Role of Angiotensin II in the Transforming Growth Factor-β1
Expression of Rat Kidney in Anti-Glomerular Basement Membrane
Antiserum-Induced Glomerulonephritis

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Induction of acute nephritis in the rat by injecting anti-glomerular basement membrane (GBM) antiserum is accompanied by a transient increase in angiotensin II generation in blood circulation within the first 24 h and a subsequent elevation of transforming growth factor-β1 (TGF-β1) mRNA levels in kidney cortex with a peak at days 7—8. Studies were carried out to determine whether the increased generation of angiotensin II plays a role in the elevation of TGF-β1 mRNA. Elevation of TGF-β1 mRNA levels 7 d after injection of antiserum was significantly inhibited by a successive daily administration of TCV-116, angiotensin II type 1 receptor antagonist, at 1 mg/kg/d from days 0 to 2 or from days 0 to 6, while it was not influenced by a single administration of this dose on day 0. In addition, angiotensin II infusion for 24 h at a rate of 50 ng/min did not alter the level of TGF-β1 mRNA which was measured 6 d after the infusion. These results suggest that the anti-GBM antiserum-induced increase in TGF-β1 expression in the kidney is not responsible for angiotensin II generated in the blood circulation during the early phase of acute nephritis, but is probably mediated by angiotensin II generated locally in the kidney.

Key words transforming growth factor-β1; angiotensin II; angiotensin II angiotensin; glomerulonephritis

Glomerulonephritis is an inflammation of the kidney characterized by the accumulation of extracellular matrix within the damaged glomeruli, impaired filtration and proteinuria.1) The pathogenesis of glomerulonephritis is only partially understood, but the eliciting factor is thought to be an immunological injury to mesangial and/or other resident cells in the glomeruli2) which results in the accumulation of glomerular extracellular matrix and thickening of the glomerular basement membrane (GBM). 3)

Recent studies have revealed that the experimentally induced nephritis in animals is associated with increased production of transforming growth factor-β (TGF-β), an inducer of extracellular matrix production, in the kidney and therefore suggested a key role of TGF-β in the transition from the acute injury phase to the later development of glomerular sclerosis.4) Expression of TGF-β by the mesangial, endothelial and vascular smooth muscle cells is stimulated by angiotensin II (ANG II).5—7) The in vitro studies of cultured mesangial cells demonstrated that ANG II-induced increase in the synthesis of extracellular matrix proteins is secondary to the induction of TGF-β1. 8

We recently found an increased activity of the renin–angiotensin system in the circulation in the experimentally induced nephritis, such as purumycin aminonucleoside- and anti-GBM antiserum-induced nephritis in the rat: plasma levels of both angiotensinogen and renin increased during the first 5 d in the former model9) and within 24 h in the latter.9) The aim of this study was to determine whether the increased generation of ANG II in the early phase of anti-GBM antiserum-induced acute nephritis is responsible for the increase in the expression of renal TGF-β1.

MATERIALS AND METHODS

Animals Male Sprague–Dawley rats (120—140 g) were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan).

Anti-GBM Antiserum-Induced Nephritis Rabbit antirat GBM antiserum was kindly supplied by Mr. K. Hayashi, Dr. T. Nogami and Dr. Y. Suzuki, Department of Pharmacology, Faculty of Pharmacy, Meijo University, Nagoya, Japan. The antiserum and normal rabbit serum were inactivated by heating at 56°C for 30 min. Glomerulonephritis was induced by a single intravenous injection of 0.2 ml of anti-GBM antiserum/animal on day 0. Control animals received the same volume of normal rabbit serum.

Collection of Urine and Assay of Urine Protein Rats were separately placed in metabolic cages, and urine samples were collected into bottles containing 0.5 ml of saturated boric acid solution as an antiseptic. Urine protein was determined by the method of Bradford using bovine γ-globulin as a standard.10)

Determination of TGF-β1 mRNA Levels in Kidney Cortex The plasmid containing rat cDNA encoding TGF-β1 was obtained from RIKEN (Saitama, Japan), and the 490-base pair of BgII/EcoRI fragment of pTGF-11 was prepared as a hybridization probe. The cDNA fragment was radiolabeled with [α-32P]dCTP (3000 Ci/mmol) by random priming.11) Animals were sacrificed by ether anesthesia, and the kidney cortex was trimmed off with scissors for RNA preparation. Total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform extraction method.12) Aliquots (15 μg) of total RNA were electrophoresed on denaturing formamide gels. The gel was transferred onto nylon membrane (Hybond N+; Amersham) by overnight capillary transfer. The membrane was cross-linked by UV-irradiation, baked, and prehybridized in a solution containing 1% bovine serum albumin, 1 mm EDTA, 7% sodium dodecyl sulfate (SDS) and 5% formamide in 0.5 M sodium phosphate, pH 7.2, for 2 h.13) The hybridization was continued overnight in

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the presence of $^{32}$P-labeled probe, at 65°C. The membrane was rinsed in 40 mM sodium phosphate, pH 7.2, containing 1 mM EDTA and 0.1% SDS. The blots were autoradiographed and evaluated using a Fuji Film Bio Imaging Analyzer BAS2000 (Fuji Photo Co., Tokyo). The membranes were reprobed with a $^{32}$P-labeled oligonucleotide (41-mer) that hybridizes with 18S rRNA as a control for loading. The TGF-β1 mRNA/18S rRNA ratio was determined for estimating tissue TGF-β1 mRNA concentration.

