TGF-β1 in glomerulosclerosis and interstitial fibrosis of adriamycin nephropathy

KIYOSHI TAMAKI, SEIYA OKUDA, TAKASHI ANDO, TAKETO IWAMOTO, MASARU NAKAYAMA, and MASATOSHI FUJISHIMA

Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan

TGF-β1 in glomerulosclerosis and interstitial fibrosis of adriamycin-induced nephropathy. The role of transforming growth factor-β1 (TGF-β1) for renal injury was investigated in the chronic model of progressive renal disease in rats induced by the injection of adriamycin. The renal cortical tissues were sampled at weeks 4, 8 and 16 for histological examination, either cortical or glomerular cell culture, and RNA extraction. A progressive increase in fibronectin synthesis was found in metabolically labeled cortical or glomerular culture at week 8 or 16, correlating with the degree of glomerulosclerosis and interstitial fibrosis. TGF-β bioassay (mink lung epithelial cell assay) showed a progressive increase in latent TGF-β secretion from cortex and glomeruli, while the amount of active TGF-β was small. The peak of latent TGF-β levels at week 16 coincided with the intense TGF-β1 staining of inflammatory cells dispersed in the interstitial and glomeruli. Northern blotting demonstrated the difference in the mRNA expression patterns of TGF-β1 and latent TGF-β binding protein (LTBP) in the cortex. TGF-β1 mRNA was constantly high throughout the experiment, while LTBP mRNA increased progressively and reached a peak at week 16. Furthermore, mRNA levels of fibronectin, procollagen α2(I), and TGF-β type II and type III receptors increased progressively in a similar pattern to the renal histological changes. These temporal and spatial relationships between the renal histological changes and the increased expression of TGF-β1 and TGF-β receptors may thus suggest that TGF-β1 plays an important role in the process of the renal fibrosis and sclerosis.

The accumulation of the extracellular matrix (ECM) [1] is a major histological change in glomerular diseases which often progresses to chronic renal failure [2]. The renal function correlates well with chronic tubulointerstitial fibrosis or glomerulosclerosis [3, 4]. Fibrosis or sclerosis is a complex process requiring the participation of several cell types and ultimately resulting in the pathological deposition of connective tissue [5]. Such deposition is probably the result of the increased synthesis and decreased degradation of ECM which can be mediated by the increased production of molecular signals such as cytokines.

Transforming growth factor-β1 (TGF-β1) has a potent effect on the proliferation and differentiation of a variety of cell types as well as a combined effect of enhancing the synthesis of ECM [6–8], while inhibiting the proteolytic degradation of newly formed matrix proteins [9, 10]. In a previous study, TGF-β1 stimulated the production of ECM by cultured rat mesangial cells [11]. In experimental glomerulonephritis, there was a close association between the elevated expression of TGF-β1 mRNA and the development of glomerulonephritis [12]. The treatment of glomerulonephritis model in rats with anti-TGF-β1 antibody prevented the histological accumulation of ECM in the glomeruli [13]. These results thus suggested that TGF-β1 may participate in glomerular matrix expansion. However, it is still unknown whether TGF-β1 may play a role in the process of a progressive renal disease.

TGF-β1 is synthesized as a TGF-β1 precursor. The C-terminal of the TGF-β1 precursor forms a mature TGF-β1, that is biologically active. The N-terminal remnant of the TGF-β1 precursor is denoted as the TGF-β1 latency associated peptide (LAP) because it is sufficient for TGF-β1 latency. TGF-β1 is usually secreted by normal cells in a biologically inactive or latent form with high molecular weights [14, 15]. The latent form of TGF-β1 is composed of three distinct components; mature TGF-β1, LAP and latent TGF-β1 binding protein (LTBP) [16, 17]. LAP is linked to a single molecule of LTBP by a disulphide bond. The dissociation of LAP from mature TGF-β1 renders TGF-β1 biologically active. Acidification, heating and protease treatment lead to activation of latent TGF-β1 complex in vitro, while it is still unknown how latent TGF-β1 complex is activated in vivo.

