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Role of Connective Tissue Growth Factor and Angiotensin II in Tubulointerstitial Fibrosis in Experimental Obstructive Nephropathy

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Angiotensin II (Ang-II) and connective tissue growth factor (CTGF) are involved in various renal disorders that lead to end-stage renal disease. Here, we determined the role of Ang-II and CTGF in the progression of tubulointerstitial injury in the rat unilateral ureteral obstruction (UUO) model. Sprague-Dawley rats (n=16) were used; 10 rats underwent UUO, and 6 control rats underwent sham operation; and rats of both groups were sacrificed on days 7 or 14. Histomorphometric analysis was performed to quantitate tubulointerstitial injuries in the experimental group. Kidney sections were stained immunohistochemically for Ang-II, CTGF, transforming growth factor-ß1 (TGF-ß1), type III collagen and α-smooth muscle actin (α-SMA). Renal CTGF expression was studied using *in situ* hybridization and reverse transcriptase-polymerase chain reaction. Double staining for Ang-II with α-SMA and CTGF with α-SMA was performed to identify cells with enhanced expression of Ang-II and CTGF. Similar dual staining of Ang-II with type III collagen and CTGF with type III collagen was performed. The correlation between Ang-II and CTGF expression and tubulointerstitial injury was examined. In obstructed kidneys, there was a significant (p<0.001) increase in expression of Ang-II, CTGF, TGF-ß1, type III collagen and α-SMA, compared with control kidneys. Tubular epithelial cells and interstitial cells were the main Ang-II- and CTGF-producing cells in the obstructed kidneys. A significantly (p<0.001) positive correlation was detected in obstructed kidneys between renal expression of CTGF and expression of TGF-ß1 (r=0.91), type III collagen (r=0.87) or α-SMA (r=0.90). Similarly a significantly (p<0.001) positive correlation was found in obstructed kidneys between Ang-II expression and expression of TGF-ß1 (r=0.88), type III collagen (r=0.79) and α-SMA (r=0.91). Finally, there were significantly positive correlations between CTGF /Ang-II expression and tubulointerstitial fibrosis in the obstructed kidneys (r=0.88, p<0.001). The results of our *in vivo* studies suggest that both Ang-II and CTGF, produced by intrarenal cells, coordinately regulate progression of renal tubulointerstitial injury, by facilitating increased accumulation of interstitial collagens in obstructed kidneys.

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Introduction

Most renal disorders, irrespective of their original cause, lead to renal tubulointerstitial fibrosis in the chronic and advanced stages.[1-4] The extent of tubulointerstitial fibrosis is a prognostic indicator in most renal diseases.[5,6] One common feature of renal fibrosis is increased deposition of matrix components due to uncontrolled turnover of matrix proteins. Although the exact molecular mechanisms of renal fibrotic diseases are not yet clear, increased synthesis and deposition of type I, III, IV and VI collagens have been detected in various chronic progressive fibrotic renal diseases.[7-9] Numerous studies have convincingly demonstrated that increased synthesis with excessive deposition of collagens is primarily responsible for the initiation and progression of renal fibrotic diseases, however, how various fibrogenic factors coordinately regulate renal fibrogenic responses is not yet clear.

Connective tissue growth factor (CTGF) is a potent mediator of matrix synthesis. Increased expression of CTGF has been shown to be associated with various human and experimental glomerular diseases.[10-12] CTGF can induce extracellular matrix (ECM) production, and mediates the profibrotic actions of transforming growth factor-ß1 (TGF-ß1).[10-13]

Recent studies have suggested the role of the renin-angiotensin system (RAS) in the pathogenesis of interstitial fibrosis.[14-18] Angiotensin II (Ang-II), the main peptide of the RAS, appears to be important in initiating and sustaining the fibrogenic destruction of renal tissue.[19]

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Its actions are likely to be mediated by both hemodynamic and non-hemodynamic components. Induction of TGF-ß1 has been documented in glomeruli after systemic infusion of Ang-II into normal rats.[15] Similarly, renal induction of CTGF following systemic infusion of Ang-II into normal rats was reported.[20]

Although numerous studies have suggested that CTGF and Ang-II may be important mediators of TGF-ß1-induced profibrotic activities, the potential correlation between Ang-II and CTGF during the progression of fibrotic diseases has not yet been investigated in similar detail. Using the unilateral ureteral obstruction (UUO) model, we determined the cellular sources of CTGF, and its association with TGF-ß1 and Ang-II during tubulointerstitial fibrosis, and tested the hypothesis that Ang-II and CTGF coordinately regulate fibrogenesis in obstructed kidneys.

Materials and Methods

Experimental protocol

Female Sprague-Dawley rats (n=16), with weights ranging from 200 to 250 g, were used. Ten rats (n=10) underwent UUO as follows: under intraperitoneal Nembutal anesthesia (25 mg/kg body weight) and through a small abdominal incision, a complete obstruction of the left ureter was produced by two ligatures, 5 mm apart, in its upper two-thirds, using 4-0 silk sutures. Ureteral obstruction was confirmed by the development of dilatation of the renal pelvis and proximal ureter and collapse of the distal ureter. The abdominal incision was then closed, and the rats were given free access to tap water and standard chow. The 16 rats were divided into two groups: Group 1 (n=6): Six rats had physical manipulations of their left kidney and ureter, but no ureteral ligations were performed. Three rats were sacrificed at day 7 and day 14, respectively. These six rats were used as the control group. Group 2 (n=10): Ten rats with UUO were sacrificed, five each on days 7 and 14, respectively. Urine was collected for bacterial culture.

Renal tissue collection

The rats were sacrificed by exsanguination under ether general anesthesia. The left kidneys were removed via a midline abdominal incision, and immediately fixed. Renal tissues were fixed in Carnoy's solution for 2 hours for immunohistochemistry. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University Graduate School of Biomedical Sciences.

