Transforming growth factor beta mediates the angiotensin-II-induced stimulation of collagen type IV synthesis in cultured murine proximal tubular cells


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Abstract

Background. Angiotensin II (Ang II) stimulates synthesis of type IV collagen in a cultured murine proximal tubular cell line (MCT cells). In addition, Ang II also induces the expression of TGF-β in these cells. Since TGF-β has well-known stimulatory effects on the transcription of various collagens, we tested whether the Ang-II-mediated stimulation of type IV collagen is due to induction of endogenous TGF-β synthesis in MCT cells.

Results. A neutralizing monoclonal anti-TGF-β antibody abolished the Ang II-stimulated release of type IV collagen in culture supernatants. The anti-TGF-β antibody also partly blocked Ang-II-mediated incorporation of [3H]proline into de novo synthesized collagens. Moreover, 5 μM TGF-β antisense oligonucleotides, but not the same concentration of sense oligonucleotides, completely blocked Ang-II-stimulated [3H]proline incorporation. MCT cells incubated with TGF-β antisense phosphorothioate-modified oligonucleotides failed to synthesize TGF-β protein after Ang II treatment as measured by a sandwich ELISA in culture supernatants. SDS-polyacrylamide electrophoresis of [3H]proline-labelled collagens and comparison with standard collagens also demonstrated that the neutralizing anti-TGF-β antibody abolished the Ang-II-mediated stimulation in type IV collagen. Semiquantitative cDNA amplification for collagen type al(IV) transcripts revealed that the anti-TGF-β antibody abrogates the increase in mRNA after Ang II treatment. Transient transfection studies in MCT cells using murine collagen α1(IV) enhancer/promoter constructs also demonstrated the suppressive effect of the neutralizing antibody on Ang-II-stimulated gene transcription.

Conclusions. Our data collectively suggest that the Ang-II-mediated increase in type IV collagen in MCT cells is mediated by endogenous synthesis and autocrine action of TGF-β. These findings may be important in changes of the tubulointerstitial architecture during the progression of renal disease.

Key words: transforming growth factor beta; angiotensin II; collagen type IV synthesis; tubular cells

Introduction

Tubulointerstitial fibrosis causing chronic renal failure is the common endpoint of many renal diseases. The degree of sclerosis of the tubulointerstitium is closely related to the progressive loss of renal function even in primary glomerular diseases (for review see [1–3]). An increased deposition of extracellular matrix proteins in the tubulointerstitial microenvironment is responsible for the tubulointerstitial fibrosis [3]. This increased deposition of extracellular matrix is the result of a stimulated synthesis of proteins like collagens as well as a decrease in their turnover rate. Immuno-histological studies in a variety of different renal diseases associated with tubulointerstitial fibrosis have demonstrated that the production of interstitial collagens type I and III as well as basement-membrane-associated collagen type IV is enhanced [1]. Collagen types I and III are preferentially produced by activated fibroblasts migrating into/or residing in the tubulointerstitial space [3]. In contrast, local tubular cells are most probably the source of the stimulated type IV collagen production in tubulointerstitial sclerosis [1]. Therefore it is interesting to analyse more closely factors that stimulate collagen type IV production in tubular epithelial cells.

We have recently described that angiotensin II (Ang II) induces cellular hypertrophy in vitro in a murine proximal tubular cell line (MCT) and in porcine LLC-PK1 cells which express some features of proximal tubular cells [4–8]. Others investigators have confirmed our findings [9,10]. The Ang-II-induced hypertrophy was associated with a stimulated transcription and synthesis of collagen type IV, but not of type I [6]. Moreover, we have also shown that Ang II induces...
transformation growth factor beta (TGF-β) synthesis in tubular cells and that the Ang-II-mediated cellular hypertrophy partly depends on the induction of TGF-β [5]. Since TGF-β is a well-known fibrogenic factor stimulating the synthesis of different collagens in a wide variety of cell types, we investigated in the present study whether the Ang-II-stimulated collagen type IV synthesis in MCT cells may be mediated by endogenous induction of TGF-β.

