

Original Article

Glomerular activin A overexpression is linked to fibrosis in anti-Thy1 glomerulonephritis

Jens Gaedeke, Torsten Boehler, Klemens Budde, Hans-H. Neumayer and Harm Peters

Department of Nephrology, Universitätsklinikum Charité, Berlin, Germany

Abstract

Background. Activin A, a member of the transforming growth factor- β (TGF- β) superfamily of proteins, shares many biological features with the pro-fibrotic cytokine TGF- β 1, which is primarily responsible for the accumulation of extracellular matrix proteins in renal disease. This study was designed to identify regulators of activin A production in glomerular mesangial cells and test if activin A acts as a pro-fibrotic cytokine in mesangial cells.

Methods. The effect of inflammatory cytokines on activin A production and the effect of exogenous activin A on mediators of fibrosis were analysed in cultured rat mesangial cells. Expression of activin A and of established mediators of fibrosis was analysed in a rat model of glomerular fibrosis (anti-Thy1 glomerulonephritis).

Results. In cultured mesangial cells, interleukin-1 and basic fibroblast growth factor, both mediators of glomerular inflammatory injury, dose-dependently increased activin A expression. Incubation with activin A significantly stimulated TGF- β 1, PAI-1 and connective tissue growth factor RNA expression and increased production of extracellular matrix proteins in mesangial cells. In rats with anti-Thy1 glomerulonephritis, expression of glomerular activin A mRNA and protein paralleled the expression of TGF- β and other indices of fibrosis, showing little change from normal on day 1, a marked, 70-fold increase of activin protein production on day 6, and a subsequent decrease at day 12. Antifibrotic therapy with the angiotensin-converting enzyme inhibitor enalapril significantly reduced glomerular activin A production.

Conclusion. Taken together, the results of this study link overexpression of activin A to glomerular matrix protein expansion *in vivo* and *in vitro*, suggesting that activin A acts as pro-fibrotic cytokine in renal disease.

Keywords: activin A; anti-Thy1 glomerulonephritis; fibrosis; glomerular disease; rat; TGF- β

Introduction

Glomerulosclerosis and tubulo-interstitial fibrosis are hallmarks of progressive renal disease and ultimately lead to end-stage renal failure [1]. The degree of sclerosis and fibrosis has been identified as an important prognostic factor for progressive nephropathies of different aetiologies, emphasizing the relevance of matrix protein expansion for the outcome of renal disease. In most, if not all, fibrotic renal diseases, activation of these pathways is closely related to overexpression of the cytokine transforming growth factor- β (TGF- β) [1]. In both the glomerular and the tubulo-interstitial compartment, TGF- β acts as a pro-fibrotic cytokine by increasing matrix protein production and by reducing matrix turnover and removal. Antagonizing TGF- β reduces matrix protein build-up in models of acute renal disease, making it a target for treatment of fibrotic renal disease [1]. However, as shown so far, antagonism of TGF- β does not normalize the renal matrix content fully [2]. This may be due to an insufficient treatment time or dose of TGF- β neutralizing agent, but may also indicate that other pro-fibrotic pathways and mediators—redundant or additional—are involved. A novel as yet unidentified mediator of renal fibrosis may be activin A, which is a member of the TGF- β superfamily of proteins and recently has been related to extracellular matrix in tissues other than kidney.

The TGF- β superfamily of proteins is a group of several related proteins that share sequence and structural motifs and whose more prominent members beside the proper TGF- β s are activins, inhibins and bone morphogenic proteins. All are multi-functional proteins which, over time, have been shown to have more biological effects than the one that led to their

Correspondence and offprint requests to: Jens Gaedeke, MD, Med. Klinik m.S. Nephrologie, Charité, Campus Mitte, Schumannstr. 20–21, D-10117 Berlin, Germany. Email: jens.gaedeke@charite.de

initial discovery. Activin A was discovered originally as a regulator of pituitary function [3]. Additional research then showed that it has a much wider range of biological functions, mediating such different effects as the induction of mesoderm during embryogenesis and the regulation of erythrocyte precursor maturation and vascular smooth muscle cell behaviour (reviewed in [4]). Activin A is comprised of two activin β A subunits and has ~60% homology at the amino acid level with TGF- β . Its intracellular signal transduction pathway is closely related to that of TGF- β , involving SMAD proteins also activated by TGF- β receptors (SMADs 2–3) [5]. Differences exist at the level of the membrane receptors where activin forms a complex with a type I and a type II receptor recruited from a larger pool of receptor isoforms than TGF- β receptors. Additional heterogeneity exists at the level of activin isoforms. While the three TGF- β isoforms expressed in mammals are related in their biological behaviour, the expression of additional activin subunits such as β B, β C and β E can lead to the secretion of various heterodimeric activin proteins (β A coupled to β B– β E) with partially agonistic action (activin AB homo- and B) or potentially antagonistic action (activin AC and AE) towards the homodimeric activin A [6,7]. Furthermore, activin bioactivity is regulated by the existence of at least two activin-binding proteins, follistatin and follistatin-like 3, that prevent activin binding to its receptor.

However, the biological action of activin A and TGF- β seem to be closely related, especially their effect on matrix proteins. In hepatocytes and fibroblasts, both cytokines upregulate the production of matrix protein synthesis [8]. Mice that are genetically engineered to overexpress activin A in the skin show an increase in dermal connective tissue, thickening of the epidermis and increased extracellular matrix formation after skin wounding [9]. Increased expression of activin A has also been described in an experimental model of pulmonary fibrotic disease.

However, both *in vivo* and *in vitro*, activin A and TGF- β 1 are not totally interchangeable. This is suggested by the marked difference in phenotype of the TGF- β 1 [10] and activin A [11] knockout mouse and the apparent failure of each molecule to complement the other in these knockout mice.

