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Albumin up-regulates the type II transforming growth factor-beta receptor in cultured proximal tubular cells[1]

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Albumin up-regulates the type II transforming growth factor-beta receptor in cultured proximal tubular cells.

Background. Clinical and experimental observations suggest that proteinuria is not merely a marker of chronic nephropathies, but may also be involved in the progression to end-stage renal failure. Filtered proteins are taken up by tubular cells, and overwhelming this system may lead to tubular synthesis of various proinflammatory and profibrogenic cytokines, including transforming growth factor-beta (TGF-β). TGF-β acts by first binding to specific receptors. We studied in an in vitro system using a well-defined mouse proximal tubular cell line (MCT cells) whether fatty acid-free bovine albumin modulates expression of specific receptors for TGF-β.

Methods. MCT (and LLC-PK1) cells were challenged in serum-free medium with different concentrations of albumin. Activation of a local renin-angiotensin system was tested by real-time polymerase chain reaction (PCR) for renin and angiotensinogen transcripts and determination of secreted angiotensin II (Ang II) by enzyme-linked immunosorbent assay (ELISA). Some cells were also treated with the AT_1 receptor antagonist losartan. TGF-β receptor types I and II mRNA levels were determined by Northern analysis whereas protein abundance was measured by Western blots. To test for a functional consequence of up-regulated TGF-β receptors, MCT cells were preincubated with albumin and subsequently treated with low-dose TGF-β that normally does not induce collagen type IV expression by itself. Downstream signaling events were detected by Western blots for phosphorylated Smad2. Scatchard assays with [^{125}I]TGF-β1 were performed to estimate affinity and number of specific binding sites. Different length TGF-β type II promoter constructs linked to CAT reporter were transiently transected into MCT cells to determine transcriptional activity.

Results. Incubation of MCT cells with 0.5 to 10 mg/mL albumin leads to an increase in type II TGF-β receptor mRNA and protein expression without influencing type I receptors. An increase in type II TGF-β receptor protein expression was detected after 12 hours of albumin incubation and was still detectable after 48 hours. The albumin-mediated increase in type II TGF-β receptor mRNA was attenuated in the presence of 1 μmol/L losartan, suggesting involvement of a local renin-angiotensin system. MCT cells treated with albumin significantly increased expression of angiotensinogen and renin transcripts and also secreted more Ang II into the culture supernatant. Analysis of transcriptional activity showed that promoter segments containing activating protein (AP-1)-binding sites are necessary for albumin-induced transcription of the TGF-β type II receptor. Binding assays revealed that albumin treatment significantly increased the overall binding sites as well as the affinity for TGF-β. This effect had functional consequences because MCT cells pretreated with albumin reacted with a stronger TGF-β–mediated phosphorylation of down-stream Smad2 and also increased collagen IV expression compared with control cells.

Conclusion. Our findings indicate that albumin up-regulates ligand-binding TGF-β receptors on cultured proximal tubular cells. Albumin-induced activation of local Ang II production appears to be responsible for this effect. This may amplify the matrix-stimulatory actions of TGF-β on tubular cells and could be a novel mechanism for how proteinuria exhibits pathophysiologic effects on tubular cells ultimately leading to tubulointerstitial fibrosis.

Proteinuria is a common finding in many renal diseases. Although traditionally considered to be a marker of renal injury, there is increasing evidence that proteinuria plays a key role in the tubulointerstitial changes that ultimately lead to renal insufficiency [1, 2]. There is a good correlation between the severity of tubulointerstitial injury and the amount of proteinuria [3]. Furthermore, clinical as well as experimental data suggest that proteinuria is closely involved in proinflammatory and profibrotic changes of the tubulointerstitium [4, 5]. In vitro studies have demonstrated that albumin, one of the major components found in proteinuria of glomerular origin, induces proinflammatory, profibrotic, and vasoactive factors in cultured proximal tubular cells [6–9]. A key factor leading to tubulointerstitial fibrosis and tubular atrophy is transforming growth factor-beta (TGF-β). It has been previously shown by in vitro and in vivo studies that albumin activates the tubular synthesis of TGF-β. [6, 9, 10]. This profibrogenic cytokine could then locally stimulate collagen biosynthesis in tubular cells and interstitial

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fibroblasts and also increase proliferation of fibroblasts [10]. Moreover, recent evidence suggests that TGF-β is important for the transdifferentiation of tubular cells into interstitial fibroblasts that may explain, at least to some extent, the tubular atrophy in chronic nephropathies [11]. Since the actions of TGF-β, including the stimulation of extracellular matrix (ECM) proteins, are mediated by specific receptors for this cytokine [12], it is possible that the overall profibrogenic activity of TGF-β is also influenced by the presence and surface density of specific receptors.

The present study was undertaken to test the hypothesis that albumin may modulate expression of TGF-β receptors in cultured murine proximal tubular (MCT) cells [13]. Our results suggest that albumin stimulates TGF-β receptor type II transcription, synthesis, and surface expression in cultured proximal tubular cells. This mechanism may contribute to the profibrogenic effects of TGF-β on proximal tubular cells under proteinuric conditions.

METHODS

Cell culture

MCT cells are a proximal tubular cell line originally isolated from adult SJL (H-2^s) mice [13]. Although MCT cells represent a permanent cell line, they do express many features of proximal tubular cells and have been used by many investigators as a model system to study the function of proximal tubules in vitro [13–16]. For some additional control experiments, LLC-PK1 cells, a porcine cell line with some properties of proximal tubular cells, were used [17]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 450 mg/dL glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L supplemental glutamine, and 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Eggenstein, Germany) at 37°C in 5% CO_2. Cells were passaged every 96 hours by light trypsination.