Adriamycin(ADR)-induced nephropathy is a model of chronic progressive focal glomerular sclerosis (FGS) in rats [18]. The epithelial degeneration consists of an initial lesion in the kidney, which later progresses into glomerulosclerosis and tubulointerstitial changes [18–21]. In the present study, we examined the role of TGF-β1 on the matrix protein synthesis and the gene expression of LTBP and TGF-β receptors in the process of glomerulosclerosis and interstitial fibrosis of ADR-nephropathy.

Methods

Experimental design

Male Sprague-Dawley (SD) rats, weighing 220 to 250 g were used in the present study. Experimental FGS was induced by the intravenous injection of ADR, 0.25 mg/100 g body weight, dissolved in 0.9% saline, twice at 20-day intervals in SD rats [18]. Control rats were given an intravenous injection of the
same volume of 0.9% saline twice at the same time. The rats were kept in individual cages and were allowed free access to water and regular rat chow ad libitum. The number of rats used for each experiment is given in the figure legends. Urinary protein was measured by the sulfosalicylic acid method at the day before injection and at weeks 4, 8 and 16. The rats were sacrificed at the day before the first injection (normal untreated rats), and at 4, 8 and 16 weeks after the second injection of ADR (the ADR rats) or only saline (the control rats). The kidneys were perfused in situ via the aorta with phosphate-buffered saline (PBS), pH 7.4 and then excised. The renal capsules were removed and the cortex trimmed off with scissors. The cortex from either the control rats or the ADR rats was saved for preparation in either a cortical or glomerular conditioned media, glomerular RNA extraction by a sieving technique, a histological analysis by fixating in formalin, as well as for immunofluorescence and cortical RNA extraction by snap freezing in liquid nitrogen. The cortex from the normal untreated rats was also saved for glomerular or cortical RNA extraction.

Histological examination

The kidneys were fixed in neutral buffered formalin and embedded in paraffin for the light microscopic study and sections of 2-μm thickness were stained with periodic acid-Schiff. To semiquantitatively glomerular matrix, 50 glomeruli were selected at random, and the degree of glomerular matrix expansion was determined using a published method [22]. The percentage of each glomerulus occupied by a mesangial matrix was estimated and assigned a score beginning with 0 = 0%, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75% and 4 = 76 to 100%. The number of glomeruli showing a lesion of 0 was set as n0, 1 + n1, 2 + n2, 3 + n3, 4 + n4, respectively. Fifty glomeruli were examined independently, and then the sclerosis index was obtained by the following formula: \( \frac{0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4}{50} \times 100 \). To estimate the relative interstitial volume of the kidney, tissue sections were examined with a 121-point (100 square) eyepiece micrometer [23]. Representative sections from the entire cortex were analyzed by means of a point-counting technique to obtain the relative interstitial volume. A minimum of five sections (605 points) were randomly selected and counted in all cases. Because the results were similar in the control rats examined at the different times of the study, the results for the control rats were pooled and compared with each experimental group with Bonferroni’s t-test for independent means. A P value of less than 0.05 was considered statistically significant.

The method of immunohistochemical staining has been described previously [24]. Paraffin sections were cut at 5-μm thickness. After deparaffinization, blocking of endogenous peroxidase in 0.3% H2O2 in methanol for 30 minutes, and permeabilization in hyaluronidase, the sections were incubated over night at 4°C with anti-LC antibody (15 to 20 μg/ml; provided by Drs. K. C. Flanders and M. B. Sporn, National Institutes of Health, Maryland, USA) or ED-I (5 μg/ml; Serotec, Oxford, UK). Anti-LC antibody is a polyclonal antibody made against a synthetic peptide corresponding to the first 30 amino acids of active TGF-β1. Anti-LC antibody stains intracellular TGF-β1 [25]. ED-I is a mouse monoclonal IgG antibody which recognizes cytoplasmic antigens in monocytes and macrophages [26]. After extensive washing, the sections for anti-LC antibody were blocked with normal goat serum, then incubated with affinity-purified goat anti-rabbit IgG and avidin-enzyme-complex (Nichirei Corp., Tokyo, Japan). The sections for ED-I were blocked with normal rabbit serum, then incubated with affinity-purified rabbit anti-mouse IgG and avidin-enzyme-complex (Nichirei Corp.). Staining was visualized by incubating with diaminobenzidine (DAB) (Nichirei Corp.) and counterstaining with 1% methyl green for one minute. Controls were obtained by replacing the primary antibody with normal rabbit IgG (Sigma Chemical Co., St. Louis, Missouri, USA) or normal mouse IgG (Sigma) at equivalent concentration. To examine the distribution of fibronectin and collagen type I, the indirect immunofluorescence study was performed. Frozen tissues were sectioned at 4-μm thickness using cryostat. Air-dried sections were fixed in cold acetone, incubated with polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, California, USA) or polyclonal rabbit anti-rat collagen type I antibody (Chemicon), and then react with fluorescein-isothiocyanate(FITC)-labeled goat anti-rabbit IgG (Organon Teknika Corp., West Chester, Pennsylvania, USA). As control experiments, tissue sections were incubated with normal rabbit sera, followed by FITC-labeled goat anti-rabbit IgG, or secondary antibody alone.