The present study was designed to characterize the role of activin A in glomerular matrix accumulation using the model of anti-Thy1 glomerulonephritis and cultured mesangial cells. Anti-Thy1 glomerulonephritis was chosen because, unlike other models of fibrotic renal disease, it is characterized by a relatively distinct sequence of injury, overexpression of extracellular matrix proteins and subsequent resolution. Thus the temporal expression pattern can give important information about how a cytokine is related to renal matrix expansion. The initial injury in anti-Thy-1 glomerulonephritis is characterized by rapid lysis of mesangial cells. The cell lysis is mediated by large amounts of nitric oxide and is followed by an increased expression of cytokines including, among others, interleukin-1 (IL-1) and basic fibroblast growth factor (bFGF).

Subsequently, the remaining mesangial cells start to proliferate and to produce large amounts of extracellular matrix proteins. TGF- β has been shown to be a central mediator of matrix accumulation in this model. This process peaks at around day 5–8 after disease induction. Subsequently, TGF- β expression decreases and glomerular matrix protein content returns to normal.

Thus, in our *in vivo* studies, expression of activin A was determined following induction of anti-Thy1 glomerulonephritis and was temporally related to injury, matrix accumulation and resolution. In the *in vitro* experiments, we studied the effect of two important mediators of glomerular damage (IL-1 and bFGF) on the expression of activin A. In addition, we analysed the effect of activin A on the production of mediators of glomerular matrix expansion [TGF- β 1, PAI-1 and connective tissue growth factor (CTGF)] and matrix proteins. Together, these experiments will provide several lines of evidence showing that activin A may act as a pro-fibrotic cytokine in renal disease.

Materials and methods

Reagents

Tissue culture supplies were purchased from Seromed, Berlin, Germany. Recombinant rat IL-1 was purchased from Serotec, UK; recombinant human bFGF and recombinant human TGF- β 1 from Serva, Heidelberg, Germany; and recombinant human activin A from R&D Systems, Wiesbaden, Germany. Enzyme-linked immunosorbent assay (ELISA) kits were from Serotec, UK (activin A) and R&D Systems, Germany (TGF- β). All other reagents were from Sigma, Deisenhofen, Germany.

Experimental design and animals

Male Sprague–Dawley (SD) rats (200–270 g) were obtained from Tierzucht Schönwalde (Schönwalde, Germany). Glomerulonephritis was induced by tail vein injection of OX-7 antibody (1 mg/kg) under light ether anaesthesia as previously described [3]. Control animals were injected with equal volumes of phosphate-buffered saline (PBS). Groups of rats ($n=8$) were sacrificed 1, 6 and 12 days after induction of disease. The kidney were removed, decapsulated and minced with razor blades, and glomeruli from individual rats were isolated forcing the minced kidney with a spatula through graded sieves (150, 125, 106 and 75 μ m mesh metal sieves) as previously described [2]. Isolated glomeruli were then counted and seeded at 5000/ml in RPMI1640 without serum. In a separate experiment, untreated ($n=8$) and enalapril-treated ($n=8$) nephritic rats were studied. Enalapril treatment was begun 1 day after induction of disease (40 mg enalapril/kg body weight/day). On day 6 after induction of disease, animals were sacrificed and their glomeruli analysed as described above. All animal experiments were performed following institutional and EU guidelines.

Determination of activin A, TGF- β 1, PAI-1 and fibronectin protein levels in supernatants of cultured glomeruli and mesangial cells

Levels of total activin A were measured using the activin A Assay Kit (Serotec, UK) according to the manufacturer's instruction. This assay measures total, i.e. free and follistatin-bound, activin A using a sandwich ELISA with monoclonal antibodies. Experimental values are compared with bovine follicular fluid containing standardized amounts of activin A. The assay has no cross-reactivity to TGF- β , inhibin- α or activin AB. TGF- β 1, PAI-1 and fibronectin were measured by ELISA as previously described [2].

Histological evaluation of nephritic kidneys

Kidneys of nephritic and control animals were analysed for glomerulosclerosis using periodic acid-Schiff (PAS)-stained slides as previously described [2]. Slides were analysed in a blinded fashion and data were recorded as the percentage of matrix occupying each glomerulus.

Mesangial cell culture

Mesangial cells were isolated by standard differential sieving of minced kidneys from male SD rats (Tierzucht Schönwalde, Germany). Cells were used from passages 6–18.

RT-PCR

RNA from rat mesangial cells, rat kidney cortex or freshly prepared glomeruli (10 000 glomeruli per sample) was isolated using the Trizol reagent (Invitrogen, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the RNA-PCR core kit (Perkin Elmer, Langen, Germany). Detailed information including primer sequences is available as Supplementary material at NDT on-line.

Northern blotting

RNA from isolated glomeruli and cultured cells was analysed by non-radioactive northern blotting, following standard electrophoresis on agarose gels and capillary transfer to nylon membranes. Sequence-verified PCR products were used as probes. The PCR product used as the activin A probe was derived from the conserved mature region of the protein using primers described in the Supplementary on-line material. Labelling and detection of probes was performed using the AlkPhos Direct Kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions. Blots were developed with CDP-star, as recommended by the manufacturer.

Effect of IL-1 and bFGF on mesangial activin A production

Mesangial cells were cultured as described above. After induction of quiescence, cytokines (recombinant human bFGF and recombinant rat IL-1) were added to a final concentration of 1–50 ng/ml. After 24 h, the tissue culture medium was aspirated, briefly centrifuged to remove cellular

debris and then stored at -70°C until further assay. To ascertain equal number of cells after cytokine treatment, cells were trypsinized and counted and the results standardized to 10^5 cells. Supernatant was assayed for activin A content as described below.