Subjects and methods

Cell cultures

MCT cells are an SV 40 immortalized murine proximal tubular cell line [11]. These cells have been extensively characterized by various investigators and express many features of proximal tubular cells [4,5,11]. Cells were maintained in Dulbecco's modified Eagle's medium containing 450 mg/dl glucose (DMEM; Gibco-BRL, Eggenstein, Germany) which was supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were kept in an incubator at 37°C with 5% CO₂ and were passaged every 72–96 h by light trypsinization.

ELISA for type IV collagen secretion

For these experiments, 10⁴ MCT cells were plated into each well of a 24-well culture plate (Nunc, Roskilde, Denmark) and were rested for 24 h in serum-free DMEM. MCT cells were stimulated for another 24 h in serum-free medium containing 50 μg/ml of ascorbic acid and β-amino- propionitrile with a single dose of 10⁻⁸ M Ang II (Sigma, Deisenhofen, Germany) in the presence or absence 30 μg/ml of a neutralizing anti-TGF-β₁,₃ monoclonal antibody (Genzyme, Boston, MA). Since this antibody is of murine origin, it does not interfere with the subsequent collagen detection using rabbit sera. At the end of the incubation period, cells were counted and 100 μl of each cell culture supernatant was transferred into a polyvinyl chloride ELISA plate (Nunc). After incubation for 12 h at 4°C, the culture supernatants were removed and possible unspecific binding sites of the plate were blocked with 5% bovine serum albumin (BSA) in 0.1 M phosphate buffer (pH 8.0) for 2 h at room temperature. A 1:100 dilution of a rabbit polyclonal monoclonal anti-collagen type IV antibody (Chemicon, Temecula, CA) in phosphate buffer with 0.5% BSA was used for the specific detection of type IV collagen secreted into the conditioned culture supernatants [6,11]. After three washes, ELISA plates were incubated with a 1:500 dilution of a peroxidase-labelled horse anti-rabbit antibody (Amersham, Braunschweig, Germany). The colour reaction was developed with tetramethylbenzidine and hydrogen peroxide for 20 min, stopped with 1 M HCl, and absorbance was measured at 450 nm in a microplate reader. Serial dilutions of mouse collagen type IV isolated from Engelbreth–Holm–Swarm sarcoma (Sigma) served as a standard. Collagen secretion is expressed in ng/ml culture supernatant. Experiments were repeated six times independently with duplicate measurements for each supernatant.

[H]proline incorporation experiments

To study [³H]proline incorporation into MCT cells, 10⁴ cells/well were plated into 24-well culture plates (Nunc) and rested in serum-free DMEM for 24 h [6]. Cells were stimulated for an additional period of 24 h with a single dose of 10⁻⁸ M Ang II in the presence or absence of 30 μg/ml of neutralizing murine anti-TGF-β₁,₃ antibody. For the last 12 h of culture, 1 μCi [³H]proline (L-2,3,4,5-³H]proline, 130 Ci/mmol; Amersham) was added to each well. At the end of the stimulation period, cells were washed twice in ice-cold PBS, and were proteolyzed in 10% TCA. After redissolving precipitates in 500 μl 0.5 M NaOH with 0.1% Triton X-100, 10 ml Rotiszint™ scintillation cocktail (Roth, Karlsruhe, Germany) was added and radioactivity was measured by liquid scintillation spectrophotometry. [³H]proline incorporations experiments were repeated independently six times.