Cortical and glomerular cell cultures

For the preparation of cortical conditioned media, pieces of cortical tissue were weighed in a petri dish and minced with a sharp blade into small pieces less than 1 mm in diameter, rinsed, and suspended in serum-free RPMI-1640 (GIBCO, Grand Island, New York, USA) at a concentration of 10 mg tissue per milliliter. For the preparation of glomerular conditioned media, the glomeruli were isolated using a graded sieving technique [27]. A spatula was used to pass the minced cortex through a 149-μm nylon screen (Spectrum Medical, Los Angeles, California, USA). The tissue which emerged through a nylon screen was then passed sequentially through a 105-μm and 74-μm sieve. Intact glomeruli retained on the 74-μm sieve were collected, and washed three times in PBS, and resuspended at 5 × 104 glomeruli per milliliter in serum-free RPMI-1640 in six-well multidwell plates. All glomerular preparations used consisted of more than 95% glomeruli with minimal tubular contamination. After 24 hours of incubation, these conditioned media were harvested and centrifuged for five minutes at 4°C. The pellet was discarded and the supernatant was collected, aliquoted, and stored frozen at -20°C until the TGF-β bioassay. The rest of the cortical and glomerular cultures were biosynthetically labeled by the addition of 200 μCi/ml of 35S-methionine for 24 hours. All isotopes were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, USA). The culture media were harvested, phenylmethylsulfonyl fluoride, pepstatin and aprotonin (Sigma) were added as protease inhibitors, and the mixtures were centrifuged for five minutes to remove cell debris. The samples were stored frozen at -20°C.

Electrophoretic technique

The samples for SDS-PAGE were mixed with a sample buffer containing 3% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10% β mercaptoethanol and heated for five minutes at 100°C [28]. Aliquots (15 μl) were equally applied to 4 to 20 gradient
gels (Daichii Pure Chemicals Co., Tokyo, Japan). The molecular size markers were from R&D Systems (Minneapolis, Minnesota, USA). Fluorography was performed by incubating gels in Enlightning (New England Nuclear, Boston, Massachusetts, USA). Immunoprecipitation of fibronectin was performed by adding 100 μl of polyclonal rabbit anti-rat fibronectin antibody (Chemicon) to 500 μl of conditioned medium as previously described [11].

**TGF-β bioassay**

Mink lung epithelial cells were maintained in DMEM (GIBCO) with 10% FCS. Subconfluent cells were used in the TGF-β growth inhibition assay as described by Danielpour et al [29] with a few modifications. The cells were trypsinized, washed with DMEM, and suspended in DMEM supplemented with 5% FCS, 10 mmol/liter HEPES, pH 7.4, penicillin (25 U/ml), and streptomycin (25 μg/ml). The cells were seeded at 2 × 10^4 cells per 200 μl in each well of 96-well dishes. After one hour, the conditioned media were added in dilutions of 1:10. After 22 hours incubation the cells were pulsed with 1.0 μCi ³H-thymidine per well for two hours at 37°C. The cells were then washed twice with 200 μl of PBS and trypsinized and harvested using a microculture harvesting device and counted in liquid scintillation counter to measure ³H-thymidine incorporation. For each assay a standard curve was obtained with 0.03 to 3 ng/ml of a purified porcine TGF-β1 (R&D Systems). The results were expressed as the counts per minutes. To neutralize TGF-β activity, a rabbit anti-TGF-β antibody (R&D systems) was added at a concentration of 10 μg/ml. To measure total (latent + active) TGF-β activity, 1 N HCl was added to the conditioned media until the pH decreased to 2.0 to 2.5. After 30 minutes at room temperature, the transiently acidified media was brought to pH 7.4 with 1 N NaOH and dialyzed against serum-free RPMI-1640 for 24 hours at 4°C. Because the results were similar in cortical or glomerular conditioned media from the control rats at the different times of the study, the results for the control rats were pooled and compared with each experimental group with Bonferroni's t-test for independent means. A P value of less than 0.05 was considered statistically significant.