Effect of activin A mesangial matrix protein production

The incorporation of radiolabelled proline into newly synthesized matrix proteins was used to analyse the effect of exogenous activin A on matrix protein production. Rat mesangial cells were seeded at 10^5 cells in 24-well plates, allowed to attach overnight and then made quiescent. Recombinant human activin A (0.3–100 ng/ml) was added and cells were treated for 24 h. During the last 6 h, $1\ \mu\text{Ci}$ of tritiated proline (Amersham-Bucler, Braunschweig, Germany) was added per well. At the end of treatment, supernatant and cells were collected together and proteins were precipitated with 1 vol. of ice-cold 12% trichloroacetic acid (TCA), centrifuged (10 000 g, 5 min), followed by two washes with 6% TCA. The pellet was lysed in 1 M NaOH/0.5% Triton X-100 and aliquots were counted in a scintillation counter.

Statistical analysis

Data are expressed as means \pm SEM. Statistical analysis between groups was performed by analysis of variance (ANOVA) and *t*-testing with Bonferroni correction. A *P*-value of <0.05 was considered as significant.

Results

Expression of activin A and its receptors in normal rat kidney

Activin A and its receptors are expressed in normal rat kidney cortex, cultured mesangial cells and normal glomeruli. Using highly sensitive RT-PCR, the expression of all components of the activin system including the activin antagonist follistatin in normal rat glomeruli could be demonstrated in glomeruli (Figure 1). Similar results were obtained when kidney cortex or cultured rat mesangial cells were analysed (data not shown).

Expression of activin A following the induction of anti-Thy1 glomerulonephritis

The regulation of activin A mRNA following induction of anti-Thy1 glomerulonephritis was analysed by northern blotting. Glomerular RNA was harvested 1, 6 and 12 days after injection of anti-Thy1 antibody. Normal non-nephritic glomerular RNA was harvested from animals injected with PBS. Figure 2 shows low amounts of activin A mRNA in control and day 1 nephritic glomeruli. However, a six-fold increase of activin A mRNA is seen 6 days after induction of disease followed by a return to close to baseline at day 12 (Figure 2b).

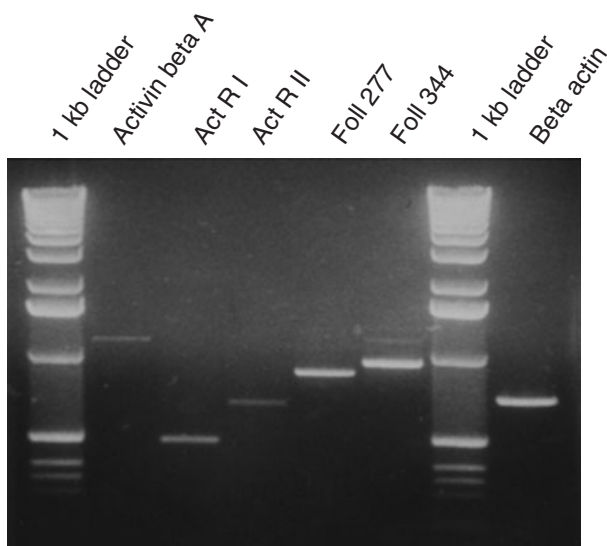


Fig. 1. Expression of activin A, its receptors and follistatin in normal glomeruli. RT-PCR for activin A, activin type I and II receptor and follistatin. From left to right: 1 kb ladder; activin A; activin receptor I; activin receptor II; follistatin 277; follistatin 315; 1 kb ladder; β -actin control.

Activin A protein expression was analysed in glomerular culture supernatants. Equal numbers of glomeruli were cultured in serum-free medium for 48 h and their culture supernatant analysed by ELISA. Activin A expression was low in normal glomeruli (0.2 ± 0.1 ng/ml, Figure 3a). Induction of mesangial cell lysis (day 1) had no significant effect on activin A production. However, a highly significant 70-fold increase of activin A protein production was seen in nephritic day 6 glomeruli (14.9 ± 3.1 ng/ml, $P \leq 0.001$, Figure 3a), followed by a decreased activin A production at day 12 (5.2 ± 0.4 ng/ml).

As shown in Figure 3, the pattern of activin A expression in anti-Thy1 glomerulonephritis closely corresponded to the changes in glomerular matrix expansion. Glomerular matrix content was low in controls ($27 \pm 2\%$) and day 1 animals ($20 \pm 3\%$, slight decrease, parallel with mesangial cell lysis), when mRNA and protein expression of activin A was low (Figures 2 and 3a). In contrast, glomerular matrix content was markedly increased at day 6 ($81 \pm 1\%$), when activin A expression peaked, while at day 12, the beginning of the resolution phase in anti-Thy1 glomerulonephritis, both glomerular matrix content ($74 \pm 2\%$) and activin expression (Figures 2 and 3a) decreased again.

Together, these data show that glomerular activin A RNA expression and protein production are closely linked to the degree of matrix expansion in anti-Thy1 glomerulonephritis.

To characterize the role of activin A in glomerular matrix expansion further, we compared its production with that of the matrix-modifying proteins TGF- β 1 and PAI-1 and the matrix protein fibronectin. Glomerular