In situ hybridization

In situ hybridization was performed as described previously with slight modifications.[7] Briefly, polymerase chain reaction (PCR) product for CTGF from rat mRNA was subcloned into the multiple cloning site of pT-NOT, flanked by T3 and T7 promoters. Antisense or sense strand cRNA probe of CTGF was synthesized using digoxigenin (DIG)-labeled UTP with T3 or T7 RNA polymerase. The renal tissues were fixed for 16 hours in 4% buffered paraformaldehyde (pH 7.4), embedded in paraffin, and sliced at 5 μm thickness. The sections were mounted onto slides treated with 2% 3-aminopropyltriethoxysilane, deparaffinized, digested with 10 μg/mL proteinase K for 10 min at 37℃, and soaked for 10 min in 0.25% acetic anhydride-0.1 mM triethanilamine hydrochloride (pH 8.0)- 0.9% NaCl. DIG-labeled probes (500 ng/mL) were added to the hybridization buffer composed of 50% formamide, 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.6 M NaCl, 0.5 mg/mL yeast tRNA, 0.25 mg/mL salmon sperm DNA, 1.5% skim milk, 0.25% sodium dodecyl sulfate (SDS), and 5× Denhardt's solution. After hybridization at 45℃ for 16 hours, the slides were washed several times in 5× standard saline citrate (SSC) and immersed in 50% formamide-2× SSC at 50℃ for 30 min. The sections were then treated with 20 μg/mL of RNase A at 37℃ for 30 min and finally washed in 0.2× SSC at 50℃ for 20 min. Hybridization signals were detected by immunohistochemistry detection with alkaline phosphatase-conjugated anti-DIG Fab fragments using nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as the chromogenic substrate.

Semiquantitative reverse transcriptase-PCR

Total RNA was extracted from kidney tissue with the RNeasy kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Reverse transcription-PCR (RT-PCR) was performed as described previously[21] using the primers listed in Table 1. The RT reaction proceeded at 42℃ for 60 min with 2 μg total RNA in a reaction mixture containing 100 ng random primer (Takara Shuzo, Otsu, Japan), 1 mM deoxy-NTPs (Clontech Laboratories, Palo Alto, CA), 10 mM dithiothreitol, 1× reaction buffer, 50 U ribonuclease inhibitor (Wako Pure Chemical Industries, Osaka, Japan), and 200 U RT enzyme (Invitrogen, San Diego, CA) in a final volume of 20 μL. The samples were heated at 45℃ for 10 min to inactive RT activity and denature RNA-complementary DNA hybrids. Equal amounts of the product of the reverse transcription reaction were subjected to PCR amplification. We co-amplified the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to allow for a semi-quantifiable comparison of the PCR products. Amplification consisted of 5 min of denaturation at 94℃ followed by 28 cycles of 60 sec at 94℃, 60 sec at 62℃, and 180 sec at 72 ℃. The final extension was set for 5 min at 72℃. In the preliminary experiments, the number of amplification cycles was examined by performing RT-PCR for 18, 23, 28 and 33 cycles. PCR products were within the linear logarithmic phase of the amplification curve until 28 cycles (data not shown). After amplification, 5 μL of each PCR reaction product was electrophoresed on a 2.0% agarose gel containing ethidium bromide (0.5 μg/mL). The mRNA levels of CTGF and TGF-ß1 were normalized with GAPDH mRNA levels. The mean mRNA level was defined as 1.0 arbitrary unit. At least three independent PCR procedures were performed to allow for statistical analysis.

Table 1. The primers used for RT-PCR

Target[a]		Primers	Size (bp)
CTGF	Forward	5'-GCCCTGTGAAGCTGACCTAGA-3'	716
	Reverse	5'-GCAAGCTATAATGTCCCTCCCC-3'	
TGF-ß1	Forward	5'-AGAACCCCCATTGCTGCTCCGT-3'	209
	Reverse	5'-CCGGGTTGTGTTGGTTGTAGAGG-3'	
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'	452
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'	

[a]CTGF=Connective tissue growth factor; TGF-ß1=Transforming growth factor-ß1; GAPDH=Glyceraldehyde-3-phosphate dehydrogenase.

Immunohistochemical studies

Immunohistochemical staining was performed as follows.[22-24] Paraffin-embedded tissue sections were deparaffinized with xylene, rinsed thoroughly with 95% ethanol, and then soaked in 0.3% H_2O_2 in methanol for 30 min at room temperature in order to inactivate endogenous peroxidase activity. After a 5-min treatment with 0.05% trypsin (Sigma, St. Louis, MO), tissue sections were incubated with either 10% goat serum or 10% rabbit serum for 30 min, and then covered with relevant primary antibodies. The following antibodies were used: anti-TGF-ß1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ang-II (1:100, Peninsula, San Carlos CA), anti-CTGF (1:100, Santa Cruz Biotechnology), anti-type III collagen (1:100, Chemicon International Inc., Temecula, CA), and anti-α-smooth muscle actin (α-SMA) (1:100, Dako, Glostrup, Denmark). The slides were washed with 0.01 M phosphate-buffered saline (PBS) and processed further using the histofine SAB-PO Kit (Nichirei, Tokyo, Japan), as recommended by the manufacturer, and then reacted with 3.3' diaminobenzidine and H_2O_2.

To quantify the grade of staining, the number of TGF-ß1-, CTGF-, Ang-II-, and α-SMA-positive cells was counted in five randomly chosen fields (×400) of the renal cortex. The average number of TGF-ß1-, CTGF-, Ang-II-, and α-SMA-positive cells from five different animals was then calculated.

Matrix scoring for type III collagen in the renal cortical interstitium was determined as described previously, with slight modifications.[25-27] Twenty random, non-overlapping fields of tissue sections stained for the above-mentioned markers were scored from 0 to 3, as follows: 0—no staining (normal kidney in control group), 1—focal staining, 2—diffuse mild to moderate staining, and 3-diffuse and strong staining.

Double immunostaining

Double immunostaining in all the combination to each other for CTGF, Ang-II, α-SMA, type III collagen and TGF-ß1 was performed, as described earlier.[28,29] We used a histostain-DS kit (Zymed Laboratories Inc., Co., San Francisco, CA). Briefly, 4 μm thick sections were firstly stained for Ang-II or CTGF using the streptavidin-alkaline phosphatase method, which produced a dark purple staining. The tissue sections were counterstained for either type III collagen or α-SMA by the streptavidin-peroxidase method, and visualized by using 3-amino-9-ethylcarbazole hydrochloride (AEC)/H_2O_2 application, which produced an intense red staining. The sections were heat-inactivated in 0.01 M citrate buffer (pH 6.0) for 10 min to minimize the cross-reactivity between the first and the second staining. For both single and double immunostaining, as a control, the primary antibodies were replaced with either 0.01 M PBS or mouse IgG or rabbit IgG diluted with PBS (with a concentration similar to that of the primary antibody used). In the sections of double immunostaining, the positive cells for CTGF, Ang-II, α-SMA and TGF-ß1 were counted in five high-power fields per a kidney of UUO rats. The numbers of positive cells were plotted in relation to those positive for the second antibodies.