**RNA extraction and Northern blot analysis**

Cortical tissue samples and glomeruli were isolated and purified as described above. Total RNA was isolated from cells using guanidine isothiocyanate, according to the method of Chirgwin et al [30]. Ten micrograms of poly (A)⁺ RNA from cortex and 10 μg of total RNA from glomeruli were subjected to electrophoresis in a 2.2% formaldehyde-1% agarose gel, transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, Illinois, USA), and then fixed by baking at 80°C for two hours.

The cDNA probes used were for rat TGF-β1 and rat latent TGF-β1 binding protein (provided by Dr. T. Nakamura, Kyushu University, Fukuoka, Japan) [16], human TGF-β type II receptor and rat TGF-β type III receptor (provided by Dr. R. A. Weinberg, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA) [31, 32], rat fibronectin (provided by Dr. R. O. Hynes, Massachusetts Institute of Technology) [33], mouse procollagen α2(I) (provided by Dr. G. Liau, J. H. Holland Laboratory, Rockville, Maryland, USA) [34], and chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as an internal control probe.

The membranes were prehybridized at least for two hours at 37°C in hybridization solution (5× SSC, 5× Denhardt’s solution, 0.1 mg/ml of salmon sperm DNA, 0.1% SDS, and 50% formamide). The cDNA probes were labeled with ³²P-dCTP by the random primer method and hybridized in the hybridization solution at 42°C overnight. The membranes were washed twice in 2× SSC, 0.1% SDS, and twice in 1× SSC, 0.1% SDS at 42°C for 15 minutes. Autoradiography was performed by the standard methods.
Fig. 2. Immunofluorescence micrographs with anti-fibronectin antibody on the renal sections from a control rat (A) and an ADR rat at week 16 (B, C); anti-rat collagen type I antibody on sections from a control rat (D) and an ADR rat at week 16 (E, F). Fibronectin and collagen type I were present in the glomeruli and interstitium in the control rat (A, D). Increased quantities of fibronectin and collagen type I were present in the tubulointerstitium of the ADR rat (B, E). The accumulation of both ECM proteins increased in the expanded mesangium and Bowman’s capsule of the enlarged glomerulus of the ADR rats (C, F). (A, B, D; ×135, C, E, F; ×270)

Results

Experimental focal glomerulosclerosis

As described previously [18], this model of FGS showed massive proteinuria immediately after ADR injection, which continued until week 16 (date not shown). In the ADR rats at week 4, a light microscopic examination revealed that the glomeruli and interstitium were fairly intact. At week 8, focal glomerulosclerosis was scatteringly observed. Tubulointerstitium showed patchy changes with flattened epithelial cells, round cell infiltration and fibrosis. At week 16, extensive glomerular sclerosis and hyalinosis, tubular atrophy and interstitial fibrosis with round cell infiltration were observed. Figure 1 shows a progressive increase in the degree of glomerular sclerosis...
and interstitial fibrosis in this model. The sections from control rats showed that the glomeruli and interstitium kept mostly intact throughout the study.

By immunofluorescence microscopy, fibronectin and collagen type I were present in the glomeruli and interstitium of the control rats. Both matrix proteins were intensely accumulated on the sclerosing glomeruli and fibrous interstitium of the ADR rats at weeks 8 and 16 (Fig. 2).