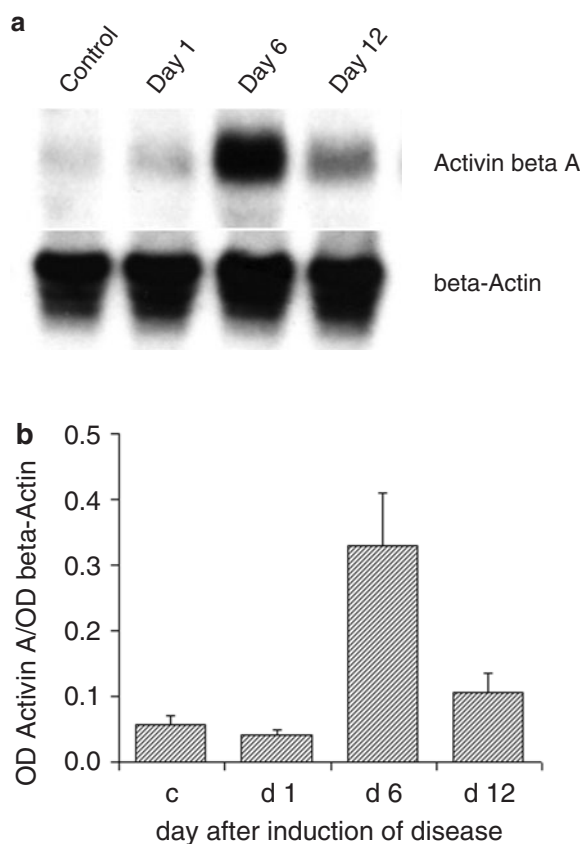


Fig. 2. Expression of activin A RNA in the time course of anti-Thy1 glomerulonephritis. (a) RNA from control and nephritic glomeruli was harvested on the indicated day after induction of disease and analysed by northern blot for activin A expression. Each lane was loaded with 20 μ g of pooled RNA from eight animals. (b) Densitometric analysis of northern blots, showing β -actin standardized OD values.

supernatants harvested on day 1, 6 and 12 after induction of disease were analysed and compared with supernatants from non-nephritic control glomeruli. As shown in Table 1, mesangial cell lysis was associated with a small but significant increase in glomerular release of TGF- β 1 (123 ± 13 pg/ml in control, 381 ± 65 pg/ml in day 1 glomeruli, $P \leq 0.05$), fibronectin (3.6 ± 0.2 to 5.1 ± 0.3 ng/ml, $P \leq 0.01$) and PAI-1 (167 ± 13 to 283 ± 65 ng/ml, $P \leq 0.001$). Similar to the expression of activin A, there was a marked and highly significant upregulation of TGF- β 1 at day 6, which showed a 10-fold increase over control values (1269 ± 77 pg/ml, $P \leq 0.001$). At day 12, the beginning of the resolution phase, TGF- β 1 levels had decreased markedly (192 ± 35 pg/ml). The expression of fibronectin and PAI-1 showed the same pattern (Table 1), with a peak expression at day 6 (2663 ± 159 pg/ml PAI-1, $P \leq 0.001$ and 14.1 ± 0.6 ng/ml fibronectin, $P \leq 0.001$) and a subsequent decline at day 12. Thus, activin A expression closely follows the expression of the pro-fibrotic cytokine TGF- β 1 and central markers of glomerular matrix expansion and matrix degradation in anti-Thy1 glomerulonephritis.

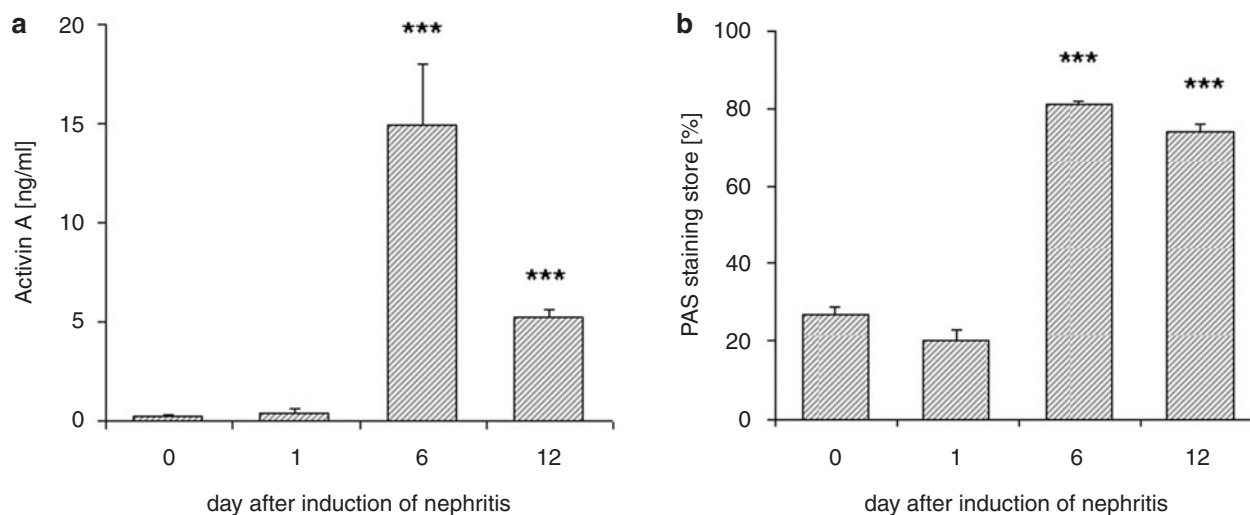


Fig. 3. Expression of activin A protein (a) and glomerular matrix content (b) following induction of anti-Thy1 glomerulonephritis. (a) Renal tissue was harvested at the indicated time points after induction of anti-Thy1 glomerulonephritis. Activin A released into the supernatant of cultured glomeruli was measured by ELISA. (b) Matrix content was scored on PAS-stained slides. Results of eight animals are shown (mean \pm SEM, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Table 1. Glomerular expression of TGF- β 1, fibronectin and PAI-1 following induction of anti-Thy1 glomerulonephritis

Parameter	Days after induction of nephritis			
	0	1	6	12
TGF- β (pg/ml)	123 \pm 13	381 \pm 65*	1269 \pm 77***	195 \pm 35**
PAI-1 (ng/ml)	167 \pm 13	283 \pm 71***	2663 \pm 159***	2425 \pm 325**
Fibronectin (ng/ml)	3.6 \pm 0.2	5.1 \pm 0.3**	14.1 \pm 0.6***	10.5 \pm 0.5***

Glomeruli from individual rats ($n = 8$) were harvested as indicated. Expression of TGF- β 1, fibronectin and PAI-1 in the culture supernatant was analysed by ELISA. Results are shown as mean \pm SEM (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Effect of enalapril on glomerular activin A production following induction of anti-Thy1 glomerulonephritis

Previous studies have shown that antagonism of angiotensin II reduces glomerular matrix expansion after induction of anti-Thy1 glomerulonephritis [2]. As shown in Figure 4b, administration of enalapril markedly reduced glomerular matrix content at day 6 after induction of disease (PAS staining score $78 \pm 1.6\%$ in untreated nephritic and $58 \pm 2.8\%$ in enalapril-treated nephritic rats, $P \leq 0.01$). Similar to the effect on glomerular matrix accumulation, enalapril treatment was associated with a marked decrease in activin A production. Compared with untreated nephritic rats, the glomerular activin A production was reduced by 62% in enalapril-treated nephritic rats (9.5 ± 2.1 ng/ml in untreated and 3.6 ± 0.9 ng/ml in treated rats, $P \leq 0.01$, Figure 4b). Thus, limiting glomerular matrix expansion goes along with reduced expression of activin A.