Angiotensin II (Ang II) secretion

To determine whether albumin may influence Ang II production and secretion, MCT cells were incubated for 24 hours with 0.5 to 10 mg/mL bovine serum albumin (BSA) (>97% albumin, essentially fatty acid-free from Sigma, Deisenhofen, Germany). This albumin was endotoxin free (<0.5 ng/mL) as determined with the endotoxin kit from Sigma. Ang II released into the supernatant was directly measured by a specific solid-phase immobilized epitope immunoassay [18] (IBL, Hamburg, Germany). The limit of detection for this assay is 0.5 pg/mL. A standard curve was constructed with cell culture medium. Cells were lysed and the protein content was determined by with the Lowry method. Ang II concentrations are expressed as pg/mg cellular protein. The experiment was independently repeated four to five times for each albumin concentration.

Receptor binding studies

For receptor binding studies, 10^4 cells were plated per well of a 24-well plate (Nunc Inc., Naperville, IL, USA), and were rested for 24 hours in serum-free DMEM. Cells were then treated for another 24 hours with 10 mg/mL albumin in DMEM or were kept in serum-free DMEM. Binding studies were done in assay buffer [serum-free DMEM with 5 mmol/L $MgCl_2$, 10 mmol/L ε-aminocaproic acid, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5% aprotinin] on a shaking platform at 4°C for 1 hour [19]. We have previously demonstrated that saturation of TGF-β binding sites occurred after 40 minutes under these conditions [19]. For saturation binding assays increasing amounts (0.5 to 100 pmol/L) of human recombinant [^{125}I]TGF-β1 (specific activity 2101 Ci/mmol) (Amersham, Braunschweig, Germany) were added [19]. Nonspecific binding was determined in the presence of 1 μmol/L human recombinant TGF-β1 (Sigma). After 1 hour, cells were gently washed three times with ice-cold phosphate-buffered saline (PBS), and proteins were precipitated with 10% trichloroacetic acid. Finally, cells were lysed in 500 μL 0.5 mol/L NaOH with 0.5% Triton X-100, and the amount of radioactivity was counted in a gamma scintillation counter. Control wells without radioactivity were also lysed and protein content was measured with the Lowry method. Nonspecific biding was subtracted and data were analyzed with the computer program Enzfitter (Elsevier-Biosoft, Cambridge, United Kingdom). Results are presented as Scatchard plots of saturation binding data and every point represents the mean of four determinations [19].

Western blots

A total of 10^6 quiescent cells were treated for 12 to 48 hours in serum-free DMEM with 1 to 10 mg/mL BSA. At the end of the stimulation period, cells were washed twice in PBS, and cell monolayers were directly lysed on ice in 150 μL of a buffer containing 2% sodium dodecyl sulfate (SDS) and 60 mmol/L Tris-HCl (pH 6.8) supplemented with a cocktail of protease inhibitors (Complete™) (Boehringer Mannheim, Germany) containing antipain-HCl, chymostatin, leupeptin, bestatin, pepstatin, phosphoramidon, aprotinin, and ethylenediaminetetraacetic acid (EDTA). The protein content was measured by a modification of the Lowry method. Protein concentrations were adjusted to 60 μg/sample, and 5% glycerol/0.03% bromophenol blue/10 mmol/L dithiothreitol (DTT) were added, then samples were boiled for 5 minutes. After centrifugation, supernatants were loaded onto a denaturing 7.5% SDS-polyacrylamide gel. High-molecular-weight markers (Rainbow markers) (Amersham), which comprise 45,000 to 200,000 D, served

as the molecular weight standards. After completion of electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (High-bond-N) (Amersham) in transfer buffer (50 mmol/L Tris-HCl, pH 7.0, 380 mmol/L glycine, 0.1% SDS, and 20% methanol). Filters were stained with Ponceau S to control for equal loading and transfer. The blots were blocked in 5% nonfat dry milk in PBS with 0.1% Tween 20 for 1 hour at 22°C. For the detection of TGF-β receptors, a 1:1000 dilution of rabbit polyclonal antibodies generated against the mouse TGF-β receptors types I and II (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Washes, incubations with horseradish peroxidase-conjugated antirabbit secondary antibodies, and detection using the enhanced chemiluminescence (ECL) reagent (Amersham) were performed according to the manufacturer's recommendations. For selected blots, membranes were washed for 30 minutes in PBS with 0.1% Tween-20 and were reincubated with a mouse monoclonal antibody against β-actin (1:2000 dilution) (Sigma) to control for small variations in protein loading and transfer. Western blots were independently repeated three times with qualitatively similar results. Exposed films were scanned with Fluor-S™ multi-imager (Bio-Rad Laboratories, Hercules, CA, USA), and data were analyzed with the computer program Multi-Analyst™ (Bio-Rad). The intensity of the bands from control cells (no albumin) was assigned an arbitrary value of 1.0. If Western blots were reincubated with an antibody against β-actin, the signal intensities were normalized to β-actin.

To investigate whether albumin-treatment of MCT cells may influence TGF-β–induced Smad signaling, cells were incubated for 24 hours in either serum-free DMEM or DMEM supplemented with 10 mg/mL albumin. Cells were then treated with 0.5 ng/mL recombinant TGF-β1 (Sigma) for 30 minutes. Western blots were performed with a rabbit antibody generated against phosphorylated Smad2 using a 1:500 dilution (Cell Signaling, Beverly, MA, USA). Membranes were reincubated with a monoclonal antibody against total Smad2 (Cell Signaling) (1:1000 dilution). Western blots for Smad2 were repeated twice with qualitatively similar changes.