Cortical and glomerular fibronectin synthesis

The control rats and ADR-injected rats were sacrificed for the experiments at weeks 4, 8, and 16 after the second injection. The cortex and glomeruli were placed in culture, and biosynthetically labeled to identify any newly synthesized fibronectin, which is the most prominent glycoprotein found in the extracellular matrix. In the cortex of the ADR rats, fibronectin synthesis was not significantly observed at week 4 as the same as in the control. At week 8, synthesis was detected, which reached a peak at week 16. In the glomeruli of the ADR rats, fibronectin synthesis increased at weeks 4 and 8. At week 16, however, the glomeruli could not be isolated from the cortex because of the marked adhesion. Similar results were obtained in the different experiments. Representative results are shown in Figure 3. No difference was found in the fibronectin synthesis between the cortical or glomerular culture from the control rats at three different times of the study. Therefore, the results of cortical or glomerular cultures from the control rats at week 4 are shown as a representative control in the figure.

Gene expression of fibronectin and collagen type I

Fibronectin mRNA and procollagen α2(I) mRNA were examined in the cortex. No difference was found in mRNA expression between each of the control rats at the different times of the study. Fibronectin mRNA in the cortex of the ADR rats increased progressively and reached a peak at week 16. This result suggests that the increased fibronectin synthesis in this model was due to an augmented transcriptional level. Procollagen α2(I) mRNA expression in the cortex of the ADR rats also increased progressively. Similar results were obtained in three different experiments. Representative results are shown in Figure 4.

Fibronectin mRNA was also examined in the glomeruli. Glomerular RNA of the ADR rats could be extracted only from glomeruli at week 4 but not from week 8 or 16, because the glomerular RNA of the ADR rats at week 8 or 16 was unstable during the isolation process. Glomerular fibronectin mRNA of the ADR rats also increased even at week 4 compared to the control (Fig. 5).

TGF-β bioassay

The conditioned media from either the cortical or glomerular cultures were assayed for their ability to inhibit the proliferation of mink lung epithelial cells in the control or ADR-nephropathy rats in order to determine whether or not the diseased kidneys were releasing increased amounts of TGF-β. To confirm the specificity of the assay, a rabbit polyclonal anti-TGF-β antibody, which neutralizes both TGF-β1 and -2, was used. Before acidification, neither the cortical nor glomerular conditioned media from the control or ADR-nephropathy had any significant inhibitory effect on the mink lung epithelial cells compared to
each medium added with anti-TGF-β antibody. After acidification, cortical conditioned media from all stages, including the control rats, showed inhibitory effects on mink lung epithelial cell proliferation. The inhibitory effects of the acidified conditioned media from the ADR rats increased progressively and reached a peak at week 16, correlating to the degree of fibrous changes and fibronectin synthesis in the cortex and this effect could be reversed by anti-TGF-β antibody (Fig. 6A). In the glomeruli, acidified conditioned media from the ADR rats at weeks 4 and 8 also showed a more inhibitory effect on mink lung epithelial cell proliferation compared to the control (Fig. 6B). Similar results were obtained in three different experiments. These results indicate that the TGF-β secreted by the cortex or glomeruli from either the control rats or the ADR rats was almost in a latent form while mature TGF-β could not be detected in our TGF-β bioassay.

**Gene expression of TGF-β1, LTBP, and TGF-β type II and type III receptors**

To verify the increase in TGF-β synthesis in the ADR-nephropathy as suggested by the mink lung epithelial cell bioassay, the amount of TGF-β1 mRNA expression was investigated in both the cortex and glomeruli. Northern blotting for latent TGF-β1 binding protein (LTBP) mRNA in the cortex was also investigated to determine the role of LTBP in TGF-β metabolism. A Northern analysis of cortical TGF-β1 mRNA for the control rats showed a weak band as a 2.5 kb species, suggesting that there was a low level expression of this growth factor in the cortex. In the ADR rats, there was a marked increase in cortical TGF-β1 mRNA at week 4, when both fibronectin synthesis and TGF-β secretion did not increase. The
high level of cortical TGF-β1 mRNA continued until week 16. LTBP mRNA which was represented by two species of 5.3 and 6.2 kb, was detectable in the cortex of the control rats and the 6.2 kb species had a higher relative intensity than the 5.3 kb species. The difference in size is attributed to the missing 5' sequence [16]. There was no correlation in the relative mRNA levels between LTBP and TGF-β1 in the cortex of the ADR rats. Cortical LTBP mRNA increased progressively and reached a maximum at week 16, and its pattern was similar to cortical fibronectin synthesis and TGF-β secretion, but not to TGF-β1 mRNA. The mRNA levels for TGF-β type II and type III receptors in the cortex of the ADR rats also increased progressively and reached a maximum at week 16, which resembled the mRNA levels for LTBP and fibronectin. The same results were obtained in three different experiments. Representative results are shown in Figure 7. No difference was found in mRNA expression between each of the control rats at the different times of the study.