Activin A regulation and action in vitro: effects of IL-1 and bFGF

IL-1 and bFGF are upregulated early after induction of anti-Thy1 glomerulonephritis and precede the increase

in glomerular matrix content. Thus, we analysed the effect of IL-1 and bFGF on activin A production in cultured rat mesangial cells. Using northern blot analysis, a 20-fold upregulation of activin A mRNA could be detected after treatment of mesangial cells with as little as 1 ng/ml IL-1 (Figure 5). Increasing doses of IL-1 led to further increases in activin A mRNA. Similar results were obtained after treatment with bFGF (data not shown). To test whether these increases in RNA result in protein production, cell culture supernatant of IL-1- and bFGF-treated cells was analysed by ELISA. As shown in Figure 6, there was a dose-dependent increase in the activin A content of mesangial cell supernatant when cells were treated with either IL-1 (Figure 6a) or bFGF (Figure 6b) for 24 h. After treatment of cells with 10 ng/ml of IL-1, the activin A content of cell culture supernatants rose from 14.3 ± 3.8 to 44.0 ± 3.7 ng/ 10^5 cells ($P \leq 0.001$, Figure 6a). A comparable increase was seen after treatment with 50 ng/ml bFGF (7.9 ± 0.2 to 29.8 ± 2.0 ng/ 10^5 cells, $P \leq 0.001$, Figure 6b).

Taken together, these data show that two known important mediators of glomerular injury directly induce the production of activin A in mesangial cells.

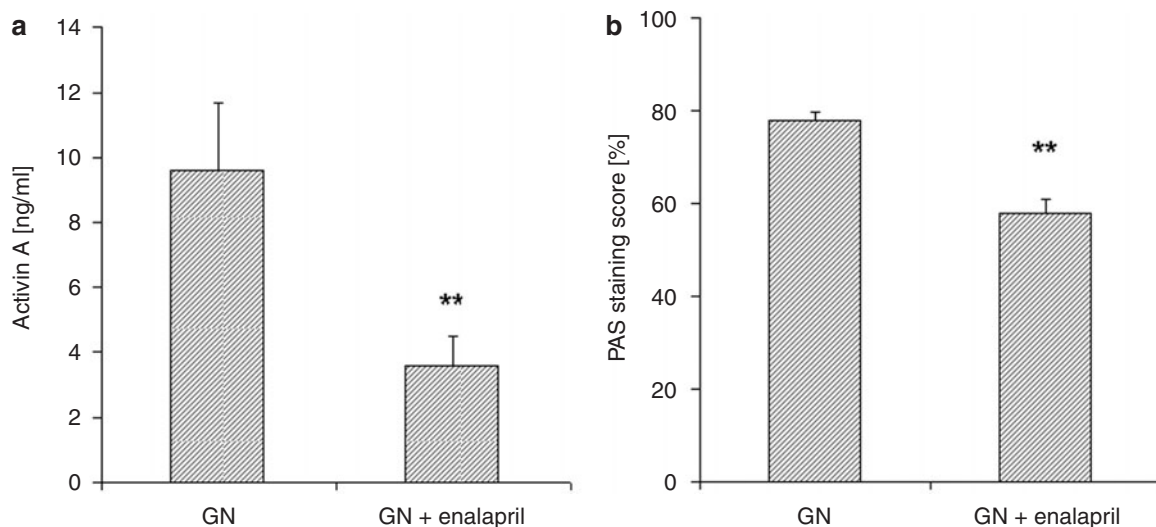


Fig. 4. Effect of enalapril treatment on expression of activin A protein (a) and glomerular matrix content (b) following induction of anti-Thy1 glomerulonephritis. (a) Glomeruli were harvested on day 6 after induction of disease from enalapril-treated rats ($n=8$, 40 mg/kg body weight of enalapril) or untreated nephritic rats ($n=8$). Activin A released into the supernatant of cultured glomeruli was measured by ELISA. (b) Matrix content was scored on PAS-stained slides. Results are shown as mean \pm SEM (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

