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Increased expression of TGF-β1 mRNA in the obstructed kidney of rats with unilateral ureteral ligation

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Increased expression of TGF-β1 in the obstructed kidney of rats with unilateral ureteral ligation. Renal interstitial fibrosis is a common consequence of chronic ureteral obstruction. While several cytokines may initiate fibrogenesis, TGF-β is considered to be a major stimulating factor. It has been reported that TGF-β1 regulates extracellular matrix (ECM) synthesis, that thromboxane (Tx) stimulates ECM protein synthesis, and that angiotensin II (Ang II) increases expression of TGF-β1 mRNA in rat aortic smooth muscle cells. Therefore, we measured TGF-β1 mRNA expression by reverse transcription coupled with polymerase chain reaction in renal cortex of rats with unilateral ureteral obstruction (UUO) to determine whether Ang II and/or Tx stimulates increases in TGF-β1 mRNA. TGF-β1 mRNA levels in contralateral kidneys of rats with UUO did not change significantly during 14 days of obstruction, while in the obstructed kidney TGF-β1 mRNA levels were increased significantly after three days as compared to the control (unoperated rats) kidneys. The increase in TGF-β1 mRNA expression in the obstructed kidney cortex was found in tubular cells rather than glomeruli. OKY-046, an inhibitor of thromboxane synthase, did not affect the changes in TGF-β1 mRNA in the obstructed kidney. Enalapril, an angiotensin I converting enzyme inhibitor, significantly blunted but did not completely abrogate the increase in TGF-β1 mRNA. These data suggest that in obstruction TGF-β1 is increased at the transcriptional level and thus may play a role in initiating fibrogenesis in obstructive nephropathy. The effect of thromboxane on extracellular matrix synthesis does not appear to be mediated by TGF-β1. Angiotensin II has a role in stimulating TGF-β1 expression in UUO.

Renal interstitial fibrosis is a common consequence of chronic ureteral obstruction. Fibrogenesis develops as a result of an imbalance between extracellular matrix (ECM) synthesis, matrix deposition and matrix degradation [1]. There are several cytokines secreted by inflammatory cells that stimulate fibroblast proliferation and regulate the synthesis of ECM components [1]. Among these cytokines TGF-β1 plays a major role in regulating ECM synthesis [1, 2]. TGF-β1 stimulates the synthesis of ECM proteins, inhibits matrix degradation by both an increase in the activity of protease inhibitors and a decrease in proteases, and stimulates synthesis of receptors for ECM proteins [3–5]. TGF-β has been shown to regulate gene transcription of collagen types I, III, and IV, fibronectin, laminin and integrins [1, 3, 5–7]. Furthermore, TGF-β is a chemoattractant for fibroblasts [4, 8] and also stimulates fibroblast proliferation [1]. Thus, it may have a role in the accumulation of ECM proteins in the renal interstitium. However, little is known about the mechanism of interstitial fibrosis during obstructive nephropathy, especially in terms of ECM synthesis and its potential regulation by TGF-β1.

Several vasoactive compounds that affect renal hemodynamics are increased in acute ureteral obstruction [9, 10]. We showed previously that two major vasoconstrictors, angiotensin II (Ang II) and thromboxane (Tx) A2, play an important role in the altered renal hemodynamics in this setting. Indeed, prior inhibition of Ang II and Tx synthesis ameliorated the decrease in GFR and renal plasma flow seen in the obstructed kidney [11]. The increased production of prostanoids that occurs in the obstructed kidney is mediated in large part by the elevated levels of endogenous Ang II [12]. Recently, Ang II has been shown to modulate proliferation of vascular smooth muscle cells by stimulating TGF-β1 synthesis [13, 14]. Stouffer and Owens [14] showed that this smooth muscle cell proliferation is regulated by Ang II through autocrine production of TGF-β. Treatment with Ang II increases TGF-β activity while TGF-β neutralizing antibody inhibits the Ang II-induced increase in DNA synthesis. Gibbons, Pratt and Dzau [13] reported that Ang II increases mRNA expression of TGF-β1 in cultured aortic smooth muscle cells and also promotes the conversion of latent forms of TGF-β1 to the active form. Majesky et al [15] showed that neointimal thickening after arterial injury is initiated by an increased synthesis of TGF-β1 through augmented gene expression. Expression of fibronectin, collagen α1(I) and collagen α(III) mRNA was increased in the neointima after the increase in cytokine. These studies on vascular smooth muscle indicate a regulatory effect of Ang II on gene expression for TGF-β1.

Bruggeman et al [16] reported that Tx stimulates ECM protein synthesis. Thromboxane analogs increased production of fibronectin, laminin and type IV collagen by human mesangial cells through increased levels of mRNA for these genes. Others have also shown that Tx stimulates synthesis of ECM proteins: type IV collagen [17–19], fibronectin [20] and laminin [17, 20]. However, it is not known whether the stimulating effect of Tx on ECM is mediated directly or through the expression of another product such as TGF-β1.

Thus, the aims of this study were to examine the levels of mRNA expression for TGF-β1 in the kidney during unilateral

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ureteral obstruction (UUO) and to determine whether or not Tx and/or Ang II stimulated the expression of TGF-β1 mRNA.