TGF-β sense and antisense oligonucleotides

TGF-β₁, sense and antisense oligonucleoside phosphorothioates were prepared on an Oligo 1000 DNA synthesizer (Beckmann Instruments, Fullerton, CA) using phosphoramidite chemistry and sulphurization with the Beaucage reagent (Glen Research, Sterling, VA). Modified oligonucleotides were deprotected with ammonium hydroxide and purified on Poly-Pak™ cartridges. The following murine sequences with the ATG initiation codon or its complement were used: TGF-β₁ sense 5'CCCCGAGGCCGCATGGGGG3', TGF-β₁ antisense 5'TCCCCCATGGCCCTCGGG3'. These sequences have been previously successfully used by others and block transcription of TGF-β₁ [12]. For sense and antisense oligonucleotide experiments, 10⁴ MCT cells were plated in each well of a 24 well plate and rested for 24 h in serum-free medium. Five μM of each oligonucleotide was added per well in serum-free DMEM and the cells were further stimulated with 10⁻⁸ M Ang II or solvent (DMEM without serum) one h after addition of the oligonucleotides [12]. [³H]proline incorporation was measured for the last 12 h as described above. Culture supernatants were harvested separately, and TGF-β₁ was measured by a specific ELISA system (Predicta™, Genzyme) according to the manufacturer's recommendations after activation of supernatants by 1 M HCl and subsequent neutralization with NaOH. TGF-β₁ concentrations in culture supernatants were expressed in pg per 10⁵ cells.

SDS–polyacrylamide electrophoresis

In 75-cm² cell-culture flasks, quiescent MCT cells were incubated for 24 h with 10⁻⁸ M Ang II in the presence or absence of 30 μg/ml neutralizing anti-TGF-β₁,₃ antibody. L-ascorbic acid and the cross-linking inhibitor β-aminopropionitrile (50 μg/ml of each) as well as 10 μCi/ml [³H]proline was added. At the end of the incubation period, culture supernatants and cells were harvested separately. To this end, proteins in the cell layer were extracted with 5% acetic acid after extensive washing with ice-cold PBS, and a cocktail of protease inhibitors was added to the supernatants (10 mM N-ethylmaleimide, 20 mM EDTA, 1 mM phenylmethylsulphonylfluoride, and 10 units aprotinine). Proteins were precipitated at 4°C with absolute ethanol (final concentration 33%; vol:vol; [6]). After precipitation, samples for supernatants and cell lysates were centrifuged, dried under vacuum and redissolved in SDS-loading buffer with dithiothreitol.
Angiotensin II and collagen synthesis

(DTT) as a reducing agent. Protein concentrations were measured by a modification of the Lowry method which is insensitive to the used concentrations of SDS and DTT. Equal amounts of protein (80 μg) were boiled for 10 min and loaded onto a 10% SDS-polyacrylamide gel with a 4% stacking gel. Standard murine collagens (Sigma), and high-molecular-weight Rainbow™ markers (Amersham, comprises 14 300–200 000 Daltons) were run in parallel lanes. After electrophoresis, the gel was stained with Coomassie blue, soaked in Amplify™ (Amersham), vacuum dried, and fluorographed for 5 days at ~80°C. Equal protein loading of each lane allowed a semiquantitative analysis of the gel. This experiment was repeated twice with similar results.

Isolation of RNA and semiquantitative cDNA amplification

Quiescent (10^6) cells were stimulated for 24 h with a single concentration of 10^{-8} M Ang II. Some cells also received neutralizing anti-TGF-β_{1,3} antibody (30 μg/ml). Total RNA was extracted according to standard protocols [13]. cDNA was synthesized from 5 μg of total RNA using 0.5 μg of poly-d(T) primer (Pharmacia, Freiburg, Germany) in the presence of 500 units of Maloney murine leukemia virus reverse transcriptase diluted in 50 μl of a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, and 500 μM dNTP. After incubation for 90 min at 37°C, the reaction mixture was precipitated with 25 μl 7.5 M ammonium acetate and 50 μl isopropanol, and pellets were recovered by centrifugation [13]. After washing in 70% ethanol, pellets were resuspended in 50 μl distilled water. Polymerase chain reactions for quantitative comparison of collagen type IV transcriptions were performed as previously described [13] and were normalized for the presence of a housekeeping transcript (GAPDH). The complete amplification reaction without primers was equivalently distributed to separate tubes with either primers for TGF-β_{1,3} or GAPDH [13, 14]. A total of 0.15 μg of each of following primers were used:

- Murine collagen type α 1(IV): sense 5'-GTGCCGGTTTGT-GAAGCACCAGG3', antisense 5'-GGTCTTCTCATGACAC-TT3';
- GAPDH: sense 5'-AATGCACTCTTGGCACCACAA3';
- antisense 5'-ATACTGGTACACCTTAAAGG3'.