Statistical analysis

The data were expressed as mean±standard deviation. Difference between experimental groups was examined for statistical significance using the *t*-test.

Results

Morphological changes in obstructed kidneys

By the end of the first week, dilatation of tubules began to appear in some areas of the kidneys of UUO rats, which was associated with mononuclear cell infiltration, as well as edematous and mild fibrotic changes in the renal interstitium, in contrast to control (Figure 1 a). In the second week, a diffuse dilatation of renal tubules, particularly the collecting ducts and distal convoluted tubules was noted. There was a complex network of interlacing fibrils surrounding each tubule, with proliferation of interstitial cells (Figure 1 b).

Expression of type III collagen in obstructed kidneys

Mild interstitial expression of type III collagen was detected in control kidneys. In contrast to control a significant increase in the

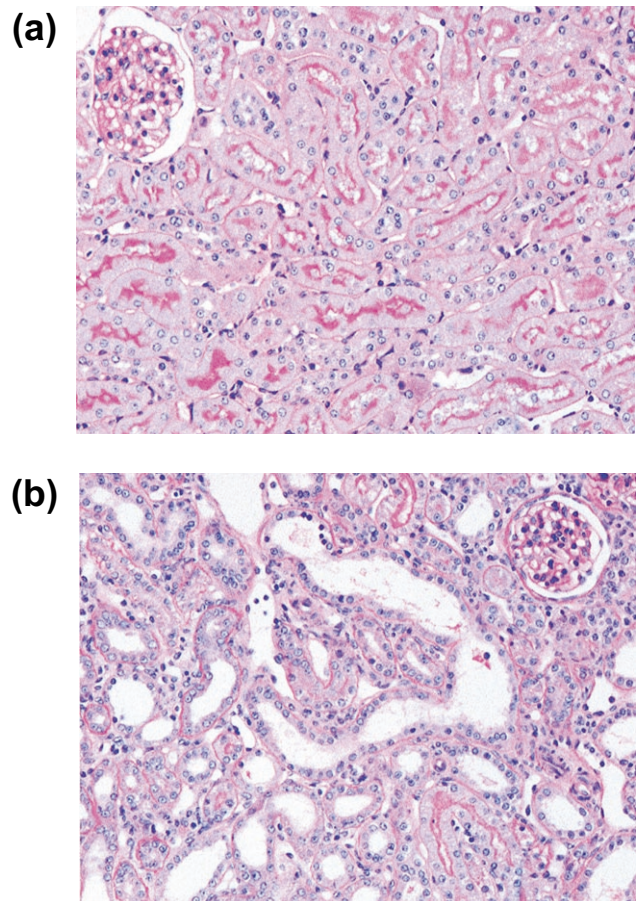

Figure 1. Morphological changes in obstructed kidneys. The images of PAS staining from control **(a)** (×200) and obstructed kidneys on day 14 in UUO rats **(b)** (×200). Note dilatation of tubules, inflammatory infiltrates, edematous changes, and focal interstitial fibrosis **(b)**.

interstitial accumulation of type III collagen was consistently detected in obstructed kidneys, from day 7 onwards. On day 7, the type III collagen matrix score was 1.07±0.07, which further increased on day 14, with the matrix score being 1.80±0.14.

Expression of α-SMA in obstructed kidneys

Compared to control, obstructed kidneys showed strong positive staining for α-SMA in the tubulointerstitium. The number of α-SMA-positive cells was significantly higher in obstructed kidneys compared to control (p<0.001) both on day 7 (102.1±5.82 vs 3.9 ±0.41) and day 14 (149.3±7.84 vs 4.2±0.43).

Expression of TGF-ß1 in obstructed kidneys

Levels of TGF-ß1 mRNA were analyzed by RT-PCR. A significant increase in expression of TGF-ß1 mRNA was found in obstructed kidneys at day 7 compared with normal controls (p<0.05) (Figure 2 a). On immunohistochemistry, mild TGF-ß1 staining was noted in control kidneys (Figure 2 b). In contrast, TGF-ß1 expression was strong in tubular epithelial cells and interstitial cells in obstructed kidneys (Figure 2 c). The number of TGF-ß1-positive cells was significantly higher in obstructed kidneys than in control kidneys (p<0.001) both on day 7 (86.0±4.85 vs 7.4±0.99) and day 14 (105.1±4.81 vs 8.7±0.78).