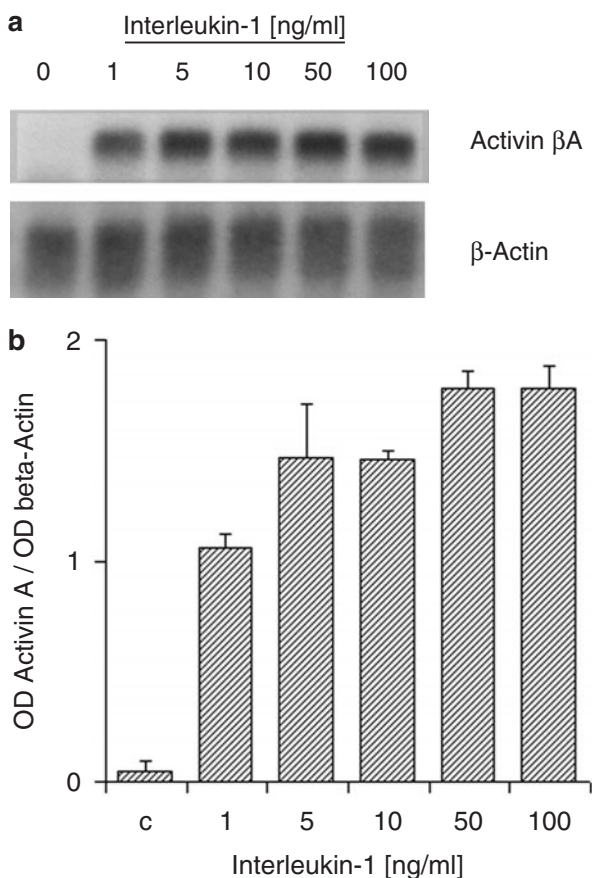


Fig. 5. Effect of IL-1 on activin A mRNA expression in mesangial cells. (a) Cells were treated with the indicated amounts of cytokine for 6 h and activin A RNA was detected by northern blot. A blot representative of four independent experiments is shown. β -Actin is shown as a loading control. (b) Densitometric analysis of blots of four separate experiments, showing OD values standardized for β -actin.

Activin A induces TGF- β and PAI-1 in cultured mesangial cells

To analyse further the interaction of factors involved in the sequence of repair after induction of anti-Thy1 glomerulonephritis, we analysed the effect of activin A on glomerular production of TGF- β 1 and PAI-1. Both are upregulated during the phase of matrix expansion in anti-Thy1 glomerulonephritis. Treatment of cultured mesangial cells with 5–50 ng/ml activin A led to a dose-dependent increase in TGF- β production which had a maximal effect at 10 ng/ml activin A ($86 \pm 16\%$, $P \leq 0.05$) (Figure 7). Similarly, mesangial PAI-1 production went up by $43 \pm 6\%$ ($P \leq 0.05$) after treatment with 50 ng/ml activin A (Figure 8). Similar, albeit lower increases in TGF- β and PAI-1 production were obtained when cultured normal glomeruli were treated with activin A (data not shown). These data show that activin A regulates the expression of mediators of renal matrix expansion in mesangial cells.

Activin A upregulates CTGF RNA and matrix protein production in mesangial cells

To analyse if activin A is directly involved in matrix protein regulation, we first analysed the expression of CTGF expression in cultured mesangial cells after activin treatment. CTGF has been shown to be a mediator of TGF- β -induced matrix protein induction and is upregulated in a variety of fibrotic renal diseases, including the anti-Thy1 disease model. Northern blot analysis shows that activin A dose-dependently increases CTGF mRNA in mesangial cells (Figure 9). Treatment of mesangial cells for 16 h with 0.3 ng/ml activin A led to a significant 2-fold increase of CTGF mRNA, and this effect was increased further at higher

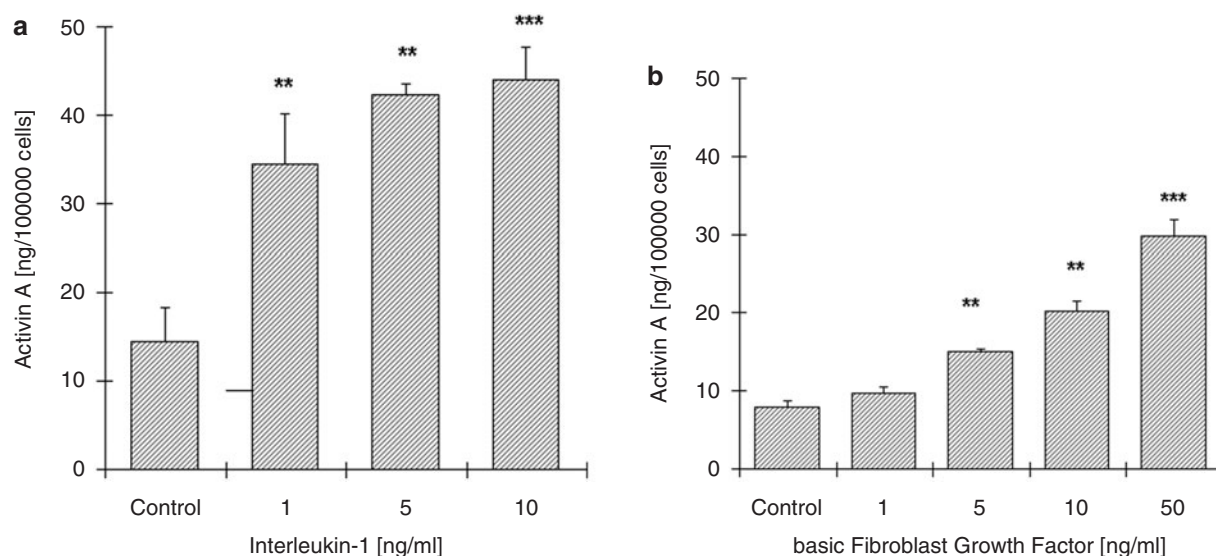


Fig. 6. Effect of IL-1 (a) and bFGF (b) on activin A production by mesangial cells. Cells were treated with the indicated amounts of cytokine for 24 h and activin A was assayed in the cell culture supernatant. Results of three independent experiments are shown (mean \pm SEM, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).



Fig. 7. Effect of activin A on mesangial cell TGF- β 1 protein production. Mesangial cells were treated with activin A for 24 h. TGF- β 1 protein levels were measured by ELISA. Results of three independent experiments are shown as mean \pm SEM (* $P \leq 0.05$, ** $P \leq 0.01$).

doses (Figure 9). To determine if activin A treatment also results in increases in matrix protein production, we analysed the incorporation of radiolabelled proline into newly synthesized matrix proteins. As Figure 10 shows, a significant increase in proline incorporation could be detected after treatment with 1.25 ng/ml activin A for 24 h. Up to a dose of 10 ng/ml, activin A increased matrix protein production by mesangial cells (Figure 10). These experiments show that activin A has pro-fibrotic properties in mesangial cells.