Methods

Experimental protocol

Female Sprague-Dawley rats, weighing 200 to 250 g, were used in this study. Under fluothane anesthesia ureteral obstruction was performed by ligating the left ureter with 4-0 silk through a midline abdominal incision. Sham-operated rats had their ureters manipulated but not ligated; kidneys from these animals and from unoperated rats served as controls. (Data from unoperated rats and sham-operated rats were indistinguishable.) Rats were sacrificed at 3, 5, 7 and 14 days after UUO. Under pentobarbital anesthesia (50 mg/kg body wt, i.p.) the aorta was clamped above the renal arteries and the kidneys were thoroughly perfused with cold Hanks' balanced salt solution (HBSS) to cool down the kidneys and to wash out blood from the renal vasculature and parenchyma. Kidneys were removed and placed in cold HBSS; the cortex was immediately dissected from each kidney and stored at −70°C.

A group of rats was given intraperitoneal enalapril (5 mg/kg body wt), an inhibitor of angiotensin I converting enzyme (ACE) or OKY-046 (20 mg/kg body wt, Ono Pharmaceutical Co., Osaka, Japan), an inhibitor of thromboxane synthase at 24 hours, 12 hours, and one hour before ligation of the ureter and every 12 hours after operation for five days, at which point they were sacrificed (total of 12 injections). A group of sham-operated rats was similarly treated with enalapril or OKY-046. These rats were also sacrificed five days after operation.

RNA isolation

Total RNA was extracted from renal cortex (approximately 50 mg to 100 mg) using guanidinium isothiocyanate (RNAzol method; Cinna/Biotex) [21] and precipitated with isopropanol. After washing twice with 70% ethanol, the RNA pellet was dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA). RNA was quantitated by UV spectrophotometry at 260 nm and 280 nm. RNA with an OD_{260}/OD_{280} ratio over 1.9 was used for cDNA synthesis. The ratio was between 2.3 and 1.9 in every RNA sample regardless of its origin: kidneys of sham-operated or normal rats, or the contralateral or obstructed kidneys of rats with UUO.

cDNA synthesis

First-strand cDNA was synthesized using a cDNA cycle kit (Invitrogen, San Diego, California, USA), 2 μg of total RNA and oligo dT for priming. The reaction was incubated at 42°C for one hour with a Perkin Elmer Cetus DNA Thermal Cycler, heated to 94°C for three minutes and chilled on ice. A second round of reverse transcription was performed after adding 5 units of avian myeloblastosis virus (AMV) enzyme.

Polymerase chain reaction

Polymerase chain reaction (PCR) coupled to reverse transcription of RNA (RT-PCR) has been popular to detect changes in mRNA levels. With this technique, cDNA synthesis from total RNA using oligo-dT primer reflects the relative amount of mRNA and PCR amplification can detect a small amount of message in a tissue or abundant message in a small amount of tissue [22]. However, PCR is so sensitive because of exponential amplification that several kinds of techniques have been required for quantitation of mRNA [23]. Recently, simpler techniques were developed to detect mRNA quantitatively by RT-PCR under optimized conditions for assay [24–27]. We used quantitative RT-PCR for this study by optimizing the conditions for the amount of total RNA and the number of PCR amplification cycles.

Three μl of each cDNA was amplified in a total volume of 50 μl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol of oligonucleotide primers and 1.25 U of Taq DNA polymerase (Promega, Madison, Wisconsin, USA). In order to quantitate the PCR products comparatively and confirm the integrity of the RNAs we coamplified a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a companion tube. The TGF-β1 primers were designed from the sequence of the rat gene [28]: 5′-AATACGTCAGACATTCGGGAAGCA (sense) from exon 2 and 3′-GTCAATGTACAGCTGCCGTACACA (antisense) from exon 6 yield a 498 bp product. The GAPDH primers were designed from the rat gene: 5′-AATGCATCCTGCACCACCAA (sense) from exon 7 and 3′-GTAGCCATATTCATTGTCATA (antisense) from exon 9 yield a 515 bp product [26, 27, 29]. PCR was carried out using a Perkin Elmer Cetus DNA Thermal Cycler with 30 cycles as follows: 94°C for one minute (denaturing), 60°C for one minute (annealing) and 72°C for two minutes (extension). PCR products were sequenced using the TA Cloning System (Invitrogen, San Diego, California, USA) and the AmpliTaq Sequence kit (Perkin Elmer, Norwalk, Connecticut, USA). The resultant sequences (not shown) confirmed the identity of the PCR products as TGF-β1 and GAPDH. Amplification without cDNA did not yield any band other than those of the primers for TGF-β1 or GAPDH (not shown). RT-PCR without AMV did not show PCR products, indicating no amplification of genomic DNA.

The sequence of the fibronectin primers is designed to flank the EDIIIA region of the rat gene: 5′-GTCAGCGTCTATGCTCAGAA (sense) and 3′-TGAGTGAACTTCAGGTTGGTC (antisense) [30–32]. cDNAs were synthesized from 4 μg of total RNA and 3 μl of each cDNA was amplified with 40 PCR cycles as follows: 94°C for one minute, 45°C for one minute and 72°C for two minutes.

Quantitative analysis of PCR products

After amplification, 15 μl of each PCR product was electrophoresed through a 1.2% agarose gel with ethidium bromide (0.5 μg/ml). The gel was photographed with Polaroid Type 665 positive/negative film (Polaroid Corporation, Cambridge, Massachusetts, USA) over UV light at the same exposure and developing time. The bands on the negative film were scanned by densitometry (Sepra Scan 2001 software, Integrated Separations System, Natwick, Massachusetts, USA) for quantification [26, 27]. The PCR product for TGF-β1 or GAPDH amplified from the same cDNA was electrophoresed in the same gel and the ratio of TGF-β1/GAPDH was determined to eliminate gel-to-gel or film-to-film variance.