A total of 0.15 μg of each of following primers were used:

Effect of a neutralizing anti-TGF-β antibody on collagen production

Since we have previously demonstrated that a single dose of 10^{-8} M Ang II stimulates synthesis of collagen type IV in MCT cells [6], this dose was consequently used for further studies. As shown in Table 1, 10^{-8} M Ang II for 24 h significantly stimulated the release of collagen type IV into the cell culture supernatant as measured by a sensitive ELISA [6]. This Ang-II-mediated production was abolished by the presence of 30 μg/ml neutralizing anti-TGF-β_{1,3} antibody. Adding the same amount of anti-TGF-β_{1,3} antibody to control cells did not significantly change collagen type IV production suggesting that the murine antibody does not interfere with the ELISA detection system.

| Table 1. ELISA for type IV collagen in culture supernatants of MCT cells |
|-----------------|------------------|-------------------|-------------------|
| Control medium | 13.0 ± 0.7       | 10^{-8} M Ang II  |
| Control medium + | 30 mg/ml anti-TGF-β_{1,3} antibody | 11.8 ± 0.8       |
| 10^{-8} M Ang II + | 30 mg/ml neutralizing anti-TGF-β_{1,3} antibody | 18.0 ± 0.4*      |
| 10^{-8} M Ang II + | 30 mg/ml anti-TGF-β_{1,3} antibody | 14.5 ± 0.2       |
| Control medium + | 2 mg/ml recombinant TGF-β_{1,3} | 16.5 ± 0.6*      |

concentrations in ng type IV collagen per ml culture supernatant, stimulation with mediators for 24 h, * P < 0.05, n = 6.
Treatment of quiescent MCT cells with 2 ng/ml exogenous recombinant TGF-β1 also stimulated, as predicted, production of collagen type IV (Table 1).

**Incorporation of 3[H]proline into MCT cells**

It has been demonstrated that [3H]proline is preferentially incorporated into newly synthesized collagens [6]. We have previously shown that Ang II stimulated [3H]proline incorporation mainly into type IV collagen in MCT cells [6]. To further determine the role of endogenous TGF-β1 synthesis in this process, we used two strategies to interfere with TGF-β1 production: First, we used the neutralizing anti-TGFβ1 antibody; second, we constructed specific modified sense and antisense TGF-β1 oligonucleotides. As shown in Figure 1, a single dose of 10^{-8} M Ang II for 24 h significantly stimulated [3H]proline incorporation into MCT cells. Addition of 30 μg/ml neutralizing anti-TGFβ1-antisense antibody partly abolished this Ang-II-stimulated incorporation (Figure 1). However, addition of 5 μM TGF-β1 antisense oligonucleotide to the medium completely abolished Ang-II-stimulated [3H]proline incorporation without significantly influencing baseline incorporation in control cells (Figure 1). The same concentration of TGF-β1 sense oligonucleotide had no significant effect on Ang-II-mediated or baseline [3H]proline incorporation. To further test the effects of the sense-antisense oligonucleotides, we measured directly total TGF-β1 in culture supernatants. Table 2 revealed that 5 μM of the TGF-β1 anti-sense, but not the sense oligonucleotide abolished the Ang II-induced release of TGF-β1 into culture supernatants of MCT cells.