Discussion

The present study shows that a marked upregulation of glomerular activin A production occurs after induction of anti-Thy1 glomerulonephritis in parallel with increases of parameters of glomerular matrix expansion such as PAS staining, and fibronectin, PAI-1 and TGF- β 1 production. We have also demonstrated that activin A induces pro-fibrotic cytokines in cultured mesangial cells, namely TGF- β 1 and PAI-1. Furthermore, in cultured mesangial cells, activin A was able to induce CTGF mRNA and increase matrix protein production. These data suggest that activin A is involved in glomerular matrix expansion in anti-Thy1 glomerulonephritis.

The role of activin A in tissue fibrosis has been studied previously in models of liver and lung fibrosis. In the liver, upregulation of activin A mRNA and protein was demonstrated in rat models of hepatic fibrosis [8]. In the lung, increased expression of activin A was noted in a murine model of bleomycin-induced lung fibrosis [12]. In our study, activin A protein was produced at low levels in normal glomeruli and production increased markedly (70-fold) on day 6 after disease induction. Direct examination of glomerular matrix by PAS staining revealed a drastic increase in PAS-positive material on day 6. At this time point, there was also maximal expression of TGF- β , fibronectin and PAI-1, which are established indicators of glomerular matrix protein accumulation. Angiotensin II blockade with enalapril, which has been shown to reduce TGF- β production and matrix accumulation in this model and others [2], markedly reduced glomerular activin A production by ~60%. Thus, there is a direct spatiotemporal correlation between glomerular matrix accumulation and increased activin A levels and between anti-fibrotic treatment and decreased

activin A levels. Further evidence for a pro-fibrotic role for activin A comes from our experiments showing an upregulation of CTGF expression and increases in matrix protein synthesis by exogenous activin A in

cultured mesangial cells. In addition, activin A upregulates mesangial production of TGF- β and PAI-1. Both proteins are directly involved in the development of glomerular fibrosis *in vivo*. Thus, our data are consistent with studies done in liver and lung models of fibrosis and suggest that activin A is a pro-fibrotic cytokine in the kidney. Recently, activin A was shown to be a potent activator of collagen secretion in rat interstitial fibroblasts [13]. Contrary to our results in mesangial cells, this study failed to show an effect of activin A on PAI-1 production by fibroblasts. Whether this suggests that activin A has different actions in the glomerular vs the tubulo-interstitial compartment needs further investigation.

The cellular source of the increased glomerular expression of activin A in anti-Thy1 glomerulonephritis is not entirely clear. We have found activin A mRNA and protein in cultured mesangial cells and isolated normal glomeruli. While this could suggest that resident glomerular cells, especially mesangial cells, are the main source of glomerular activin A production, the contribution of other cell types (resident and non-resident glomerular cells) cannot be ruled out. Macrophages can produce activin A, and this production is upregulated by inflammatory cytokines [14]. In glomerular inflammatory diseases associated with macrophage infiltration, resident glomerular cells might therefore not be the only source of activin A. In the anti-Thy1-model, early glomerular infiltration of ED-1⁺ cells (i.e. macrophages) has been described

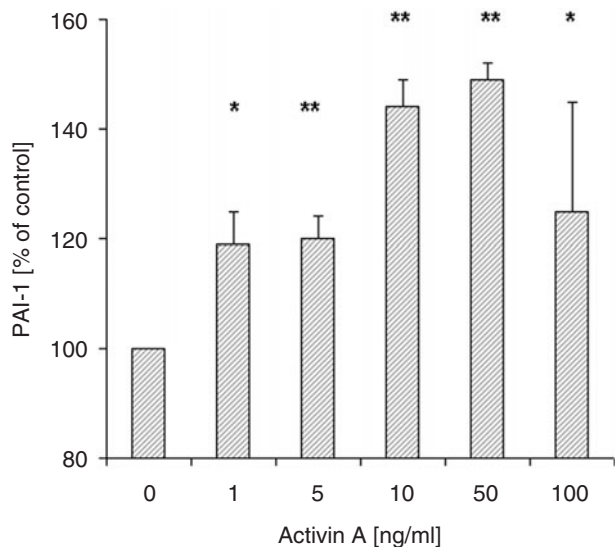


Fig. 8. Effect of activin A on mesangial cell PAI-1 protein production. Mesangial cells were treated with activin A for 24 h. PAI-1 protein levels were measured by ELISA. Results of three independent experiments are shown as mean \pm SEM (* P < 0.05, ** P < 0.01).