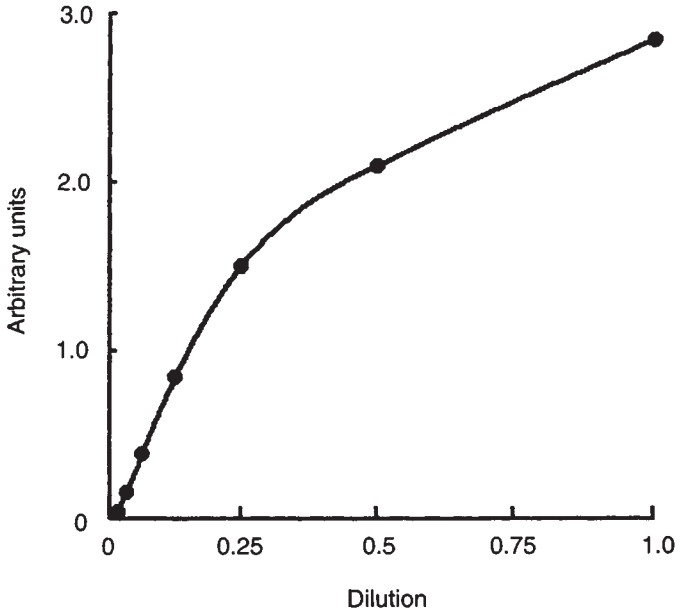


Fig. 1. *Comparison of densitometric signal against dilution of a PCR product.* A PCR product from bacteriophage lambda DNA (500 bp) was diluted serially from × 1 to × $1/2^6$. Fifteen microliters of each dilution were electrophoresed in a 1.2% agarose gel with ethidium bromide, photographed with a Polaroid negative film and subjected to densitometry. The densitometric signal (arbitrary units) is plotted against dilution.

Statistical analysis

Data were generated as mean ± SD and were analyzed by the unpaired *t*-test. Comparisons between values for the contralateral and the obstructed kidneys were performed using an unpaired *t*-test with Welch's correction.

Results

Quantitation of PCR products

To assess the sensitivity and linear response of the densitometry and the Polaroid film we obtained PCR product from bacteriophage lambda DNA provided as a control template (Perkin Elmer) and diluted the PCR product by a twofold method (1 to $1/2^6$). Each diluted aliquot was electrophoresed and analyzed quantitatively. Figure 1 demonstrates a good correlation between the dilution and the relative value of the density of each band.

To develop optimal conditions for PCR we first synthesized cDNAs from 8, 4, 2, and 1 μg of total RNAs of control kidney. Three μl of each cDNA was amplified at 28, 30 and 32 cycles. A 15-μl aliquot of each PCR cycle was electrophoresed through a 1.2% agarose gel and analyzed quantitatively. PCR products for TGF-β1 were well correlated with the amount of RNA used and the number of PCR cycles (Fig. 2A). PCR products for GAPDH showed a slight increase according to the amount of RNA and PCR cycles. The densitometric signal for TGF-β1 or GAPDH approached but was not maximal at 32 cycles (not shown). Figure 2B demonstrates the relationship between the amount of total RNA and the relative TGF-β1 mRNA expression (the ratio of TGF-β1/GAPDH). At 32 cycles the ratio showed a plateau at 4 μg of RNA. At 28 cycles the ratio was linear but the sensitivity was so low that the variance of the ratio between 1 and 4 μg RNA was small. At 30 cycles there was a clear increase in the ratio between 1 and 2 μg RNA. For this study we used 2 μg of total RNA for cDNA synthesis and 30 cycles for PCR amplification.

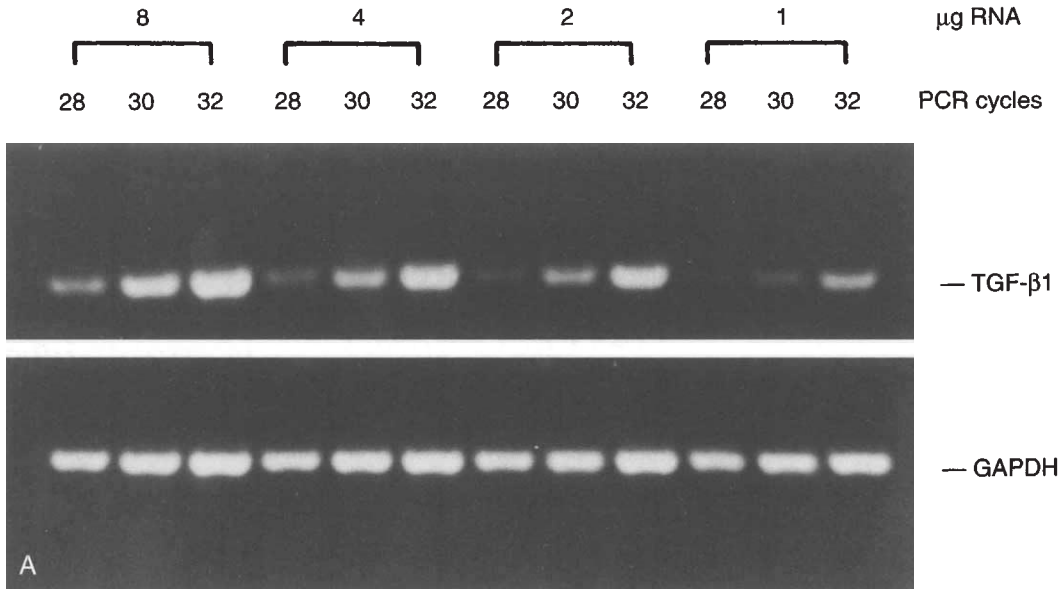
Effects of unilateral ureteral obstruction on the expression of TGF-β mRNA