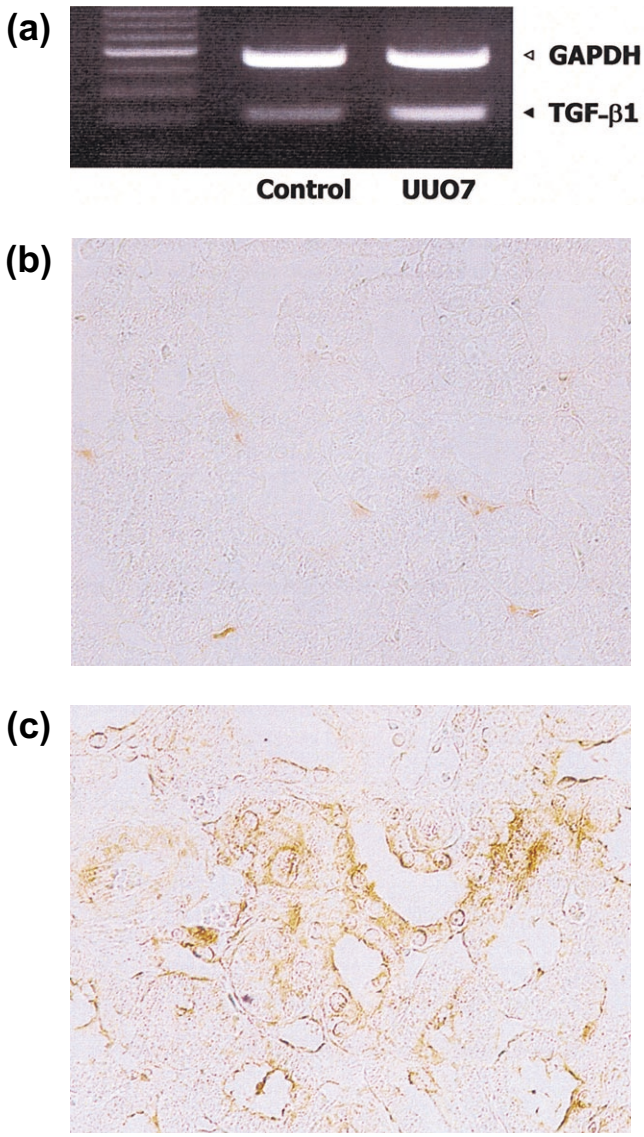

Figure 2. TGF-ß1 expression in obstructed kidney. RT-PCR showed a significant increase of TGF-ß1 mRNA in obstructed kidneys at day 7 compared to normal control **(a).** On immunohistochemistry, mild TGF-ß1 staining was noted in normal control kidneys **(b)** (×400). Overexpression of TG F-ß1 was seen in tubulointerstitial areas of obstructed kidneys **(c)** (×400).

Expression of Ang-II and its correlation with tubulointerstitial fibrosis in obstructed kidneys

Ang-II positively stained endothelial cells of blood vessels, afferent arterioles, in control kidneys (Figure 3 a). In obstructed kidneys, Ang-II was strongly positive in tubular epithelial cells, and interstitial cells (Figure 3 b). The number of Ang-II-positive cells in the tubulointerstitial area was significantly higher in obstructed kidneys than in the control kidneys (p<0.001) both on day 7 (247.6±21.3

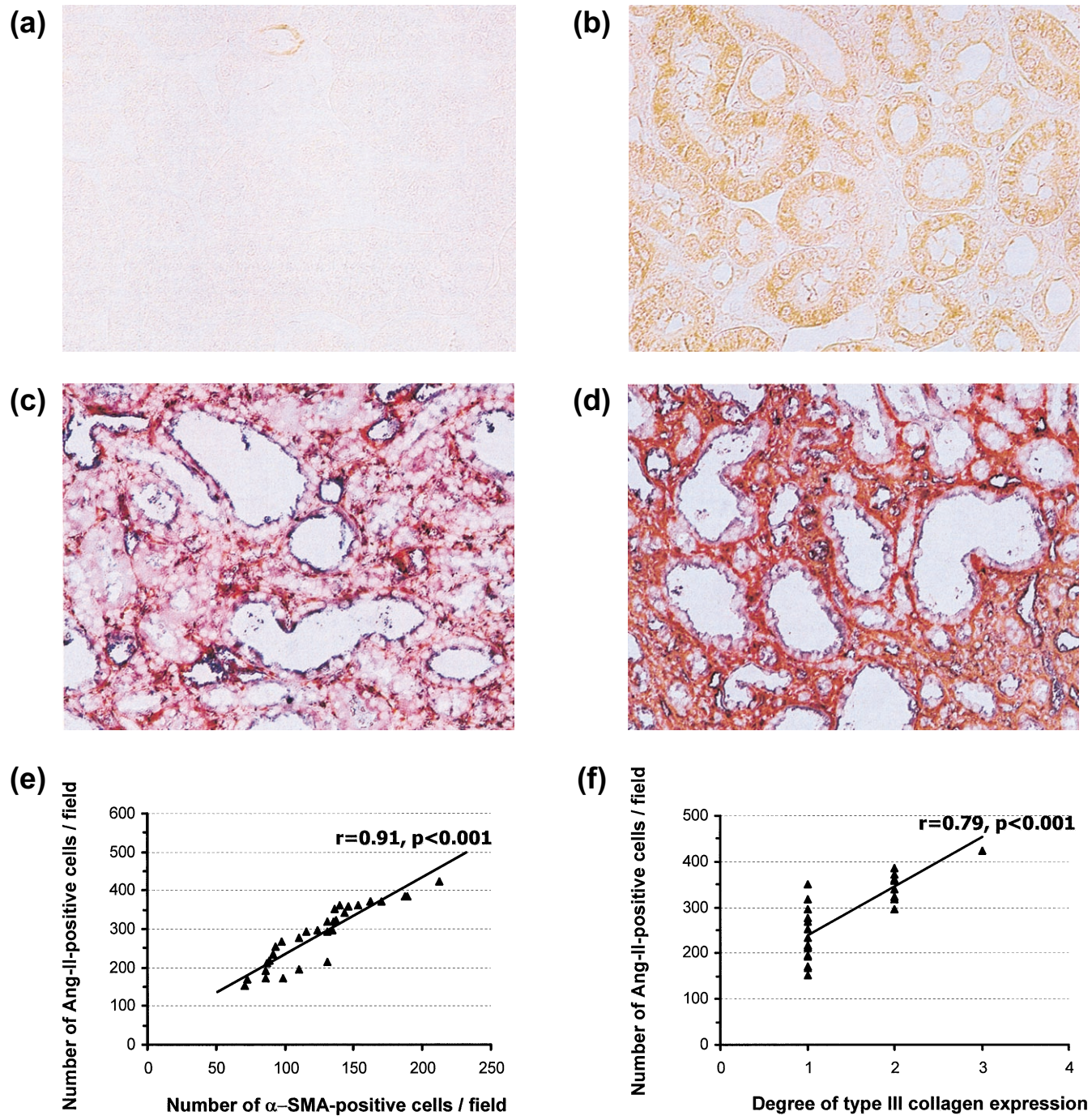

Figure 3. Ang-II expression in obstructed kidney and correlation between Ang-II and tubulointerstitial fibrosis. Ang-II positive cells were only seen in blood vessels in control kidneys **(a)** (×400). In obstructed kidneys, tubular epithelial cells and interstitial cells secreted Ang-II in obstructed kidneys **(b)** (×400). The co-localization of Ang-II and α-SMA or type III collagen was shown in tubulointerstitial spaces; dark purple represents Ang-II, red represents either α-SMA or type III collagen **(c, d)** (×400). A significantly (p<0.001) positive correlation was detected in obstructed kidneys between Ang-II and α-SMA (r=0.91) or type III collagen (r=0.79) **(e, f)**.