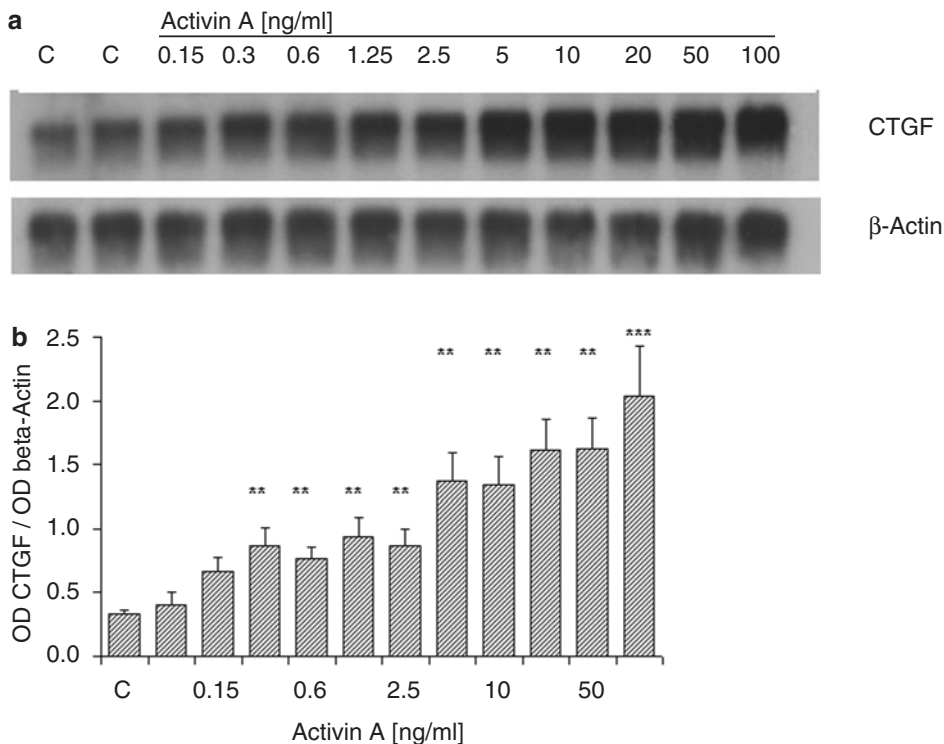


Fig. 9. Effect of activin A on connective tissue growth factor (CTGF) expression in mesangial cells. (a) Mesangial cells were treated with activin A for 16 h. CTGF expression was analysed by northern blot. A blot representative of four independent experiments is shown. β -Actin is shown as a loading control. (b) Densitometric analysis of blots of four separate experiments, showing OD values standardized for β -actin (** P \leq 0.01, *** P \leq 0.001).

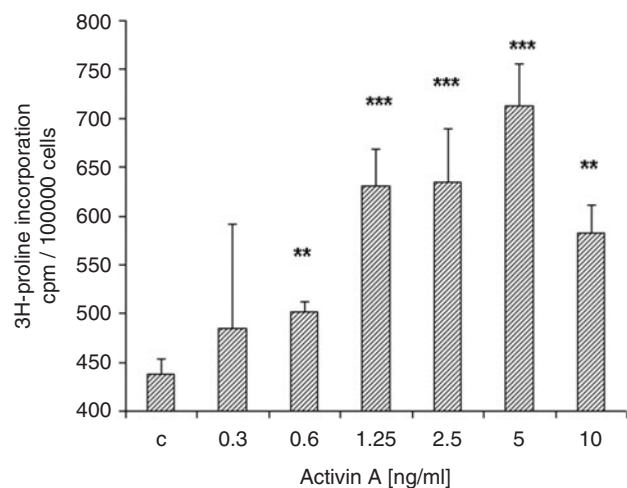


Fig. 10. Effect of activin A on matrix protein production in mesangial cells. Mesangial cells were treated with activin A for 24 h. Matrix protein production was measured as proline incorporation into TCA-precipitable material after a 6 h pulse with radiolabelled proline. Results of three independent experiments are shown as mean \pm SEM (* P < 0.05, ** P < 0.01, *** P \leq 0.001).

with a peak at days 1 and 2 and a decline thereafter [15]. We have found very little activin A production at day 1 and a drastic increase at day 6. This may suggest that glomerular macrophage content and activin A production are not correlated in this model and that resident glomerular cells are responsible for the increased production of activin A.

Rat mesangial cells in culture were used to identify some of the regulators of glomerular activin A production by analysing the effect of IL-1 and bFGF on mesangial activin A production. The production of both IL-1 and bFGF is upregulated in anti-Thy1 glomerulonephritis, and blockade of these cytokines has some beneficial effect in this model. In addition, IL-1 and bFGF have been shown to be activators of activin A production in a variety of cells of epithelial and mesenchymal origin [14,16]. Our data show that both IL-1 and bFGF dose-dependently upregulate activin A production in mesangial cells. Our finding of a maximal activin A production on day 6 correlates with data showing maximal bFGF production on day 6 after induction of anti-Thy1 glomerulonephritis. Thus, cytokines that activate mesangial cells in disease states also regulate mesangial activin A production.

As mentioned above, angiotensin II blockade was found to reduce glomerular matrix protein accumulation as well as activin A production. A strong induction of activin A by angiotensin II could be demonstrated recently in rat aortic smooth muscle cells [17], which have some similarity to mesangial cells. In the model of anti-Thy1 glomerulonephritis, angiotensin II blockade with enalapril has been shown to reduce the degree of matrix expansion during the fibrotic phase of the disease [3]. Parts of these effects seem to be mediated by a reduction of TGF- β , which is induced by

angiotensin II in mesangial cells. Our data suggest that the anti-fibrotic effect of angiotensin II blockade on glomerular matrix expansion may also be attributed to a reduction of activin A production. In addition to angiotensin II blockade, reducing activin A effects with its natural antagonist follistatin could prove another approach to limit fibrosis in anti-Thy1 glomerulonephritis. Follistatin binds activin A with a high affinity and thus limits its bioavailability. The *in vivo* application of follistatin has been shown to reduce activin A-induced inhibition of hepatocyte proliferation successfully [18]. Recently, activin C and activin E have been described as novel members of the activin protein family which are capable of forming complexes with activin A, leading to activin AC and AE heterodimers [19]. The biological actions of activin C and E expression are as yet unclear. Some groups describe an antagonistic (i.e. follistatin-like) action of activin C towards activin A [6], yet others have shown that in liver cells the C and E isoform behave similarly to activin A [20]. So far, no studies have been reported that use follistatin or activin C or E to modulate activin A-induced matrix expansion *in vivo*, an area of research that could offer additional insight into the role of activin A in fibrotic renal disease.

In summary, the present study shows evidence that activin A acts as a pro-fibrotic cytokine in anti-Thy1 glomerulonephritis by demonstrating (i) a close temporal link between matrix expansion and activin A production; (ii) a reduction of activin A production after antifibrotic therapy; and (iii) an activin A-induced upregulation of mesangial matrix protein production as well as production of mediators of fibrosis (TGF- β , PAI-1 and CTGF) *in vitro*. Activin A seems to be a part of the cytokine repertoire that mediates glomerular matrix expansion and fibrosis.

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