Figure 3A demonstrates the PCR product at the different time intervals of UUO. The GAPDH bands of both the contralateral and the obstructed kidneys showed approximately the same strength at every time point as that of the control kidney. The amount of TGF-β1 mRNA of the contralateral kidneys of UUO rats was essentially the same as that of the control kidneys during the first seven days. The amount of mRNA decreased slightly at 14 days of ureteral obstruction. The kidneys with ureteral obstruction showed an increased amount of the TGF-β1 band at every time point tested as compared to control kidneys or the contralateral kidney of the same rats. Figure 3B demonstrates the changes in the relative level of TGF-β1 mRNA expression during UUO. The mRNA expression in the obstructed kidneys relative to control kidneys or contralateral kidneys from UUO animals was significantly increased at three days (2.2-fold of control kidneys), five days (2.4-fold), at seven days (2.1-fold) and 14 days (2.2-fold). TGF-β1 mRNA level in the contralateral kidneys of rats with UUO showed a slight (not significant) increase at three days and at five days. However, it was slightly decreased at seven days and significantly decreased at 14 days as compared to that of the control kidneys. We measured the TGF-β1 mRNA at 4, 8, and 24 hours after UUO. The TGF-β1 mRNA level was approximately the same between the contralateral and obstructed kidneys after eight hours. However, mRNA levels of the housekeeping gene, GAPDH, were decreased in the obstructed kidney at 24 hours and mRNA levels of TGF-β1 were inconsistent in most cases (not shown).

To determine which cells might contribute to the increased expression of TGF-β1 mRNA in the obstructed kidney cortex, we measured TGF-β1 mRNA in isolated glomeruli and tubules. We isolated glomeruli from the renal cortex by mechanical sieving (mesh sizes 250, 150 and 75 μm) [12]. Tubules were isolated on the 150 μm mesh, while glomeruli were isolated on the 75 μm mesh. The tubule population includes proximal, distal, connecting, and collecting tubules. RNA isolation and RT-PCR were performed as described in the **Methods**. PCR amplification was performed using 1 μg of total RNA and 28 PCR cycles for glomeruli and 2 μg of RNA and 30 cycles for tubules. Figure 4 demonstrates the PCR products obtained from the glomeruli and tubules of rats with UUO for five days. There was no difference in the level of GAPDH mRNA of the glomeruli or the tubules between the contralateral and the obstructed kidneys. TGF-β1 mRNA levels in glomeruli from the obstructed kidney were essentially the same as those obtained in glomeruli from the contralateral kidney. However, TGF-β1 mRNA in the tubules was obviously increased in the obstructed kidney.

mRNA expression of fibronectin during UUO

To determine whether the increased TGF-β1 contributed to stimulation of ECM gene expression we carried out PCR to detect the mRNA expression for fibronectin at five days of



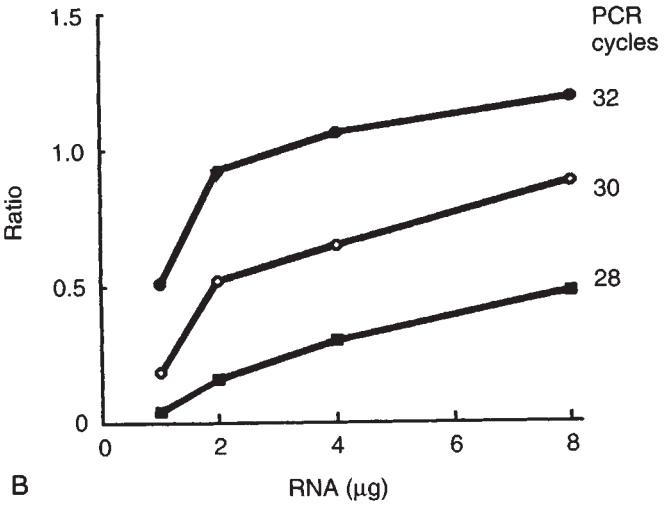


Fig. 2. *Optimization of PCR conditions.* cDNA was synthesized from 8, 4, 2, and 1 μg of total RNA obtained from control kidneys and amplified at 28, 30 and 32 PCR cycles. A 15 μl aliquot of each product was electrophoresed and analyzed quantitatively. **(A)** The TGF-β1 band shows a significant increase according to the amount of RNA and number of PCR cycles whereas that of GAPDH shows a very slight increase. **(B)** Relative level of TGF-β1 mRNA is determined by the ratio TGF-β1/GAPDH. At 32 cycles the ratio reaches a plateau at 4 μg of RNA. At 28 cycles the ratio is low but measurable. At 30 cycles there is a good correlation between the amount of RNA and the ratio. The optimum conditions were determined to be 2 μg of RNA and 30 cycles of PCR.