To further characterize [3H]proline-labelled collagens produced by MCT cells after Ang II stimulation, culture supernatants as well as cell lysates were separated by SDS-polyacrylamide electrophoresis and bands were compared after autoradiography with standard collagens run in parallel. In accordance with previous studies [6,16], MCT cells synthesize mainly type IV collagen (Figure 2). The majority of collagen proteins

![Fig. 1. Incorporation of [3H]proline as a marker of collagen novo synthesis in MCT cells. A single dose of 10^{-8} M Ang II for 24 h significantly stimulated [3H]proline incorporation. This stimulation was partly abolished in the presence of 30 μg/ml neutralizing anti-TGFβ1-antisense antibody. In addition, 5 mM of TGF-β1 antisense, but not sense, phosphorothioate-modified oligonucleotides completely blocked the Ang-II-stimulated incorporation of [3H]proline. The anti-TGFβ1-antisense antibody as well as the sense and antisense oligonucleotides had no significant effect on basal [3H]proline incorporation. (n = 6, *P < 0.01 versus unstimulated controls, #P < 0.01 versus cells treated only with Ang II.)](image1)

![Fig. 2. Separation of [3H]proline labelled collagens by SDS-PAGE. Culture supernatants and cellular lysates were separately harvested and compared with standard collagen chains run in parallel. 10^{-8} M Ang II significantly stimulated the synthesis of both type IV collagen chains. This effect was partly blocked in the presence of 30 μg/ml neutralizing anti-TGFβ1-antisense antibody. Ang II stimulation had no effect on [3H]proline incorporation in cell lysates indicating that almost all type IV collagen is secreted in the presence of cross-linking inhibitors and [3H]proline is mainly incorporated into collagens. This gel is representative of two experiments with qualitatively similar results.](image2)

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<th>TGF-β1 concentrations in culture supernatants</th>
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<td>Control medium</td>
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<td>10^{-8} M Ang II</td>
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pg TGF-β1/10^5 cells, stimulation for 24 h, *P < 0.01, n = 10 measured in duplicate.
Angiotensin II and collagen synthesis were secreted into the culture supernatant in the presence of cross-linking inhibitors. MCT cells incubated with $10^{-8}$ M Ang II released more type (IV) collagen into the cell culture supernatant. This stimulated synthesis of type IV collagen was partly abolished in the presence of 30 μg/ml neutralizing anti-TGF-β1,3 antibody (Figure 2).

**Semiquantitative cDNA amplification**

To assess transcript levels for α1(IV)collagen in MCT cells, we used semiquantitative cDNA amplification. We have previously demonstrated the reliability of this method [13,14]. As shown in Figure 3, stimulation of quiescent MCT cells with $10^{-8}$ M Ang II significantly increased transcripts for collagen type IV. This increase in mRNA was abolished in the presence of the neutralizing anti-TGF-β1,3 antibody (Figure 3).

**Transient transfections and reporter gene studies**

We have previously demonstrated that the Ang II-induced increase in steady-state mRNA for type IV collagen depends on transcriptional activity [6]. To test the influence of TGF-β in this process, MCT cells were transiently transfected with reporter gene constructs containing regulatory elements of murine α1(IV) collagen. CAT activity was measured after separation of acetylated products by thin-layer chromatography. A dose of $10^{-8}$ M Ang II more than doubled the CAT activity suggesting stimulation of type IV transcription (Figure 4). The Ang-II-stimulated CAT activity in MCT cells transfected with the collagen type IV construct was significantly reduced by the presenceence of anti-TGF-β1,3 antibody in the medium. The antibody had only little effect on basal transcriptional activity in MCT cells incubated in control medium (Figure 4). Since the neutralizing anti-

TGF-β1,3 antibody was added after the transfection, the inhibition of transcriptional activity was due to its specific neutralizing effect and not due to interference with the transfection. In addition, the anti-TGF-β1,3 antibody had no effect of the activation of a control β-galactosidase construct driven by SV 40 enhancer/promoter elements (data not shown).