Glomerular RNA could be extracted only from the ADR rats at week 4 but not from week 8 or 16, because the glomerular RNA of the ADR rats at week 8 or 16 was unstable during the isolation process. Glomerular TGF-β1 mRNA of the ADR rats also increased even at week 4 compared to the control (Fig. 8).

Cells synthesizing TGF-β1

Anti-LC is an antibody made against a synthetic peptide from TGF-β1 that reacts with cells that are thought to be synthesizing TGF-β1. We used anti-LC antibody to detect TGF-β1 producing cells in the cortical tissue. Anti-LC positive cells were not observed in the control rats, while intracellular staining with anti-LC antibody was detected in the tubular epithelial cells and a small number of infiltrating cells in the interstitium of the ADR rats at week 8. An increasing number of infiltrating cells which were stained with the anti-LC antibody were seen associated with the area of increased cellularity in the interstitium and glomeruli of the ADR rats at week 16 (Fig. 9).

To identify the type of infiltrating cells in the interstitium, cortical tissues were also stained with ED-1, which is a mouse monoclonal IgG antibody to monocytes and macrophages. ED-1 positive cells were not detected in the glomeruli or the interstitium of the control rats. A number of ED-1 positive macrophages were observed in both the glomeruli and the interstitium of the ADR rats at weeks 8 or 16 (Fig. 9). Although the distribution and the morphological appearance of anti-LC positive cells in the interstitium were similar to those of the ED-1 positive macrophages, the number of anti-LC positive cells was smaller than that of the ED-1 positive macrophages.

Discussion

The histological and physiological studies on adriamycin-induced nephropathy have been extensively done and reported from our laboratory [18–21]. This nephropathy is a reproducible model of glomerulosclerosis and chronic interstitial fibrosis associated with nephrotic syndrome. Histological changes in the kidney include the marked accumulation of extracellular matrix (ECM) in relation to the increase of the parenchymal cells, which is an important feature of this model to end-stage renal destruction. Extensive fibronectin and collagen type I deposition was revealed in the sclerosing glomeruli and fibrous interstitium in this model. Both matrix proteins are key molec-
ular components found in ECMs of interstitium. Fibronectin first appears prior to other matrix proteins in scarring tissues [35] and provides a scaffold for the deposition and fibrogenesis of interstitial collagens [36]. The present study also demonstrated that an increased fibronectin synthesis in glomerular or cortical culture was correlated with the glomerulosclerosis or interstitial fibrosis in this model.

Although many cytokines may be implicated in sclerotic and fibrotic responses consequent to adriamycin-induced renal injury, TGF-β1 seems to be a main factor in regulating sclerosis and fibrosis because of its widespread effects on ECMs. TGF-β1 actions on ECMs are mediated through regulatory effects on: (a) ECM synthesis [6–8], (b) enzymes that degrade ECM [9, 10], and (c) the expression of ECM receptor on cells [37]. In fact, TGF-β1 has a dramatic effect on the production of ECM in the cultured rat glomerular mesangial cells [11]. An experimental model of glomerulonephritis, anti-Thy-1 nephritis in the rats, was associated with the increased production and activity of TGF-β1 [12]. The suppression of the experimental disease achieved with anti-TGF-β1 antibody treatment indicates the importance of TGF-β1 in regulating ECM production in glomerulonephritis [13].