UUO. PCR products were electrophoresed in a 2% agarose gel. Figure 5 demonstrates the PCR products of fibronectin at five days after UUO. The predicted size for EDIIIA+ and EDIIIA− was 370 bp and 102 bp, respectively. The EDIIIA+ message was obviously increased in the obstructed kidney compared to that message in the control or the contralateral kidney. However, the amount of the EDIIIA− message was approximately the same in the three types of kidneys tested. There was also a PCR product of 290 bp which by limited restriction enzyme digestion was also shown to be a product of the fibronectin gene (not shown). The amount of this product was not significantly changed by ureteral obstruction.

Effects of enalapril or OKY-046 on the expression of TGF-β1 mRNA

We treated rats with enalapril or OKY-046 for one preoperative and five postoperative days to determine whether or not increased angiotensin II or thromboxane levels had a role in the increase in TGF-β1 mRNA in obstruction. These two inhibitors did not affect the levels of mRNA GAPDH in any experiment. Figure 6 demonstrates the effects of enalapril or OKY-046 on the mRNA levels of TGF-β1 in obstructed kidneys of UUO rats relative to contralateral kidneys from those animals. The increased level of TGF-β1 mRNA present in the obstructed kidney was significantly blunted though not completely abrogated by perioperative treatment with enalapril (mRNA for TGF-β decreased from 2.2-fold to 1.7-fold of the level of the control kidneys). There was still a significant difference in the mRNA level between the contralateral and the obstructed kidneys of rats given enalapril. TGF-β1 mRNA levels in the contralateral kidneys were not affected by enalapril. Treatment with enalapril had no effect on the mRNA levels of TGF-β1 in kidneys of sham-operated rats (data not shown).

The mRNA levels of TGF-β1 in the obstructed kidneys of untreated rats were not significantly affected relative to levels in kidneys of rats treated with OKY-046 (Fig. 5). Thus, OKY-046 did not suppress the increased levels of TGF-β1 mRNA present in the obstructed kidneys. The mRNA level of TGF-β1 in the contralateral kidneys was also not affected by OKY-046 administration. Sham-operated rats treated with OKY-046 showed