Northern blots

Quiescent MCT cells or LLC-PK1 cells were stimulated for 24 hours with 1 to 10 mg/mL BSA. Some cells also received 1 μmol/L of losartan, an AT_1 receptor antagonist (gift of Merck, Sharp & Dohme, Munich, Germany). To test for a functional influence of upregulated TGF-β receptors, MCT cells were incubated for 24 hours with 1 mg/mL albumin. After washing of cells with serum-free medium, 0.5 to 2 ng/mL recombinant TGF-β1 (Sigma) were added for another 12 hours. After washing in RNAse-free PBS, cells were directly lysed with acid guanidinium thiocyanate, and total RNA was isolated. Equal amounts of total RNA (15 μg per lane) were denatured in formamide-formaldehyde at 65°C and electrophoresed through a 1.2% agarose gel containing 2.2 mol/L formaldehyde. Blotting, hybridization, and washing conditions were exactly as previously described [19]. cDNA probes for the mouse TGF-β receptors types I and II were constructed by reverse transcription-polymerase chain reaction (RT-PCR) based on the published sequences and were cloned into the TA vector (Invitrogen, Leek, The Netherlands). The probes used were a 306 bp *Eco*RI fragment encoding the mouse TGF-β receptor type I, and a 353 bp *Eco*RI fragment from the mouse TGF-β receptor type II. These cDNA probes have been previously used [19, 20]. For collagen type IV levels, a 1300 bp mouse α1(IV) PstI cDNA fragment was used [21]. For control hybridizations, a 2.0 kb cDNA insert of the plasmid pMCI encoding the murine ribosomal 18S band was used. Exposed films were scanned and analyzed as described for Western blots. A ratio between the intensities of the hybridization signals for TGF-β receptors or collagen type IV versus 18S was calculated. Signals from cells incubated in serum-free medium without albumin were considered a relative value of 1.0. Northern blots were repeated three times for TGF-β receptor expression and twice for collagen type IV hybridizations with qualitatively similar results.

Real-time PCR

To assess potential changes in angiotensinogen and renin mRNAs expression in MCT cells induced by albumin, real-time PCR was performed. After isolation of total RNA using the acid guanidinium thiocyanate method, possible remaining DNA was digested with RNAse-free DNAse I (Ambion, Austin, TX, USA). After addition of DNAse inactivator reagent (Ambion) and centrifugation, 1 μg total RNA from each sample was used for reverse transcription using the SYBR™ Green RP-PCR kit from Applied Biosystems (Foster City, CA, USA) with oligod$(T)_{16}$ primers and 1.25 U MultiScribe™ reverse transcriptase. Amplifications for angiotensinogen/renin and glyceraldehyde-3-phosphatase (GAPDH) were performed for each cDNA sample with SYBR™ Green PCR Master mix (Applied Biosystems) using the hot start technique with AmpliTaqGold™ DNA polymerase. The following primer were used: mouse angiotensinogen (5′-CCTACT TTTCAACACCTACGTTCACT-3′, 5′-GCTGTTGTCC ACCCAGAATTC-3′) [22], mouse renin (Ren-1^d) (5′-CTCAGCCAGGACTCGGTGAC-3′, 5′-CCAGCATG AAAGGGATCAGG-3′) [23], GAPDH (5′-ACCACA GTCCATGCCATCAC-3′, 5-′TCCACCACCCTGTTG

CTGTA-3′). The Abi Prism™ 7000 sequence detection system (Applied Biosystems) was used. After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, 40 cycles were performed at 95°C for 15 seconds, 58°C (angiotensinogen) or 59°C (renin, GAPDH) for 60 seconds, and 65°C for 60 seconds. After 40 cycles, the temperature was increased from 60 to 95°C to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using comparative C_T method with $2^{-\Delta\Delta CT}$. The results are given as relative expression of angiotensinogen or renin normalized to the GAPDH housekeeping gene. Signals from cells incubated without albumin were considered a relative value of 1.0. Real-time RT-PCR experiments were independently (stimulation of cells, RNA isolation, reverse transcription, and PCR) performed six times. In pilot experiments, PCR products run on agarose gels revealed a single band.

Transient transfections and reporter gene assays

To test for potential Ang II–mediated transcriptional activity of the TGF-β receptor type II, transient transfection of MCT cells were performed using Lipofectin™ (Gibco-BRL) according to the manufacturer's recommendations. Quiescent MCT cells (final density 10^5 cells) were cotransfected with 10 μg of plasmids containing different regions of the human TGF-β receptor type II connected to the CAT genes [24] (generous gift from Dr. Seong-Jin Kim, National Cancer Institute, Bethesda, MD, USA) and the same amount of the plasmid pSV-β galactosidase in which the β-galactosidase gene is under control of SV40 promoter and enhancer. After 12 hours, the serum-free medium was renewed and cells were incubated for another 24 hours with serum-free medium containing 0 to 10 mg/mL albumin. For selected experiments, MCT cells were transfected with the 1240 construct/pSV-β galactosidase and stimulated with 10 mg/mL albumin in the presence of 1 μmol/L losartan. Some cells were also treated with 50 ng/mL of a pan-specific neutralizing anti-TGF-β1-3 antibody (R&D Systems, Minneapolis, MN, USA). At the end of the experiments, cells were washed three times in PBS, cell layers were lysed, and protein concentrations of supernatants were adjusted. CAT and β-galactosidase protein concentrations were measured with specific enzyme-linked immunosorbent assay (ELISAs) (Boehringer Mannheim, Mannheim, Germany). A ratio between CAT and β-galactosidase concentrations was calculated, and data from unstimulated control cells were considered as 1.0. Transient transfections and reporter gene assays were independently performed four times for each construct.

Statistical analysis

All data are presented as the means ± SEM. Statistical significance between different groups was first tested with the nonparametric Kruskal-Wallis test. Individual groups were subsequently tested using the Wilcoxon-Mann-Whitney test. A P value of <0.05 was considered significant.