vs 7.0±0.69) and day 14 (319.3±24.2 vs 7.8±0.65). Furthermore, the number of Ang-II-positive cells in obstructed kidney was significantly higher on day 14 of UUO than on day 7 of UUO. On dual staining, co-localization of Ang-II with α-SMA was observed in tubulointerstitial cells, suggesting that interstitial myofibroblasts are the main source of Ang-II in the obstructed kidney (Figure 3 c). Furthermore, increased expression of Ang-II was detected in and around increased interstitial accumulation of type III collagen in obstructed kidneys (Figure 3 d). A close correlation between increased expression of Ang-II and increased expression of TGF-ß1 was

seen in obstructed kidneys from day 7 onwards (data are not shown). In obstructed kidneys, a significantly (p<0.001) positive correlation was observed between expression of Ang-II and interstitial accumulation of either α-SMA or type III collagen (Figure 3 e, f).

Expression of CTGF and its correlation with tubulointerstitial fibrosis in obstructed kidneys

To determine which cells were producing CTGF, we performed *in situ* hybridization on renal sections prepared from control and obstructed rat kidneys. CTGF mRNA was rarely expressed only by interstitial cells in control (Figure 4 a). However, in obstructed kidneys, the periglomerular cells, tubular epithelial cells and interstitial cells expressed signals for CTGF mRNA (Figure 4 b). The increased CTGF expression noted on RT-PCR complemented the results of *in situ* hybridization. A significant increase in CTGF mRNA was found in obstructed kidneys compared with control (p<0.05) (Figure 4 c). A very mild staining for CTGF was noted in control (Figure 4 d). In obstructed kidneys, we found increased expression of CTGF in tubular epithelial cells and interstitial cells (Figure 4 e). A significantly higher number of CTGF-positive cells were detected in obstructed kidneys compared with control (p<0.001) both on day 7 (145.2±5.11 vs 8.1±0.72) and day 14 (210.4±13.8 vs 8.2±0.73). As expected, the pattern of CTGF expression on immunohistochemistry was in agreement with the distribution of CTGF mRNA as shown by *in situ* hybridization (Figure 4 b).

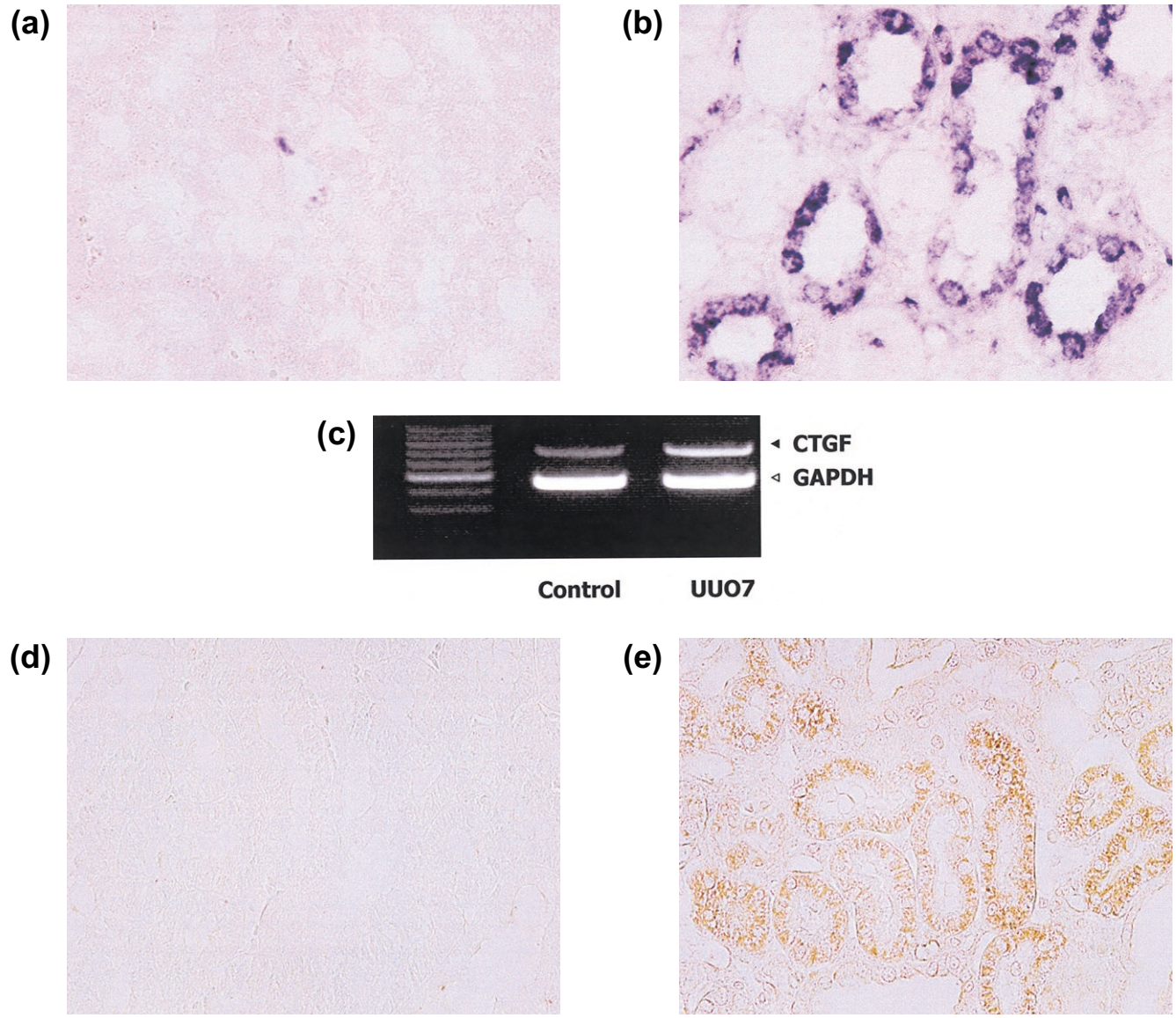

Figure 4. CTGF expression in obstructed kidney. In situ hybridization showed CTGF mRNA was rarely expressed only in interstitial cells in control kidneys **(a)** (×400). However, overexpression of CTGF mRNA was seen in tubular epithelial cells and interstitial cells of obstructed kidneys **(b)** (×400). RT-PCR demonstrated a significant increase of CTGF mRNA in obstructed kidneys at day 7 compared to normal control **(c)**. Immunohistochemistry also showed mild staining of CTGF in normal control kidneys **(d)** (×400), and overexpression of CTGF in tubular epithelial cells and interstitial cells in obstructed kidneys **(e)** (×400).