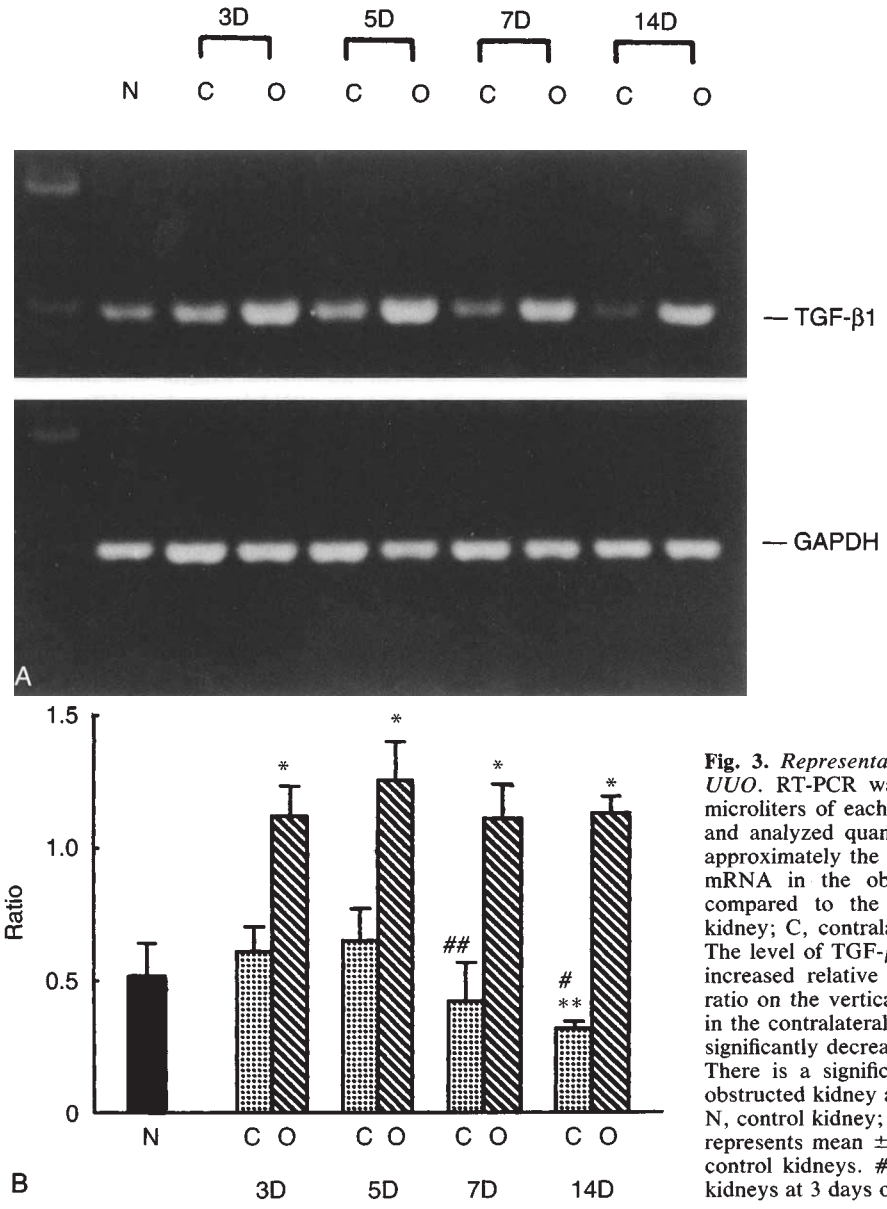


Fig. 3. *Representative changes of TGF-β1 mRNA expression during UUO.* RT-PCR was performed under optimum conditions. Fifteen microliters of each PCR product was electrophoresed, photographed and analyzed quantitatively. **(A)** The amount of GAPDH mRNA is approximately the same at every time point. The amount of TGF-β1 mRNA in the obstructed kidney is increased after UUO when compared to the control kidney. Abbreviations are: N, control kidney; C, contralateral kidney; O, obstructed kidney; D, days. **(B)** The level of TGF-β1 mRNA in the obstructed kidney is significantly increased relative to contralateral kidneys of obstructed rats. The ratio on the vertical axis refers to TGF-β1/GAPDH. TGF-β1 mRNA in the contralateral kidney is slightly decreased at seven days, and is significantly decreased at 14 days as compared to the control kidney. There is a significant difference between the contralateral and the obstructed kidney at every time point ($P < 0.001$). Abbreviations are: N, control kidney; C, contralateral kidney; O, obstructed kidney. Bar represents mean ± SD. $N = 5$. * $P < 0.001$ and ** $P < 0.05$ versus control kidneys. # $P < 0.01$ and ## $P < 0.05$ versus contralateral kidneys at 3 days or 5 days.

similar levels of TGF-β1 mRNA in their kidneys as those of sham-operated animals which did not receive OKY-046 (data not shown).

Discussion

The steady-state levels of mRNA for TGF-β1 in rat kidney have been shown to be high in glomeruli, intermediate in papilla and low in cortex and whole kidney [33]. We used RT-PCR to detect mRNA for TGF-β1 in renal cortex. We first explored the conditions that would optimize the detection of TGF-β1 mRNA (ratio of TGF-β1/GAPDH) in control kidneys and kidneys with ureteral obstruction. In preliminary experiments we determined parameters to obtain a correlation between serially diluted PCR products and their densitometric signals. Although the exact amount of amplified DNA cannot be determined in an ethidium bromide stained gel, we believe this technique is useful to measure relative mRNA levels under optimized conditions from small amounts of kidney tissue.

We showed that in the obstructed kidney the expression of TGF-β1 mRNA was increased significantly at 3, 5, 7 and 14 days after UUO. Nagel and Bulger [34] reported that in rabbits with UUO the interstitial space was widened at seven days after obstruction; there were increased collagen fibers and numerous fibroblasts detected at this time, and by the 16th day collagen was greatly increased and was arranged in large bundles. These pathological findings indicate that the process of renal interstitial fibrosis after ureteral ligation may be initiated at seven days or earlier after the onset of obstruction. Khalil et al [35] showed in a rat model of bleomycin-induced pulmonary fibrosis that the peak level of TGF-β, which occurred at seven days after bleomycin administration, preceded the maximum collagen protein synthesis, which occurred seven days later. Others [15]

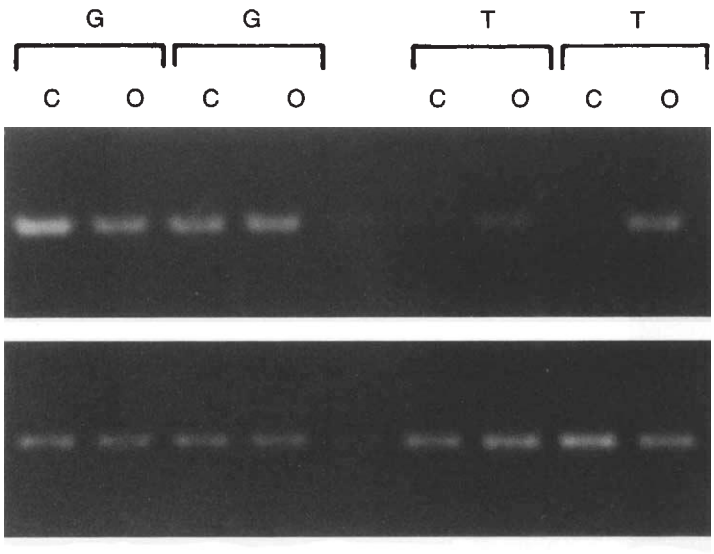


Fig. 4. *TGF-β1 mRNA expression in glomeruli and tubules after five days of UUO.* Glomeruli and tubules were isolated with a sieve technique and mRNA expressions for TGF-β1 and GAPDH were measured. Results are from two different experimental animals. The TGF-β1 mRNA level did not differ in glomeruli from the contralateral and obstructed kidneys but increased in tubules from obstructed kidneys. The center lane presents a size marker of DNA. Abbreviations are: G, glomeruli; T, tubules; C, contralateral kidney; O, obstructed kidney.