**Experiments on the Effect of TCV-116** The selective ANG II type 1 receptor antagonist TCV-116 (donated by Takeda Chemical Industries, Ltd., Osaka, Japan) was suspended in 30% gum arabic and administered orally at a dose of 1 mg/kg to 3 groups of animals. Group 1 received a single dose of TCV-116 2 h before an injection of anti-GBM antiserum. Groups 2 and 3 received this drug by successive administrations once daily from days 0 to 2 or from days 0 to 6, respectively. On day 7, animals were sacrificed to determine the levels of TGF-β1 mRNA in kidney cortex.

**Experiments on the Effect of ANG II Infusion** An Alzet osmotic minipump (Palo Alto, CA, U.S.A.) filled with ANG II or with vehicle only (0.01 N acetic acid) was implanted subcutaneously through a 1-cm midline incision of the dorsal skin of rats under ether anesthesia; the calculated infusion rate of ANG II was 50 ng/min. Twenty-four hours after implantation, minipumps were removed under ether anesthesia. Six days thereafter, animals were sacrificed, and total RNA was extracted from kidney cortex.

**Statistical Analysis** The results are expressed as the means±S.E. of five animals. Statistics between sample means were initially compared using the one-way analysis of variance (ANOVA), and if this proved significant, the differences were tested by Bonferroni's method.

**RESULTS**

**Anti-GBM Antiserum-Induced Nephritis in the Rat** A single intravenous injection of anti-GBM antiserum produced proteinuria during the first 24 h (130±19 mg of urine protein/day) which reached a plateau (200 mg/d) between day 3 and 5, then decreased to less than 100 mg/d on day 10 (Fig. 1). A single oral administration of TCV-116, a selective nonpeptide antagonist for ANG II type 1 receptor, 2 h before injection of antiserum significantly inhibited the appearance of proteinuria (Fig. 1). A similar ameliorative effect of TCV-116 on proteinuria was observed by successive administrations of TCV-116 from day 0 to 9.

**TGF-β1 mRNA Levels in Kidney Cortex after Injection of Anti-GBM Antiserum** Expression of TGF-β1 gene was determined by Northern blotting using TGF-β1 cDNA as a probe. As shown in the representative autoradiogram in Fig. 2, the message corresponding to the size of TGF-β1 mRNA in kidney cortex of non-treated animals was faint. TGF-β1 mRNA level in kidney cortex was increased by injection of antiserum on day 1 and reached a maximum on days 7 and 8, whereas an injection of preimmune rabbit serum had no effect.

**Effect of TCV-116 on the Anti-GBM Antiserum-Induced Increase in TGF-β1 mRNA** To evaluate whether the elevated expression of TGF-β1 mRNA by injecting anti-GBM antiserum is secondary to increased levels of ANG II in the circulation, we examined the effect of TCV-116 on the renal level of TGF-β1 mRNA 7 days after injection of anti-GBM antiserum. As shown in representative Northern blots in Fig. 3, the antiserum-induced elevation of TGF-β1 mRNA was not inhibited by a single administration of TCV-116 2 h before the injection of antiserum, whereas it was significantly inhibited by successive administrations of this drug from days 0 to 2 or from days 0 to 6. These results were confirmed by quantifying TGF-β1 mRNA levels by determining the ratio of TGF-β1 mRNA to 18S rRNA (Fig. 3).
Effect of TCV-116 on the Anti-GBM Antiserum-Induced Increase in TGF-β1 mRNA

TCV-116 (1 mg/kg, p.o) was administered to rats in a single dose 2 h before (●) or in successive daily doses from days 0 to 2 (□) or from days 0 to 6 (▲) after the injection of anti-GBM antiserum. Two other groups of animals received normal rabbit serum (□) and anti-GBM antiserum (▲) without treatment of TCV-116. Animals were sacrificed to extract total RNA from kidney cortex 7 d after injection of antiserum or normal rabbit serum. Representative Northern blots of TGF-β1 mRNA and 18S rRNA are shown in the upper panel. Quantitative analysis is shown in the lower panel in which the ratio of TGF-β1 mRNA/18S rRNA is normalized relative to the mean value of the ratio in the samples from animals treated with normal rabbit serum. Values are the means ± S.E. of five animals in each group. *: Significantly different from the values of animals treated with anti-GBM antiserum alone (p < 0.001).

Effect of ANG II Infusion on TGF-β1 mRNA Levels in Kidney Cortex

ANG II was infused for 24 h at a rate of 50 ng/min, then TGF-β1 mRNA levels were measured 6 d after ANG II infusion. As shown in Fig. 4, TGF-β1 mRNA levels were not influenced by ANG II infusion.