By dual staining, co-localization of CTGF with α-SMA was demonstrated in the obstructed kidneys, suggesting that interstitial myofibroblasts are the main source of CTGF in the obstructed kidney (Figure 5 a). Also, co-localization of CTGF and type III collagen was seen in the tubulointerstitium (Figure 5 b). A significantly positive correlation was also detected in obstructed kidneys between the expression of CTGF and α-SMA or interstitial type III collagen was also detected in obstructed kidneys (p<0.001) (Figure 5 c, d). In addition, we detected the co-localization of CTGF and Ang-II on tubular epithelial cells, peritubular epithelial cells, and interstitial cells in obstructed kidneys by dual staining (data are not shown). In obstructed kidneys, the number of CTGF-positive cells showed a

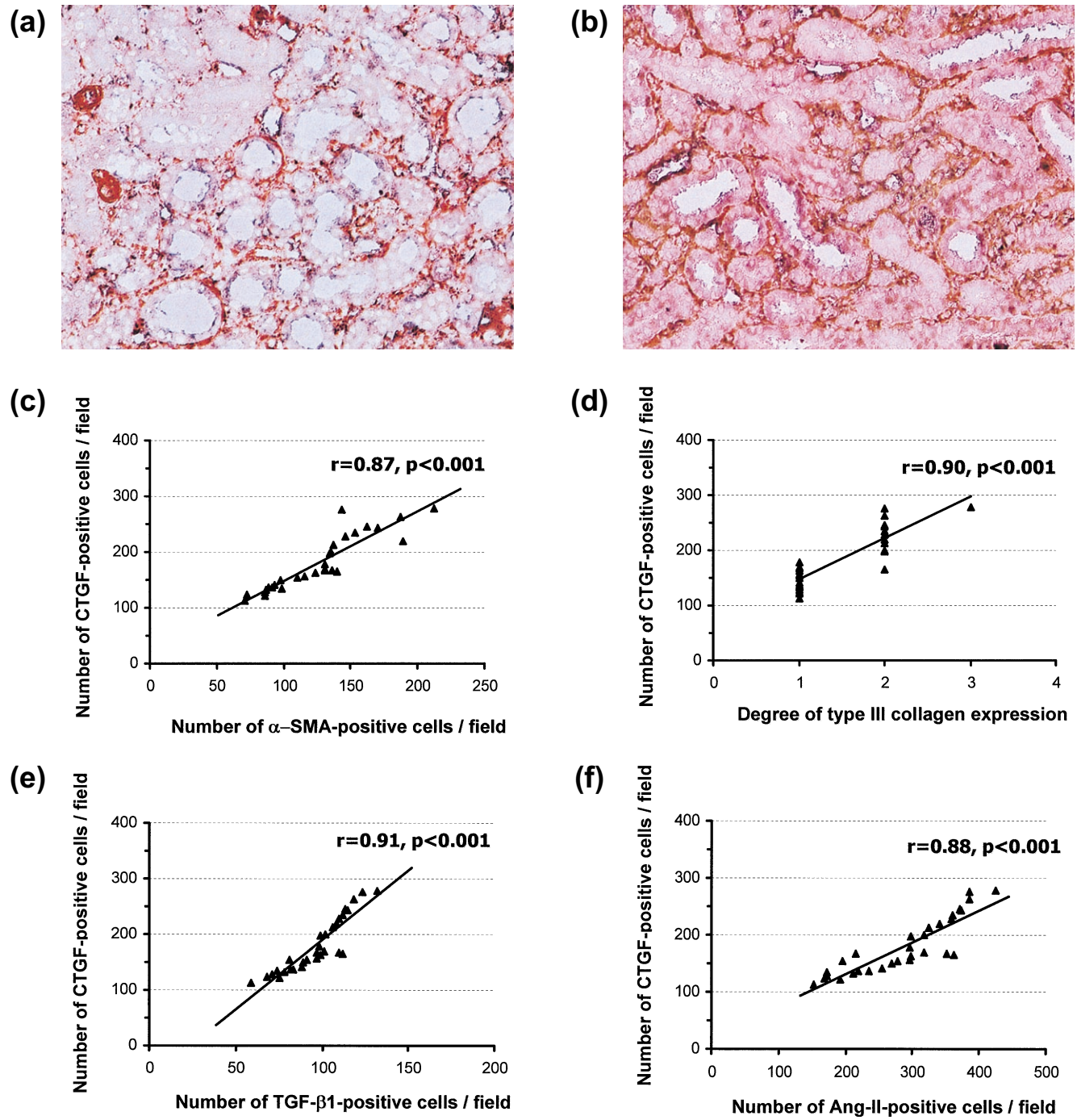

Figure 5. Correlation between CTGF and tubulointerstitial fibrosis. Co-localization of CTGF and α-SMA or type III collagen was demonstrated in tubulointerstitial spaces (dark purple represents CTGF, red represents either α-SMA or type III collagen) **(a, b)** (×400). A significantly (p<0.001) positive correlation was detected in obstructed kidneys between CTGF and α-SMA (r=0.87) or type III collagen (r=0.90) **(c, d)**. The expression of CTGF correlated significantly (p<0.001) with that of TGF-ß1 (r=0.91) and Ang-II (r=0.88) in obstructed kidneys **(e, f)**.

significantly positive correlation both with TGF-ß1-positive cells and Ang-II-positive cells ($p<0.001$) (Figure 5 e, f), suggesting close relationship among these fibrogenic factors of Ang-II, TGF-ß1, and CTGF to regulate tubulointerstitial fibrosis in obstructed kidneys.