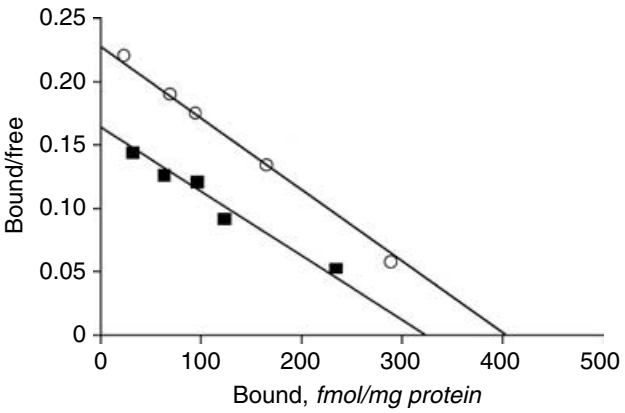

Fig. 1. Scatchard transformation of binding data. [^{125}I] transforming growth factor-β1 (TGF-β1) saturation binding was performed with MCT monolayers as described in the **Methods** section. Nonspecific biding was determined in the presence of 1 μmol/L TGF-β1 and was subtracted from all data points. Preincubation of cells for 24 hours with 1 mg/dL albumin increased the number of specific TGF-β surface binding sites (controls 320 fmol/mg protein, 1 mg/mL albumin for 24 hours: 400 fmol/mg protein) and the affinity of the binding site (K_d of controls 2.00 pmol/L, K_d of cells treated with albumin for 24 hours 1.6 pmol/L). Every data point represents the mean of four independent determinations (■) control medium, (○) albumin for 24 hours.

RESULTS

Receptor binding studies

We initially performed saturation binding studies to investigate whether albumin stimulation may influence expression of TGF-β receptors on MCT cells. As shown by Scatchard analysis of binding data (Fig. 1), MCT cells expressed a single class of high affinity receptors for TGF-β with characteristics similar to previous observations [19]. Preincubation of cells for 24 hours with 1 mg/mg albumin enhanced the affinity of specific receptors for TGF-β (K_d of controls 2.00 pmol/L, K_d of cells treated with albumin for 24 hours 1.6 pmol/L). In addition, growing MCT cells in the presence of albumin for 24 hours also increased the overall number of specific binding sites for TGF-β (controls 320 fmol/mg protein, 1 mg/mL albumin for 24 hours 400 fmol/mg protein). Since total binding sites were calculated per mg protein, this increase reflects a true upregulation of TGF-β receptors and not only an effect mediated by albumin-induced cell growth.

TGF-β receptor protein expression

Western blots from whole cell lysates were performed to evaluate TGF-β receptor protein expression. Figure 2A demonstrates that the polyclonal rabbit anti-TGF-β receptor type I detected one single band of

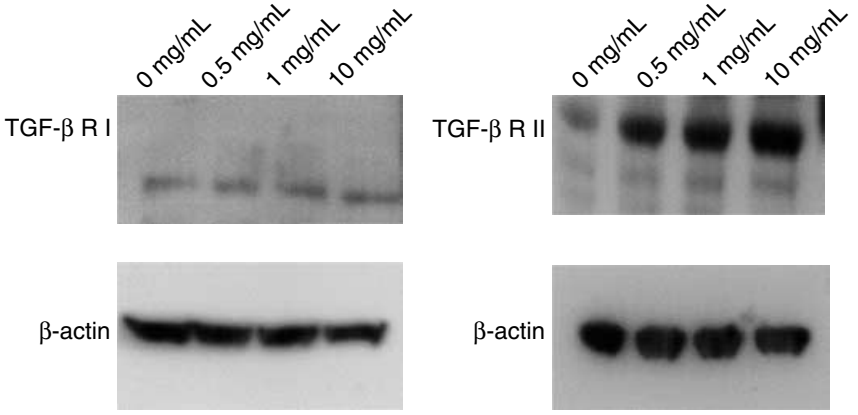

Fig. 2. Western blots for transforming growth factor-β (TGF-β) receptor type I (*A*) and II (*B*) protein expression. (A) A single band is shown of approximately 55 kD identical to the size of the type I TGF-β receptor as previously described in MCT cells as well as in other cell types. This blot was reincubated with an antibody against β-actin to control for small variations in loading and protein transfer. Albumin (0.5 to 10 mg/mL) for 24 hours failed to significantly increase protein expression of the TGF-β receptor type I in MCT cells. (B) In contrast, albumin strongly stimulates protein expression of the 70 kD TGF-β receptor type II in MCT cells. Blots are representative for three independent experiments.

approximately 55 kD, identical to the size previously described in MCT cells as well as in other cell types [19, 25]. This blot was reincubated with an antibody against β-actin to control for small variations in loading and protein transfer. However, albumin (0.5 to 10 mg/mL) for 24 hours failed to increase protein expression of the TGF-β receptor type I in MCT cells (Fig. 2A) (0 mg/mL 1.0, 0.5 mg/mL 0.99 ± 0.03, 1 mg/mL 1.10 ± 0.04, and 10 mg/mL 1.12 ± 0.05 relative changes in TGF-β receptor type I expression normalized to β-actin expression, not significant) ($N = 3$). In contrast, albumin stimulates protein expression of the 70 kD TGF-β receptor type II in MCT cells (Fig. 2B) (0 mg/mL 1.00, 0.5 mg/mL 4.52 ± 0.13*, 1 mg/mL 4.80 ± 0.22*, and 10 mg/mL 5.12 ± 0.29* relative changes in TGF-β receptor type II expression normalized to β-actin expression) (*$P < 0.05$ versus controls) ($N = 3$). An increase of TGF-β receptor type II protein expression was already detectable after 12 hours incubation with 10 mg/mL albumin and was still found after 48 hours (Fig. 3).

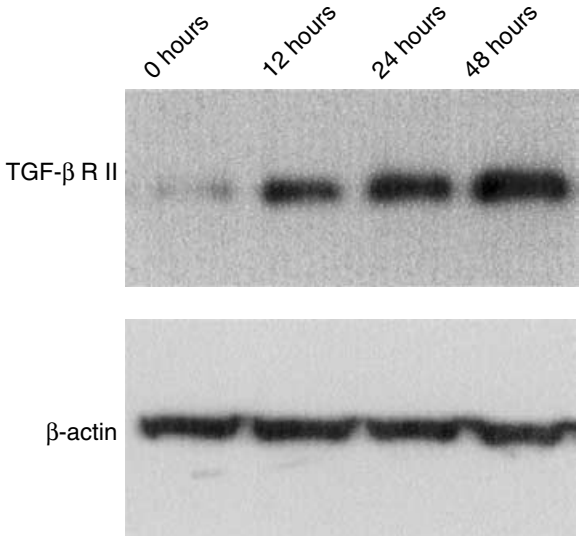

Fig. 3. Time course for transforming growth factor-β (TGF-β) receptor type II protein expression in MCT cells. Incubation with 10 mg/mL albumin up-regulated TGF-β receptor type II after 12 to 48 hours. This blot is representative for two independent experiments.