DISCUSSION

ANG II is an important factor regulating glomerular hemodynamics, and hence the peptide has been recognized to be involved in the progression of renal injury, as evidenced by the beneficial effects of angiotensin converting enzyme inhibitors in human and animals. Recently, we reported that the reinit-angiotensin system in the circulation was activated in the rat during the first 24 h after an injection of anti-GBM antiserum. We recently reported that the injection of anti-GBM antiserum resulted in a 3-fold increase in plasma ANG II at ·16 h. Thus, there is a possibility that a transient increase in ANG II levels in the circulation contributes to the development of antiserum-induced glomerulonephritis. In fact, a blockade of ANG II type 1 receptor by a single administration of TCV-116 during this period prevented the deterioration of renal function such as proteinuria, suggesting that the transient elevation of circulating ANG II contributes to the development of nephritis, presumably due to an inhibition of ANG II-induced changes in renal hemodynamics such as increase in glomerular capillary pressure.

Recently, ANG II has been found to stimulate extracellular matrix synthesis by vascular smooth muscle cells and mesangial cells through autocrine production of TGF-β1. Enhanced synthesis of renal TGF-β1 has been demonstrated in various models of experimentally induced renal injury in the rat, such as the anti-thymocyte antiserum-induced mesangial proliferative glomerulonephritis, remnant kidney and unilateral ureteral obstruction. Injection of an antiserum capable of neutralizing the activity of TGF-β1 into nephrotic rats suppresses the production of matrix components by the glomeruli and prevents the buildup of mesangial matrix, indicating an important role of this cytokine in the development of kidney diseases. As shown in this study, induction of acute nephritis by anti-GBM antiserum caused an elevation of TGF-β1 mRNA levels in kidney cortex with a peak at 7—8 d. This result is consistent with a report on the crescentic glomerulonephritis induced in rabbits by the injection of anti-GBM antiserum. A single administration of TCV-116 prior to an injection of antiserum did not inhibit the subsequent elevation of TGF-β1 mRNA, suggesting that an antiserum-induced elevation of circulating ANG II during the first 24 h is not responsible for the increased expression of TGF-β1 mRNA in the kidney, since the ANG II-induced pressor
response in the rat is potently inhibited by a single oral administration of 1 mg/kg of TCV-116 for at least 24 h.\textsuperscript{14} This was confirmed by the observation that ANG II infusion for 24 h at a dose which elevates the blood ANG II level by 3-fold\textsuperscript{23} did not alter the renal level of TGF-\(\beta\)-1 mRNA after 7 d. Thus it is unlikely that a transiently elevated ANG II in the circulation following injection of antiserum triggers the subsequent elevation of TGF-\(\beta\)-1 mRNA. In addition, despite the amelioration of nephrotic syndrome by a single administration of TCV-116, failure to inhibit the subsequent elevation of TGF-\(\beta\)-1 mRNA in the kidney suggests that the TGF-\(\beta\)-1 expression following glomerular injury is independent of the severity of nephrotic syndrome, such as proteinuria.

The expression TGF-\(\beta\)-1 mRNA in kidney cortex was inhibited by successive administrations of TCV-116 for the first 3 d. No further inhibition was obtained by the administration of this drug for 7 d, suggesting that the 1 to 3 d period after antiserum injection is crucial for the inhibition of TGF-\(\beta\)-1 expression by TCV-116. Nevertheless, the generation of ANG II in blood circulation is apparently low during this period, since plasma concentrations of both renin and angiotensinogen fell below normal levels 36 h after injection of anti-GBM antiserum\textsuperscript{30} and remained low until day 7 (unpublished data). Therefore, it seems unlikely that the antiserum-induced expression of TGF-\(\beta\)-1 is mediated by ANG II in blood circulation. Kim \textit{et al.} recently reported that TCV-116 and enalapril, an angiotensin-converting enzyme inhibitor, inhibited the enhanced expression of TGF-\(\beta\)-1 mRNA in the kidney of deoxycorticosterone acetate-salt hypertensive rats without lowering blood pressure; they therefore suggested that the intrarenal ANG II has an important function in renal injury of this hypertension by stimulating the gene expression of TGF-\(\beta\)-1.\textsuperscript{24} Thus, ANG II generated locally in the kidney rather than that generated in the blood circulation may contribute to the expression of TGF-\(\beta\)-1 mRNA.

In conclusion, although a transient activation of the renin–angiotensin system in the early phase of anti-GBM antiserum-induced nephritis may contribute to the deterioration of nephrotic syndrome, it is not responsible for the subsequent increase in the gene expression of TGF-\(\beta\)-1 in the kidney. Nevertheless, the fact that the increase in TGF-\(\beta\)-1 mRNA was inhibited by successive administrations of TCV-116 following antiserum injection appears to indicate the key role of AGN II, probably generated locally in the kidney, in the gene expression of TGF-\(\beta\)-1 in glomerulonephritis.

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\textbf{REFERENCES}