Discussion

Unilateral ureteral obstruction (UUO) leads to tubulointerstitial fibrosis, as determined by an increased accumulation of type III collagen with an increased number of matrix-producing tubulointerstitial fibroblasts. Interstitial fibrosis is mediated by inflammatory and various fibrogenic factors, which stimulate fibroblast proliferation, and extracellular matrix (ECM) accumulation. Recently, fibrogenic effects of the renin-angiotensin system (RAS), especially the effects of Ang-II, the major peptide of the RAS, have been demonstrated.[15,20] The results of our study also clearly demonstrate that locally generated Ang-II plays an important role in the pathogenesis of the experimental model of obstructive nephropathy. Ang-II is usually present in endothelial cells of blood vessels; however, in obstructed kidneys Ang-II was detected in tubular epithelial cells, peritubular epithelial cells, and interstitial cells.

Several studies have suggested a role for Ang-II during inflammatory events in the kidney. Ang-II activates inflammatory cells by direct chemotaxis and production of proinflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) and TGF-ß1.[14] Systemic Ang-II infusion into normal rats caused mononuclear cell infiltration into the interstitium.[14,30] Ang-II also regulates mesangial cell growth and increases the expression and synthesis of ECM proteins, including fibronectin, laminin and collagens.[31] Using *in vitro* studies, Wolf et al.[17] have demonstrated that Ang-II can be a single factor event capable of inducing hypertrophy in proximal tubular cells. In experimental obstructive nephropathy, the inflammatory process, proliferation of interstitial fibroblasts, and excessive deposition of interstitial ECM are important events contributing to the fibrotic process. Johnson et al.[30] suggested that rats infused with Ang-II developed tubulointerstitial injury by inducing tubular injury, interstitial monocyte infiltration, and interstitial deposition of collagens. Cultured renal interstitial fibroblasts express AT1 receptors, and after Ang-II stimulation, there was an increase in cell proliferation and expression and synthesis of ECM proteins such as fibronectin, by a TGF-ß1-mediated process.[14]

We also found increased expression of both CTGF mRNA and protein in obstructed kidneys. In normal kidney, CTGF mRNA has been detected in visceral and parietal epithelial cells, and in some periglomerular and peritubular interstitial cells, in endothelial cells of peritubular capillaries, and in interstitial fibroblasts, by *in situ* hybridization.[32] In our study, we also found similar overexpression of CTGF mRNA and its translated protein in tubulointerstitial areas in obstructed kidneys.

In a recent study, Duncan et al.[33] indicated that CTGF is a likely mediator of TGF-ß1-stimulated fibronectin production in a rat model of interstitial fibrosis and in cultured renal interstitial fibroblasts. Using *in situ* hybridization and immunohistochemistry, we demonstrated the overexpression of CTGF mRNA and its protein in the cells around interstitial fibrotic areas, as well as in tubular epithelial cells in obstructed kidneys at day 14 of UUO. Myofibroblasts and fibroblasts were the main sources of CTGF expression in tubulointerstitial fibrosis in human renal biopsy specimens.[32]

The key role of TGF-ß1 in renal tubulointerstitial fibrosis is well documented.[3] TGF-ß1 enhances the synthesis of ECM proteins such as collagens, fibronectin, and laminin. TGF-ß1 also promotes ECM accumulation by increasing the production of protease inhibitors such as plasminogen activator inhibitor-1, and by decreasing the activity of proteases such as matrix metalloproteinase.[1,2,10,12,32,34] Furthermore, TGF-ß1 stimulates fibroblast migration and proliferation and also is chemotactic for monocytes and macrophages. In our study, in accord with earlier studies, we also found overexpression of not only TGF-ß1 mRNA, but also TGF-ß1 protein in obstructed kidneys.

Ang-II, TGF-ß1, and CTGF have a selective, yet important role in the fibrotic process in renal tissues; however, the interrelation among these molecules is not yet fully elucidated. Recent studies have shown that in cultured fibroblasts, CTGF gene expression is strongly induced by TGF-ß1, and the addition of CTGF in turn potently stimulates fibroblast proliferation and ECM protein synthesis.[11-13] Other emerging evidence suggests that CTGF may be an important downstream mediator of TGF-ß1 profibrotic activities.[1,10-13,34] Systemic infusion of Ang-II into normal rats increases glomerular expression of TGF-ß1[15]; the blockade of Ang-II actions by ACE inhibitors and AT1 antagonists reduced TGF-ß1 expression and fibrosis.[14,15]

Systemic infusion of Ang-II into normal rats for three days resulted in increased renal expression of CTGF mRNA and protein levels.[20] This finding suggests that CTGF could be a mediator of the profibrogenic effects of Ang-II in the kidney. Our results indicate that CTGF and Ang-II apparently coordinately regulate the tubulointerstitial fibrotic process. van Kats et al.[16] pointed out that in addition to circulatory Ang-II, this peptide is produced in various tissues, including the kidney. We believe that the level of circulatory and renal production of Ang-II is markedly increased in obstructed kidneys in the UUO model. The increased level of Ang-II in obstructed kidneys leads to an increase in inflammatory infiltration, potentiated by the effects of TGF-ß1 and CTGF, which induce matrix proteins resulting in renal fibrosis.

In conclusion, overexpression of CTGF, Ang-II and TGF-ß1 coordinately regulate the fibrotic process in the obstructed kidney. Ang-II appears to be an initiatory factor in the process, mediating the downstream events that involve TGF-ß1 inducing CTGF, which in turn propagates the fibroproliferative process in obstructed kidneys. CTGF and TGF-ß1 could be the major mediators of the profibrogenic effects of Ang-II in tubulointerstitial fibrosis in experimental obstructive nephropathy.