Activation of a local renin-angiotensin system

Since it has been previously shown that proteinuria stimulates a local renin-angiotensin system in tubular cells [26], we first studied whether similar mechanisms are operative in our cell culture system. As shown in Table 1, incubation of MCT cells for 24 hours with albumin significantly increases angiotensinogen and renin transcripts and also stimulated the secretion of Ang II into the culture supernatant. We and others have also previously shown that Ang II up-regulates TGF-β receptor expression [19, 25]. Consequently, we next tested a potential role of albumin-induced Ang II production in mediating TGF-β receptor expression. Figure 4 demonstrates that treatment with 1 μmol/L of the AT_1-receptor blocker losartan almost completely abolished albumin-induced expression of TGF-β receptor type II protein expression without influencing the type I receptor.

Table 1. Effect of albumin on tubular renin-angiotensin system activation

	Angiotensinogen mRNA (relative increase)	Renin mRNA (relative increase)	Angiotensin II secretion (pg/mg protein)
Controls	1.00 ± 0.00	1.00 ± 0.00	1.26 ± 0.10
0.5 mg/dL	1.26 ± 0.08[a]	1.32 ± 0.05[a]	1.69 ± 0.17
1 mg/dL	2.28 ± 0.99[a]	2.59 ± 1.25[b]	1.77 ± 0.13[b]
10 mg/dL	9.73 ± 6.32[b]	8.40 ± 3.99[b]	2.01 ± 0.12[a]

[a]$P < 0.01$ versus controls without albumin ($N = 4$ to 5); [b]$P < 0.05$ versus controls without albumin.

mRNA expression

Northern blots were performed to investigate whether albumin may also increase mRNA expression of TGF-β receptors in MCT cells. Albumin (0.5, 1, and 10 mg/mL) for 24 hours failed to change the amount of TGF-β receptor type I transcripts in MCT cells (Fig. 5) (0 mg/mL 1.00,

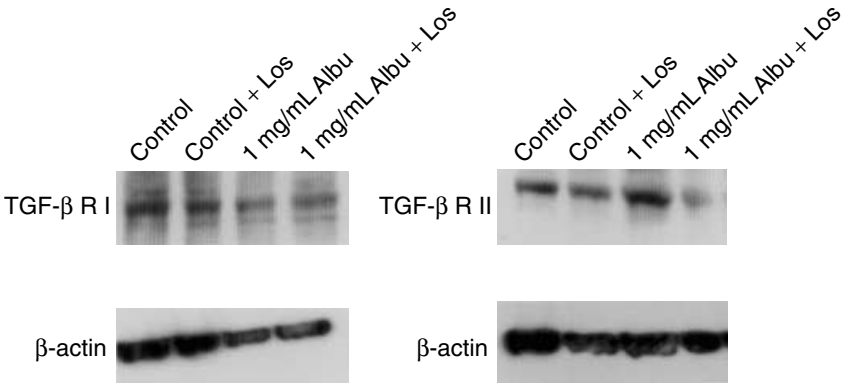

Fig. 4. Western blots for transforming growth factor-β (TGF-β) receptor types I and II. Treatment with 1 μmol/L losartan (Los) has no influence on TGF-β receptor type I expression (left panel). However, the AT_1-receptor antagonist almost completely attenuated the albumin-induction of TGF-β receptor type II protein expression (right panel). Blots are representative for two independent experiments.

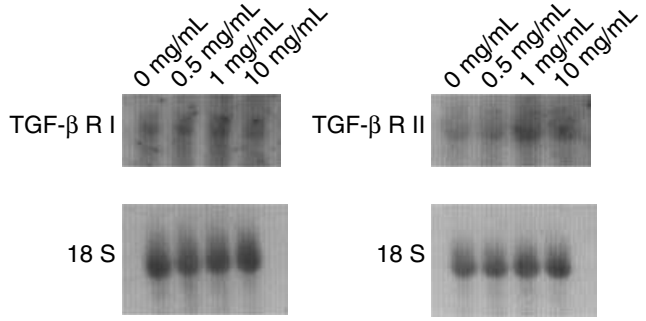

Fig. 5. Northern blots for transforming growth factor-β (TGF-β) receptor type I and II mRNAs prepared from MCT cells. Treatment with albumin for 24 hours failed to change the amount of TGF-β receptor type I transcripts (left panel). However, albumin clearly up-regulates TGF-β receptor type II mRNA expression in MCT cells. These blots are representative of three independent experiments with qualitatively similar changes.

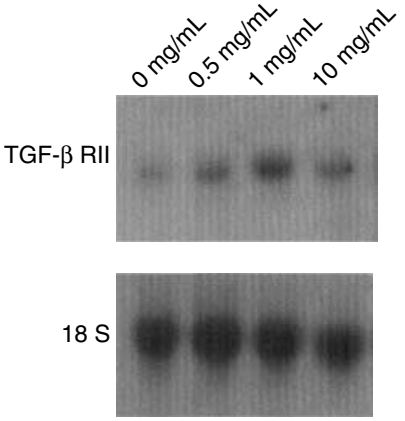

Fig. 6. Northern blot for transforming growth factor-β (TGF-β) receptor type II. Treatment with albumin also increased TGF-β receptor type II mRNA expression in LLC-PK1 cells, a porcine cell line with some properties of proximal tubules. This blot is representative for three independent experiments with qualitatively similar changes.