**Discussion**

Our data collectively suggest that the Ang-II-stimulated transcription and synthesis of type IV collagen in MCT cells is mediated by endogenous production of TGF-β. Previous studies of our group have demonstrated that Ang II induces expression of TGF-β in this cell line [5,9]. The TGF-β-mediated induction of type IV collagen expression in MCT cells is not limited to the vasoconstrictive factor Ang II. It has been previously demonstrated that MCT cells grown in the presence of high glucose containing medium produce more TGF-β which in turn stimulates type IV collagen synthesis [17]. Thus, it is tempting to speculate that in diabetes mellitus, a situation in which the local renin-angiotensin system may be activated, Ang II and hyperglycaemia have additional amplifying effects on the synthesis of type IV collagen. Indeed, an increase in tubular type IV collagen is a common feature in diabetic nephropathy [18].

Ang-II-stimulated synthesis of extracellular matrix components is not a feature unique to MCT cells. In contrast to epithelial MCT cells, the vasoactive peptide stimulates collagen type I synthesis in mouse and rat mesangial cells [16,19]. There is some evidence that
these increased production of interstitial collagen is due to TGF-β1 [19].

Overwhelming evidence has accumulated over the last decade indicating that changes in the tubulointerstitial architecture are closely related with the loss of renal function and the progression of disease [1–3]. Although the interstitial accumulation and activation of fibroblasts which subsequently result in the interstitial deposition of collagen types I and IV remains a cornerstone in the pathogenesis of interstitial fibrosis [3], there is also clear evidence of increased production and deposition of type IV collagen [1,20–22]. For example, in a model of interstitial extracellular matrix accumulation induced by acute puromycin aminonucleoside nephrosis in rats, Jones and co-workers found not only an increase in interstitial deposition of type I collagen, but also a significant increase in steady-state mRNA and staining for collagen type IV [21]. Interestingly, collagen type IV increased along the tubular basement membranes and appeared even in small amounts in the interstitial space whereas glomerular collagen type IV did not change in this model [21]. Moreover, TGF-β1 expression was significantly enhanced in chronic aminonucleoside nephrosis and treatment with an angiotensin-converting enzyme (ACE) inhibitor abolished the increase in TGF-β1 and reduced the interstitial deposition of collagens [20]. It has been also demonstrated in a model of chronic unilateral ureteral obstruction (UUO) which results in interstitial fibrosis of the affected kidney, that enalapril reduces TGF-β1 and collagen type IV mRNA expression in affected kidneys [22]. In addition, immunofluorescence staining for type IV was significantly reduced in rats with UUO treated with the ACE inhibitor [22]. An ACE inhibitor also reduced interstitial fibrosis in the remnant kidney after nephrectomy, these effects were not observed by an equal reduction of systemic blood pressure by triple therapy, suggesting that Ang II is necessary for the development of interstitial fibrosis in this model [23]. Finally, Johnson and associates demonstrated that Ang II-infused rats developed focal tubulointerstitial injury with mild fibrosis and increased type IV collagen deposition [24]. However, there exists also clear evidence that soluble antigen-binding proteins like a T-helper factor (ThF) actually downregulate collagen type IV synthesis in proximal tubular cells, which may contribute directly to the weakening of the basement membrane and ensuing tubular atrophy [25]. Thus the situation is more complex and tubular cells may not be unequivocally the source of increased collagen type IV deposition in interstitial fibrogenesis. Moreover, it is possible that tubular-derived collagen type IV synthesis may change during the course of the disease, with a stimulation early during compensatory hypertrophy and a suppression later when immuno-competent cells infiltrate the interstitium and release ThF which suppresses collagen type IV transcription.

Our antisense TGF-β1 experiments provide convincing evidence that TGF-β1 synthesis and its subsequent action can be inhibited by this approach. Although this strategies may be of considerable interest to counteract the effects of TGF-β1 in vivo, there are still many problems with the application of antisense oligonucleotides [26,27]. Moreover, our model system using SV 40 transformed tubular cells grown on plastic dishes may not accurately reflect the complex role of the extracellular matrix in vivo [28].

In conclusion, we have demonstrated that the Ang-II-mediated stimulation of collagen type IV transcription and synthesis depends on the endogenous production of TGF-β1. These findings may be important in the pathogenesis of interstitial fibrosis in situations with an activated renin-angiotensin system.

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