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References

1. Razzaque MS, Taguchi T. Cellular and molecular events leading to renal tubulointerstitial fibrosis. *Med Electron Microsc* 35: 68-80, 2002
2. Razzaque MS, Taguchi T. Factors that influence and contribute to the regulation of fibrosis. *Contrib Nephrol* 139: 1-11, 2003
3. Azouz A, Razzaque MS, El-Hallak M, Taguchi T. Immunoinflammatory responses and fibrogenesis. *Med Electron Microsc* 37: 141-148, 2004
4. Chevalier RL. Molecular and cellular pathophysiology of obstructive nephropathy. *Pediatr Nephrol* 13: 612-619, 1999
5. Bohle A, Mackensen-Haen S, von Gise H et al. The consequences of tubulo-interstitial changes for renal function in glomerulopathies. A morphometric and cytological analysis. *Pathol Res Pract* 186: 135-144, 1990
6. Bohle A, Wehrmann M, Eissele R, von Gise H, Mackensen-Haen S, Muller C. The long-term prognosis of AA and AL renal amyloidosis and the pathogenesis of chronic renal failure in renal amyloidosis. *Pathol Res Pract* 189: 316-331, 1993
7. Razzaque MS, Koji T, Kawano H, Harada T, Nakane PK, Taguchi T. Glomerular expression of type III and type IV collagens in benign nephrosclerosis: immunohistochemical and in situ hybridization study. *Pathol Res Pract* 190: 493-499, 1994
8. Razzaque MS, Koji T, Harada T, Taguchi T. Localization in situ of type VI collagen protein and its mRNA in mesangial proliferative glomerulonephritis using renal biopsy sections. *Histochem Cell Biol* 111: 1-6, 1999
9. Razzaque MS, Koji T, Harada T, Taguchi T. Identification of type VI collagen synthesizing cells in human diabetic glomerulosclerosis using renal biopsy sections. *Anal Cell Pathol* 15: 175-181, 1997
10. Yokoi H, Mukoyama M, Sugawara A et al. Role of connective tissue growth factor in fibronectin expression and tubulointerstitial fibrosis. *Am J Physiol Renal Physiol* 282: F933-F942, 2002
11. Yokoi H, Mukoyama M, Nagae T et al. Reduction in connective tissue growth factor by antisense treatment ameliorates renal tubulointerstitial fibrosis. *J Am Soc Nephrol* 15: 1430-1440, 2004
12. Yokoi H, Sugawara A, Mukoyama M et al. Role of connective tissue growth factor in profibrotic action of transforming growth factor-beta: a potential target for preventing renal fibrosis. *Am J Kidney Dis* 38: S134-S138, 2001
13. Razzaque MS, Foster CS, Ahmed AR. Role of connective tissue growth factor in the pathogenesis of conjunctival scarring in ocular cicatricial pemphigoid. *Invest Ophthalmol Vis Sci* 44: 1998-2003, 2003
14. Ruiz-Ortega M, Lorenzo O, Suzuki Y, Ruperez M, Egido J. Proinflammatory actions of angiotensins. *Curr Opin Nephrol Hypertens* 10: 321-329, 2001
15. Border WA, Noble NA. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. *Hypertension* 31: 181-188, 1998
16. van Kats JP, Schalekamp MA, Verdouw PD, Duncker DJ, Danser AH. Intrarenal angiotensin II: interstitial and cellular levels and site of production. *Kidney Int* 60: 2311-2317, 2001
17. Wolf G, Neilson EG. Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. *Am J Physiol* 259: F768-F777, 1990
18. Yoo KH, Norwood VF, el-Dahr SS, Yosipiv I, Chevalier RL. Regulation of angiotensin II AT1 and AT2 receptors in neonatal ureteral obstruction. *Am J Physiol* 273: R503-R509, 1997
19. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 52: 11-34, 2000
20. Ruperez M, Ruiz-Ortega M, Esteban V et al. Angiotensin II increases connective tissue growth factor in the kidney. *Am J Pathol* 163: 1937-1947, 2003
21. Naito T, Razzaque MS, Nazneen A et al. Renal expression of the Ets-1 proto-oncogene during progression of rat crescentic glomerulonephritis. *J Am Soc Nephrol* 11: 2243-2255, 2000
22. Razzaque MS, Nazneen A, Taguchi T. Immunolocalization of collagen and collagen-binding heat shock protein 47 in fibrotic lung diseases. *Mod Pathol* 11: 1183-1188, 1998
23. Razzaque MS, Taguchi T. Collagen-binding heat shock protein (HSP) 47 expression in anti-thymocyte serum (ATS)-induced glomerulonephritis. *J Pathol* 183: 24-29, 1997
24. Razzaque MS, Hossain MA, Kohno S, Taguchi T. Bleomycin-induced pulmonary fibrosis in rat is associated with increased expression of collagen-binding heat shock protein (HSP) 47. *Virchows Arch* 432: 455-460, 1998
25. Liu D, Razzaque MS, Cheng M, Taguchi T. The renal expression of heat shock protein 47 and collagens in acute and chronic experimental diabetes in rats. *Histochem J* 33: 621-628, 2001
26. Cheng M, Razzaque MS, Nazneen A, Taguchi T. Expression of the heat shock protein 47 in gentamicin-treated rat kidneys. *Int J Exp Pathol* 79: 125-132, 1998
27. Razzaque MS, Shimokawa I, Nazneen A et al. Life-long dietary restriction modulates the expression of collagens and collagen-binding heat shock protein 47 in aged Fischer 344 rat kidney. *Histochem J* 31: 123-132, 1999
28. Razzaque MS, Shimokawa I, Nazneen A, Higami Y, Taguchi T. Age-related nephropathy in the Fischer 344 rat is associated with overexpression of collagens and collagen-binding heat shock protein 47. *Cell Tissue Res* 293: 471-478, 1998
29. Razzaque MS, Kumatori A, Harada T, Taguchi T. Coexpression of collagens and collagen-binding heat shock protein 47 in human diabetic nephropathy and IgA nephropathy. *Nephron* 80: 434-443, 1998
30. Johnson RJ, Alpers CE, Yoshimura A et al. Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19: 464-474, 1992
31. Egido J. Vasoactive hormones and renal sclerosis. *Kidney Int* 49: 578-597, 1996
32. Ito Y, Aten J, Bende RJ et al. Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 53: 853-861, 1998
33. Duncan MR, Frazier KS, Abramson S et al. Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *Faseb J* 13: 1774-1786, 1999
34. Razzaque MS, Taguchi T. Pulmonary fibrosis: Cellular and molecular events. *Pathol Int* 53: 133-145, 2003