0.5 mg/mL 1.36 ± 0.12, 1.0 mg/mL 1.16 ± 0.61, and 10 mg/mL 1.40 ± 0.51) (relative changes in mRNA expression, not significant) ($N = 3$). In contrast, albumin clearly upregulates TGF-β receptor type II mRNA expression in MCT cells with maximal effect at 1 to 10 mg/mL albumin (Fig. 5) (0 mg/mL 1.00, 0.5 mg/mL 1.60 ± 0.10*, 1.0 mg/mL 1.71 ± 0.36*, and 10 mg/mL 1.72 ± 0.17*) (relative changes in mRNA expression) (*$P < 0.05$) ($N = 3$). Albumin-induced expression of TGF-β receptor type II transcripts was not restricted to mouse MCT cells, but was also found in LLC-PK1 cells, a porcine tubular cell line (Fig. 6). In these cells, 0.5 mg/mL albumin induced a strong upregulation of TGF-β receptor type II mRNA expression (0 mg/mL 1.00, 0.5 mg/mL 2.43 ± 0.50*, 1.0 mg/mL 1.52 ± 0.10*, and 10 mg/mL 1.44 ± 0.21*) (relative changes in mRNA expression) (*$P < 0.05$) ($N = 3$). As shown in Figure 7, losartan treatment also completely attenuated albumin-mediated expression of TGF-β receptor type II mRNA.

To further investigate whether the increase in TGF-β receptor type II transcript expression after challenge with albumin was due to a stimulated gene transcription various lengths of 5′-specific promoter/enhancer elements of the human TGF-β receptor type II gene linked to CAT were transiently transfected into MCT cells. These constructs have been previously described [19, 24]. Construct 504 contains one activating protein-1 (AP-1)–binding site whereas the full-length construct 1240 exhibits two AP-1–binding sites. Cells were subsequently treated for 24 hours with 0.5 to 10 mg/mL albumin. Although higher concentrations of albumin (1 mg/mL and 10 mg/mL) numerically reduced transcription of the shortest construct 47, these changes were not significant (Fig. 8A). Transfection with the intermediate constructs 274 and 504 revealed no significant transcriptional activity after stimulation with 0.5–10 mg/mL albumin (Fig. 8A). However, MCT cells transfected with the longest construct 1240, containing two putative AP-1 sites, showed a strong significant stimulation of the reporter gene CAT after stimulation with various albumin concentrations (Fig. 8A). Treatment of MCT cells with 1 μmol/L losartan totally attenuated albumin-induced transcription of the longest construct 1240 (Fig. 8B). However, coincubation with a neutralizing pan anti-TGFβ1-3 antibody did not

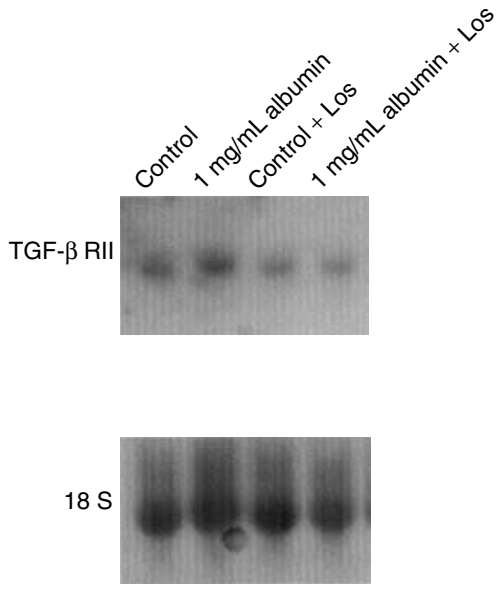

Fig. 7. Influence of losartan on albumin-mediated transforming growth factor-β (TGF-β) receptor type II mRNA expression in MCT cells by Northern blot. Treatment with 1 μmol/L losartan (Los) almost completely attenuated the albumin-induced increase in TGF-β receptor type II mRNA. This experiment was independently performed twice with qualitatively similar results.

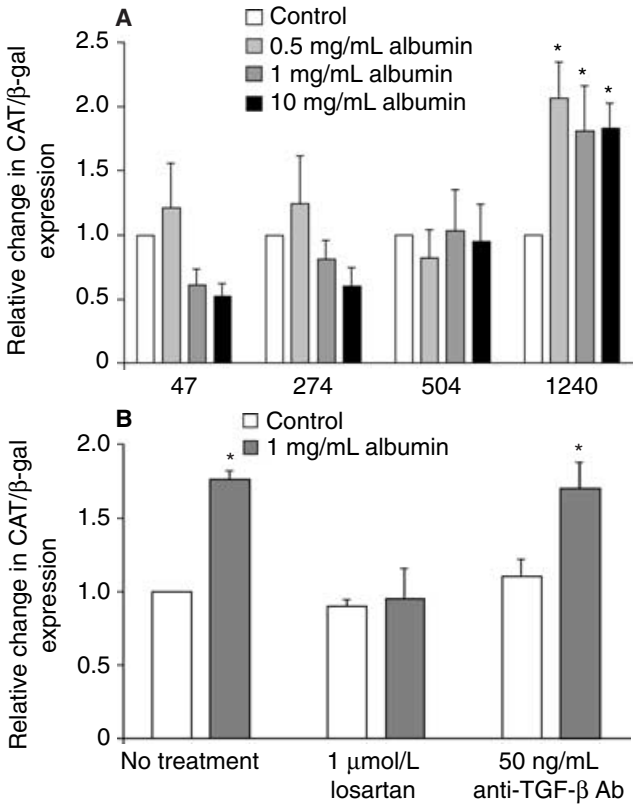

Fig. 8. Results of reporter gene studies. (*A*) Different length constructs encoding the transforming growth factor-β (TGF-β) receptor type II promoter were transiently cotransfected with SV40-β-galactosidase in MCT. Treatment with albumin (0.5 to 10 mg/mL) for 24 hours did not influence transcription of constructs 47, 274, and 504 compared with unstimulated controls. However, MCT cells transfected with the longest construct 1240 that contains two activating protein-1 (AP-1) binding sites showed a strong significant stimulation of the reporter gene CAT after albumin treatment. (*B*) Regulation of the longest construct 1240 was further investigated in detail. Administration of μmol/L losartan completely abolished the albumin-induced activation. In contrast, a neutralizing pan-anti-TGF-β antibody (50 ng/mL) had no effect. Mean values of three to five independent transfections, stimulations, and reporter gene assays.

change albumin-induced activation of the 1240 constructs suggesting that transcription is independent of TGF-β (Fig. 8B).

