an important recognition motif for a number of extracellular matrix proteins such as fibronectin, collagen, laminin and vitronectin for their specific integrin receptors. Thus, the presence of endoglin in the kidney could also serve to organize extracellular matrix in the sites of extracellular matrix deposition.

In conclusion, this is the first study demonstrating that endoglin, a TGF-β-binding protein, is up-regulated in the kidneys of rats with renal fibrosis induced by RMR. The mechanism of endoglin up-regulation and the elucidation of its pathological or physiological significance deserve to be investigated further.

Acknowledgements. The authors wish to thank Dr Neil Docherty for correcting the language of the manuscript. This study was partially supported by a grant from Dirección General de Enseñanza Superior e Investigación Científica (grant SAF1998-0095), Comision Interministerial de Ciencia y Tecnología (CICYT), Junta de Castilla y Leon (grant SA70/008B) and Comunidad de Madrid (CAM). A.R.-P. is a fellow of Fondo de Investigaciones Sanitarias (FIS). M.P. is a Fellow of Junta de Castilla y Leon and Fondo Social Europeo.
References

2. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-β (TGF-β). Growth Factors 1993; 8: 1–9
10. MacAllister KA, Grogg KM, Johnson DW et al. Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nature Genet 1994; 8: 345–351