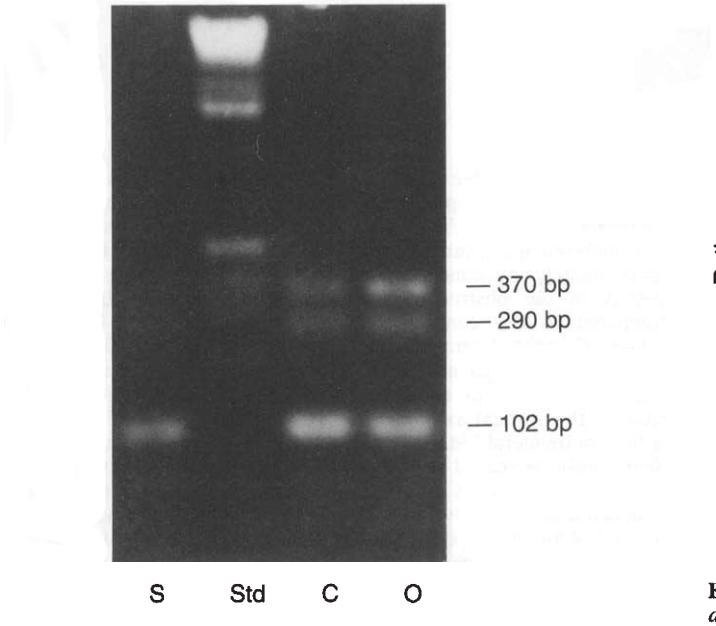


Fig. 5. *mRNA expression of fibronectin after five days of UUO.* PCR was carried out using the primers flanking the EDIIIA region of fibronectin gene. The 370 bp band (EDIIIA+) was increased in the obstructed kidney while the 290 and 102 bp bands (EDIIIA−) were essentially unchanged. Abbreviations are: S, kidney from sham-operated rats; C, contralateral kidney; O, obstructed kidney.

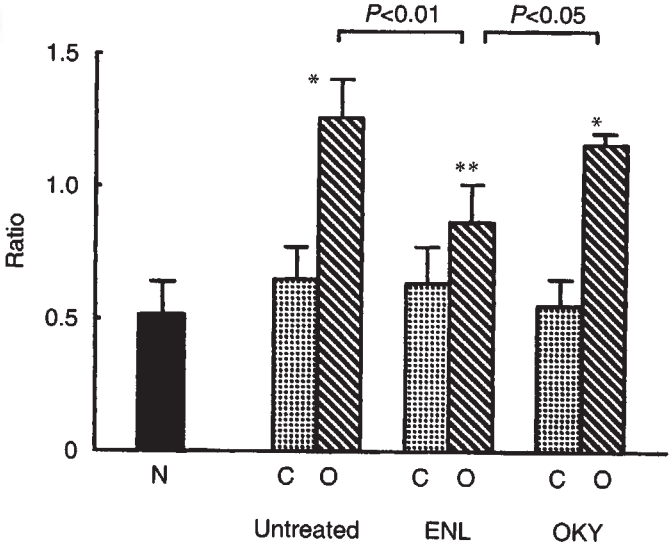


Fig. 6. *Effects of enalapril or OKY-046 on TGF-β1 mRNA expression after five days of UUO.* The ratio on the vertical axis refers to TGF-β1/GAPDH. Enalapril significantly blunted the increased level of TGF-β1 mRNA seen in the obstructed kidney while it did not affect the level in the contralateral kidney. The difference in TGF-β mRNA expression between contralateral and obstructed kidneys was still significant in enalapril-treated rats. OKY-046 did not affect the level of TGF-β1 mRNA seen in either the obstructed or contralateral kidneys. There was a significant difference between the two kidneys in untreated rats and in those treated with OKY-046 ($P < 0.001$). Abbreviations are: N, control kidney; C, contralateral kidney; O, obstructed kidney; ENL, enalapril; OKY, OKY-046. Bar represents mean ± SD. $N = 5$. * $P < 0.001$ and ** $P < 0.01$ versus control kidneys.

have demonstrated that during vascular injury the increase in mRNA for TGF-β1 preceded the increase of mRNAs for collagen and fibronectin and the thickening of the vascular wall. We demonstrated that the expression of the EDIIIA region of fibronectin mRNA was increased in the obstructed kidney at five days after UUO. Fibronectin mRNA is alternatively spliced to yield several isoforms of fibronectin [32]. Among several splicing alternates of fibronectin mRNA the EDIIIA region has been shown to be regulated by TGF-β [30, 36]. Thus, it is conceivable that the increased expression of TGF-β1 mRNA in the obstructed kidney may result in an increase in ECM protein synthesis in the renal cortex during UUO.

Walton et al [37] reported that the expression of mRNA for TGF-β was increased at 48 hours after UUO and speculated that this change may have a bearing on the development of apoptosis of renal tubular cells due to a role of TGF-β in suppressing cell growth. At present there is no evidence that could explain how TGF-β1 might participate in inhibiting cell growth and/or stimulating ECM synthesis in obstructive nephropathy. Howe, Cunningham, and Leof [38] demonstrated

that c-*cis* and c-*myc* mRNA expression in cultured fibroblasts following TGF-β1 stimulation was abolished by pertussis toxin, while the increase in collagen and fibronectin mRNA were not affected. These data suggest that TGF-β1 may play a role in the stimulation of gene expression through multiple pathways. Thus, it remains to be explored whether gene expression of ECM proteins is stimulated after the increase in TGF-β1 mRNA during UUO.

We found that the contralateral kidney of rats with UUO showed a decrease in TGF-β1 mRNA level over seven days, and the level at 14 days was significantly lower than that in the control kidneys. TGF-β1 is considered to be one of the factors that modulates renal hypertrophy. However, molecular mechanisms of renal hypertrophy and hyperplasia have not been well elucidated and several growth factors and protooncogenes may modulate renal enlargement [reviewed in 39]. Thus, at present it is not clear whether or not TGF-β1 has a role in the renal compensatory hypertrophy observed in the contralateral kidney after UUO. It may be that the increase in TGF-β1 synthesis is localized to the obstructed kidney and that increased TGF-β1 levels in this kidney may act both as an autocrine and a paracrine factor.