Functional relevance

On TGF-β binding to TGF-β receptor type II, the type I receptor is recruited into the complex and activated [27, 28]. Activated TGF-β receptor type I then signals to downstream intracellular substrates called Smads [29]. In order to test a functional relevance of up-regulated TGF-β receptors, we studied the phosphorylation of Smad2. Figure 9 shows that a relatively low concentration of exogenous TGF-β1 (0.5 ng/mL) induced a much stronger phosphorylation of Smad2 in MCT cells incubated for 24 hours in 10 mg/dL albumin compared to cells grown in control medium without albumin. The protein expression of total Smad2 was not different.

In addition, MCT cells were stimulated with exogenous TGF-β and mRNA expression for α1(IV) collagen, a typical TGF-β–induced ECM protein, was measured to investigate whether up-regulation of TGF-β receptors and the associated increase in Smad2 phosphorylation translates into increased target gene activation. As shown in Figure 10, TGF-β–induced α1(IV) collagen expression was enhanced in MCT cells previously incubated for 24 hours in 1 mg/mL albumin compared to controls. Under this condition even a relatively low TGF-β concentration strongly induced α1(IV) collagen mRNA expression (Fig. 10) (controls 1.00, 0.5 ng/mL TGF-β1 1.12 ± 0.34, 2 ng/m TGF-β1 1.14 ± 0.41, controls + albumin 1.20 ± 0.73, 0.5 ng/mL TGF-β1 + albumin 3.80 ± 0.2*, 2 ng/mL TGF-β1 + albumin 5.21 ± 0.85*) (relative changes in mRNA expression) (*$P < 0.05$ versus corresponding groups without albumin) ($N = 2$).

DISCUSSION

The present study tested whether albumin in a concentration found in proteinuria may influence expression of TGF-β surface receptors on cultured proximal tubular cells. We found that albumin leads to transcriptional up-regulation of the TGF-β receptor type II. This stimulated gene transcription was reflected in increased TGF-β receptor type II protein expression and was also demonstrated by increased binding of TGF-β to cell surfaces

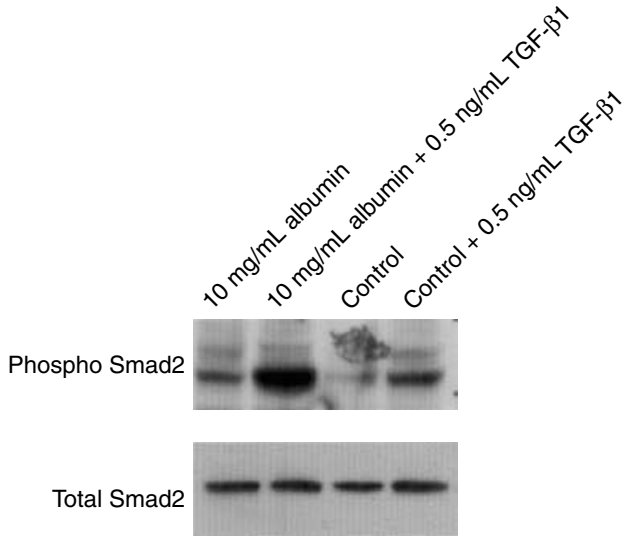

Fig. 9. Western blot for Smad2. MCT cells were either grown in 10 mg/dL albumin for 24 hours or in Dulbecco's modified Eagle's medium (DMEM) without albumin. Cells were then challenged with 0.5 ng/mL exogenous transforming growth factor-β (TGF-β1) for 30 minutes. MCT cells preincubated in albumin revealed a stronger phosphorylation of Smad2 after TGF-β1 treatment than those cells incubated in albumin-free medium. Expression of total Smad2 did not change. This blot is representative for two independent experiments with qualitatively similar results.

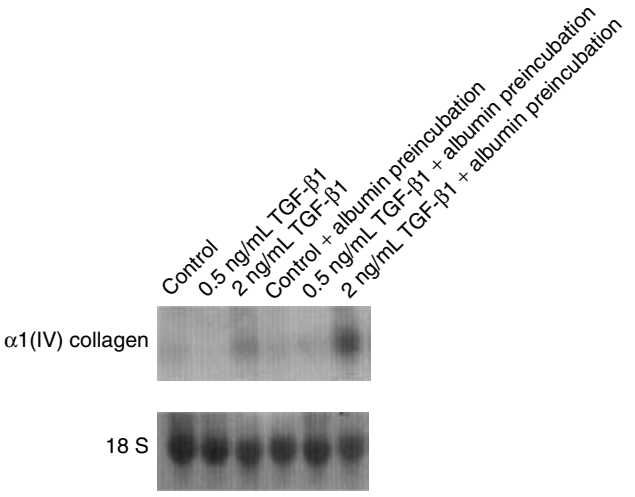

Fig. 10. Northern blot for α1(IV) collagen mRNA expression. MCT cells were either directly stimulated with 0.5 or 2 ng/mL recombinant transforming growth factor-β (TGF-β1) for 12 hours or were preincubated for 24 hours in 1 mg/mL albumin with subsequent TGF-β1 challenge. Cells pretreated with albumin showed a stronger increase in α1(IV) collagen mRNA induced by exogenous TGF-β1 compared with cells grown in serum-free medium without albumin. This blot is representative of three independent experiments with qualitatively similar changes.

with a slightly increased affinity. Finally, preincubation of MCT cells with albumin leads to a subsequent accelerated TGF-β–mediated signal transduction pathways such as Smad2 and also resulted in induction of α1(IV) collagen mRNA suggesting a functional effect of this receptor up-regulation. Since TGF-β receptor type II is primarily engaged in the initial binding of TGF-β, an increased receptor expression may result in amplification of the TGF-β effects on tubular cells.