In the present study of adriamycin-induced nephropathy, the progressive increase in TGF-β synthesis obtained by a bioassay using mink lung epithelial cells resembled the pattern of cortical or glomerular fibronectin production. It is noteworthy that the elevation of TGF-β1 mRNA preceded the increase in the fibronectin synthesis and mRNA. Immunohistologically, TGF-β1 was identified in tubular epithelial cells and infiltrating cells in the interstitium at week 16, which coincided with the peak TGF-β secretion by kidney tissues. These results suggest that the increased expression of TGF-β1 may be related to the

Fig. 7. Northern blotting of TGF-β1 mRNA, LTBP mRNA and TGF-β type II and type III receptors mRNA in the cortex. Ten micrograms of poly (A)⁺ RNA from the cortex of the normal untreated rats (N; rats sacrificed at the day before the first injection) or the control rats (C) and the ADR rats (A) at 4, 8, 16 weeks after the second injection was loaded to each lane. Poly(A)⁺ RNA was analyzed with the EcoRI/BglII fragment of rat TGF-β1 cDNA. LTBP mRNAs were detected by the cDNA insert of LTBP clone RM3. Hybridization with human TGF-β type II receptor cDNA and rat TGF-β type III receptor cDNA was also performed. Finally blots were rehybridized with GAPDH cDNA to confirm that approximately equal amounts of RNA were loaded in each lane. The arrows indicate the sizes of the major transcripts for TGF-β1 (2.5 kb), LTBP (6.2 and 5.3 kb), TGF-β type II receptor (5.5 kb), TGF-β type III receptor (6.0 kb) and GAPDH (1.3 kb).
Another possible explanation for the discrepancy of TGF-β1 mRNA increased progressively with a similar pattern to latent TGF-β secretion and fibronectin synthesis, may play an important role in the activation of latent TGF-β1 complex in this model of renal fibrosis.

The latent TGF-β secretion by the cortex was preceded by an increase in TGF-β1 mRNA at week 4. The discrepancy between the kinetics of TGF-β synthesis and the increased message expression is unknown at present but may be explained by the discrepancy between TGF-β1 mRNA and LTBP mRNA. In rat tissues, there is usually a good correlation between LTBP mRNA and TGF-β1 mRNA in various tissues except for the thymus [16]. This suggests that LTBP mRNA is synthesized in parallel with the expression of TGF-β1 mRNA in a normal tissue. The present study indicates the possibility that LTBP, of which LTBP is necessary for TGF-β1 latency and the activation of the latent TGF-β complex, probably due to concentrating the latent growth factor on the cell surface where activation occurs [43]. Epidermal growth factor (EGF)-like domains and the internal repeats with eight cysteins in LTBP may act as a functional domain to release mature TGF-β1 from the latent complex by the interaction with a cell surface matrix macromolecule [16, 43]. Our present study indicates the possibility that LTBP, of which mRNA increased progressively with a similar pattern to latent TGF-β secretion and fibronectin synthesis, may play an important role in the activation of latent TGF-β1 complex in this model of renal fibrosis.

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fibroblast) may contribute to expression of messenger RNA. A recent study using an immunohistochemical examination in carcinoma and granulation tissue in the human gastrointestinal tract, indicates that fibroblasts produce both TGF-β1 precursor and LTBP, and that cancer cells and macrophages produce only TGF-β1 precursor [45]. Further studies using immunohistochemistry or in situ hybridization will be needed to better identify the type of cells expressing TGF-β1 precursor or LTBP in ADR-induced nephropathy.

In the present study, we also examined mRNA levels for TGF-β type II and type III receptors, which increased progressively with a similar pattern to fibronectin mRNA and LTBP mRNA. TGF-β1 bind to at least three different receptors type I, II and III. Type I and type II receptors are most important for the signal transduction whereas the function of type III receptor is thought to act as a reservoir or capacitor of TGF-β1 and to regulate either the ligand-binding ability or surface expression of the type II receptor [32, 46]. TGF-β1 type II receptor has been recently cloned and has a cytoplasmic serine/threonine kinase domain which is not yet fully understood [31]. Our results may thus indicate the possibility that the TGF-β1 and TGF-β receptors are progressively augmented in the diseased tissue and are therefore involved in fibrosis in this model.

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Reprint requests to Kiyoshi Tamaki, Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan.

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