In this study we have not identified the mechanisms by which TGF-β1 mRNA is increased during UUO. It is well known that inflammatory cells have an important role in the fibrogenic process; they activate proliferative and migratory fibrogenic responses in resident cells and secrete several cytokines which regulate ECM synthesis [1]. Macrophages have been shown to produce and secrete TGF-β1 [35, 40, 41], which might have chemotactic activity for monocytes [42] and have a role in strong stimulation of fibroblast proliferation [1]. Jones et al [43] reported that the number of interstitial macrophages correlated with the increased ECM protein synthesis in purine aminonucleoside nephrosis. Our previous report demonstrated that leukocytes, predominantly macrophages, infiltrate the renal interstitium after UUO and at 24 hours the influx stabilized at a level approximately 10-fold higher than normal [44]. We were not able to consistently measure mRNA expression for TGF-β1 at 24 hours after UUO because both the GAPDH and the TGF-β1 mRNA expression were unstable in the obstructed kidney at this time point. The expressions of TGF-β1 and GAPDH, however, were the same between the contralateral and obstructed kidneys for at least eight hours. It may be that general RNA transcription declines and/or an increased exposure to nucleases occurs during the first 24 hours of ureteral obstruction. Nagel, Johnson and Jervis [45] also demonstrated that both mononuclear cells and fibroblasts are clearly increased at 72 hours after UUO. From these data it is conceivable that macrophages may be responsible for the increased expression of TGF-β1 mRNA during UUO. Epithelial tubular cells or glomerular cells may be other sites of gene expression for TGF-β1 because steady-state mRNA for TGF-β1 was confirmed in the control kidneys, where macrophages should not have infiltrated, and because gene expression of TGF-β1 has been shown in cultured proximal tubular cells [46] and in rat glomeruli [33]. We found that TGF-β1 mRNA expression in the obstructed kidney was increased in renal cortical tubules rather than in glomeruli after five days of UUO. This suggests that renal tubular cells may contribute to the increased TGF-β1 expression in the obstructed kidney. While TGF-β1 is considered to be one of the causative cytokines of increased ECM synthesis in glomerular sclerosis [47], the fact that the glomeruli in the obstructed kidney did not show an increased TGF-β1 mRNA level may be consistent with the fact that the glomeruli appear normal by light microscopy through seven days of obstructive nephropathy [48]. In a ureteral obstructed rat kidney model glomeruli appeared normal by light microscopy at day 15 although there was marked interstitial fibrosis [49]. The collagen content of the ureteral obstructed kidney of this rat model was increased threefold. These suggestive findings notwithstanding, it remains to be determined whether the increased gene expression of TGF-β1 during UUO is accounted for by macrophages and/or resident renal cells. Our determinations measured TGF-β1 mRNA content over the whole renal cortex. *In situ* hybridization would precisely locate the cells where TGF-β1 expression is increased.

We found that enalapril, an inhibitor of ACE, significantly blunted the increase in TGF-β1 mRNA in obstructed kidneys although it did not return the levels to those seen in kidneys of sham-operated or normal rats. In vascular injury Ang II has been shown to induce increased levels of TGF-β1 mRNA and promote the conversion of latent TGF-β1 to its biologically active form [13]. Thus, Ang II may have a role in stimulating expression of TGF-β1 mRNA in UUO and in increasing the number of active forms of TGF-β1 in this setting. Horikoshi et al [50] reported that water deprivation in mice, which induced an increase of plasma renin activity, did not increase TGF-β1 in juxtaglomerular apparatus or renal tubular cells but did increase TGF-β2 expression specifically in the juxtaglomerular apparatus. This suggests that elevated levels of circulating renin or Ang II might not affect TGF-β1 expression in kidney. Therefore, localized production of Ang II may contribute to the stimulation of TGF-β1 mRNA during UUO. Recent studies from the laboratory of Border and Ruoslahti have implicated TGF-β as an initiating factor in the development of glomerular sclerosis [reviewed in 51]. Whether Ang II is a causative factor in the increase in TGF-β in those models of glomerulonephritis is presently not known. It should be pointed out, however, that a low-protein diet apparently decreases the expression of TGF-β within the glomerulus [52]. This dietary maneuver in turn has been shown to decrease the apparent amount of renal Ang II [47]. However, other mechanisms may also regulate TGF-β1 mRNA expression during obstruction because the increased levels of TGF-β1 mRNA were not suppressed by enalapril to the levels seen in kidneys of control rats.

We also showed that OKY-046, an inhibitor of Tx synthase, did not modify the increased level of TGF-β1 mRNA present in the kidney with ureteral obstruction of five days duration. This implies that the stimulatory effect of Tx on ECM synthesis [16] may not be mediated through TGF-β1 transcription. However, Tx may modulate TGF-β1 synthesis at a post-transcriptional level or Tx may stimulate directly the gene expression of ECM proteins [16, 17].

In summary, we have demonstrated that mRNA levels of TGF-β1 are significantly increased in the obstructed kidney three days after UUO as compared to the contralateral kidney of the same rats or control kidney. An ACE inhibitor significantly blunted the increased level of TGF-β1 mRNA present in the obstructed kidney at five days after UUO. Tx synthase inhibitor did not affect the increased level of TGF-β1 mRNA.

These data suggest that TGF-β1 might be increased at the transcriptional level soon after UUO, that the effect of Tx on ECM synthesis might not be induced at the gene transcriptional level of TGF-β1, and that Ang II might have a role in stimulating TGF-β1 mRNA expression in UUO.

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