TGF-β signaling involves two distantly related transmembrane serine/threonine kinases named receptors I and II [27, 28]. The cytokine binds first to the type II receptor which is constitutively expressed in an oligomeric form with an activated kinase [28, 29]. Subsequently, the TGF-β receptor type I which alone cannot bind TGF-β is recruited into the complex and becomes phosphorylated by receptor II. Phosphorylation finally allows receptor I to propagate the signal to downstream signals involving the Smad family of signal transducer proteins as well as Smad-independent pathways [12]. These findings suggest that the abundance of surface expression of TGF-β receptor type II is the limiting factor in the initial activation of the signal transduction pathway because the type I receptor alone cannot bind TGF-β. In agreement with our findings, several cytokines and factors such as Ang II, previously found to induce TGF-β receptor expression on various cells, principally up-regulate the type II receptor [25].

Proteinuria is associated with stimulated tubular TGF-β synthesis [10, 30–32]. This effect may be partly mediated by Ang II [31]. It has been previously described that exposing cultured proximal tubular cells to 0.1 to 10 mg/dL albumin leads to an increased tubular production of TGF-β in a culture system using human proximal tubular cells [9]. In the remnant kidney model, Abbate et al [10] observed that ultrafiltered proteins are taken up by proximal tubules. Tubular TGF-β1 mRNA expression, as detected by in situ hybridization, was stimulated concomitantly with protein overreabsorption. Later, peritubular accumulation of α-smooth muscle actin–expressing myofibroblasts was found in this area [10]. It is obvious that a parallel increase in TGF-β receptors would further amplify autocrine TGF-β effect after induction of this fibrogenic cytokine in tubular cells by albuminuria.

Proximal tubular cells have a local renin-angiotensin system that operates independently from its systemic counterpart [33]. More than a decade ago, we have demonstrated that MCT cells posses all components of a functioning renin-angiotensin system [34]. In vitro and in vivo investigations have demonstrated that tubular cells exposed to protein revealed an increased transcription of angiotensinogen and up-regulation of angiotensin-converting enzyme (ACE) [26, 35]. This results in a local increase in Ang II. Proximal tubular cells produce Ang II in the nanomolar range and secrete it into the urine as well as into the interstitial space [36, 37]. Renal tubulointerstitial damage by persistent proteinuria is attenuated in AT_1-receptor–deficient mice suggesting a direct effect of

Ang II in this process [39]. We and others have previously shown that Ang II up-regulates TGF-β receptor expression [19, 25]. We found in the present study that albumin treatment of cultured MCT cells induced an increase in angiotensinogen and renin transcription that resulted in a significantly enhanced production of Ang II. Thus, our observation that losartan almost completely attenuated the albumin-mediated increase in TGF-β type II receptor expression in MCT cells together with the stimulated Ang II formation strongly suggests that albumin leads to tubular synthesis of Ang II which, in turn, stimulates transcription of TGF-β type II receptors. The reporter gene experiments also suggest a pivotal role for Ang II in mediating albumin-induced transcription of the TGF-β type II receptor. The promoter region for this TGF-β receptor contains multiple components, including two positive regulatory elements and two negative regulatory sites in addition to the core promoter [24]. The core promoter, which is the 47 fragment, as well as the 504 construct that contains an AP-1 site at −195 bp showed in our study no significant increase in transcriptional activity after stimulation with albumin. In contrast, a strong transcriptional activity was induced by various concentrations of albumin with the long 1240 construct that contains a second AP-1 site at −1213 bp. The protein products of *c-fos* and *c-jun* form a heterodimer that binds to AP-1 sites. Since we have previously demonstrated that Ang II stimulates expression of *c-fos* in MCT cells [16], it is intriguing to speculate that albumin induces angiotensin II that, in turn, activates *c-fos* and *c-jun* expression in MCT cells. Ultimately, this complex may bind to AP-1 sites and stimulate the transcription of the TGF-β receptor type II.

How albumin may activate the tubular renin-angiotensin system is currently unclear. Albumin is taken up into proximal tubular cells through specific transport processes [39]. Megalin and cubulin are two receptors likely involved in this specific tubular uptake of albumin [40, 41]. Recent studies demonstrate that albumin uptake in proximal tubular cells induces the formation of reactive oxygen species (ROS) and also activates certain signal transduction pathways such as the STAT/Jak 2 system [42, 43]. Since ROS could up-regulate angiotensinogen expression [44], ROS may be an important intermediate in albumin-induced renin-angiotensin system activation. On the other hand, Ang II stimulates the formation of ROS through the membrane-bound NAD(P)H oxidase suggesting a possibility for a positive feedback loop [45]. Further studies beyond the scope of the present manuscript are necessary to investigate how such a tubular uptake of albumin could then activate local renin-angiotensin system components.

However, our study has some limitations. We used human TGF-β receptor type II promoter constructs in a heterologous system in mice that may pose a limitation to some of our findings. In addition, all our data were obtained from cell culture studies. Although these MCT cells express many properties of proximal tubular cells in vivo [13–16], and we were able to demonstrate that several effects discovered in these cells such as the Ang II–induced induction of the cell cycle regulator $p27^{Kip1}$ are also operative in vivo [46, 47], further in vivo studies are certainly necessary to corroborate our cell culture findings. We are currently studying whether TGF-β receptor type II is up-regulated on tubules in puromycin nephrosis to further test our present findings in vivo. Finally, the role of albumin as the main noxious agent in proteinuria has been questioned [48], and other proteins such as complement may be more important in mediating the pathomorphologic changes in tubulointerstitial architecture associated with proteinuria.

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