Original Article

Transforming Growth Factor- β Expression in Cardiovascular Organs in Stroke-Prone Spontaneously Hypertensive Rats with the Development of Hypertension

Yoshiko TAHIRA, Noboru FUKUDA, Morito ENDO, Ryo SUZUKI, Yukihiro IKEDA, Hiroto TAKAGI, Koichi MATSUMOTO, and Katsuo KANMATSUSE

Transforming growth factor (TGF)- β activity is involved in several cardiovascular diseases owing to its effects on the growth of vascular smooth muscle cells and induction of extracellular matrix formation. We evaluated expression of TGF- β in cardiovascular organs from stroke-prone spontaneously hypertensive rats (SHR-SP) which show severe cardiovascular damages with the development of hypertension. Twelve-week-old Wistar-Kyoto (WKY)/Izm rats and SHR-SP/Izm were loaded with 1% salt for 4 weeks. Aorta, heart and kidney were removed and evaluated histologically by hematoxylin-eosin staining. Expression of TGF- β 1 mRNA was evaluated by reverse transcription and polymerase chain reaction analysis in mRNA extracted with oligo dT-cellulose. Expression of TGF- β 1 protein was evaluated by Western blot analysis and immunohistochemical study in renal cortex. Whereas expression of TGF- β 1 mRNA was detected only in the heart of SHR-SP before salt loading, it was detected in the aorta, left ventricle of heart and renal cortex from both rat strains, and it was stronger in the renal cortex of SHR-SP than in the renal cortex of WKY rats. Expression of TGF- β 1 protein was markedly higher in the renal cortex of SHR-SP than in the renal cortex of WKY rats after salt loading. TGF- β was localized at glomeruli and capillary arteries in the renal cortex, and immunostaining was stronger in SHR-SP than in WKY rats. Expression of TGF- β 1 was increased in glomeruli and capillaries of the renal cortex with the development of hypertension in SHR-SP. These results implicate TGF- β in the renal damage observed in hypertension. (*Hypertens Res* 2002; 25: 911–918)

Key Words: transforming growth factor- β , stroke-prone spontaneously hypertensive rats, hypertension, glomerulosclerosis

Introduction

Hypertension is associated with severe complications such as stroke, ischemic heart disease and glomerulosclerosis, and these conditions are thought to result from cardiovascular organ growth induced by high blood pressure, tissue angiotensin II, and cytokine (1). Transforming growth factor (TGF)- β is a multifunctional cytokine that is associated with cell growth, matrix formation, tissue repair, and inflamma-

tion (2, 3). TGF- β has been reported to be involved in several cardiovascular diseases, such as stroke, ischemic heart disease and glomerulosclerosis, owing to its effects on the growth of vascular smooth muscle cells (VSMC) and extracellular matrix formation (4–7).

We have shown that VSMC from spontaneously hypertensive rats (SHR), an animal model of essential hypertension, increasingly express TGF- β and show TGF- β receptor abnormality (8, 9). Stroke-prone spontaneously hypertensive rats (SHR-SP) show severe cardiovascular organ damages

From the Second Department of Internal Medicine, Nihon University School of Medicine, Tokyo 173–8610, Japan.

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Address for Reprints: Noboru Fukuda, M.D., Ph.D., Second Department of Internal Medicine, Nihon University School of Medicine, Ooyaguchi-kami 30–1, Itabashi-ku, Tokyo 173–8610, Japan. E-mail: nhukuda@med.nihon-u.ac.jp

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such as stroke, ventricular hypertrophy and glomerulosclerosis with the development of hypertension. The glomerulosclerosis that occurs with hypertension is associated with vascular damages and extracellular matrix formation (10). TGF- β is considered to be involved in the cardiovascular organ damage observed in cases of hypertension.

We therefore investigated TGF- β 1 expression in the cardiovascular organs of SHR-SP in association with the development of hypertension.

Materials and Methods

Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experimental Design

Twelve-week-old male Wistar-Kyoto (WKY)/Izm ($n = 8$) rats and SHR-SP/Izm ($n = 8$) (SHR Corp., Funabashi, Chiba, Japan) were used in this study. Both strains were housed, fed standard rat chow, and loaded with 1% salt water for 4 weeks. Systolic blood pressure (SBP) was determined by the tail cuff method using a blood pressure monitor for volume-oscillometry (UR-5000, Ueda Seisakusho, Tokyo, Japan) in the morning under conditions of quiet immediately before and immediately after the salt loading. The average of three successive readings was recorded. Body weight was determined before and after salt loading. All rats were anesthetized with pentobarbital after the 4-week salt loading, and the aorta, heart and kidney were removed so that expression of TGF- β could be evaluated in these cardiovascular organs.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) Analysis for TGF- β 1 mRNA in Cardiovascular Organs

mRNA was extracted from approximately 100 mg of aorta, left ventricle and renal cortex from WKY rats and SHR-SP by means of the QuickPrep micro mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Tissues were homogenized completely in 400 μ l of extraction buffer (buffered aqueous solution containing guanidinium thiocyanate and *N*-lauroyl sarcosine), then combined with 800 μ l of elution buffer (10 mmol/l Tris-HCl, pH 7.5, and 1 mmol/l EDTA). After centrifugation at 13,500 $\times g$ at 4°C for 1 min, homogenates were gently mixed with oligo (dT)-cellulose suspended in 0.15 % Kathon CG/1CP Biocide. After centrifugation at 16,000 $\times g$ at 4°C for 10 s, pellets were mixed with 1 ml of high salt buffer (10 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA and 0.5 mol/l NaCl) and resuspended in oligo (dT)-cellulose buffer. After centrifugation again at 16,000 $\times g$ at 4°C for 10 s, pellets were mixed with 1 ml of low salt buffer (10 mmol/l Tris-HCl, pH 7.5, 1 mmol/l

EDTA and 0.1 mol/l NaCl). After further centrifugation at 16,000 $\times g$ at 4°C for 10 s, residue was resuspended in 0.3 ml of low salt buffer and transferred to a polypropylene MicroSpin column (Amersham Pharmacia Biotech) that was fitted with a frit. After centrifugation at 16,000 $\times g$ at 4°C for 5 s, effluents were discarded in collection tubes. Ten microliter of glycogen solution (5–10 mg/ml in RNase-free water) and 40 μ l of potassium acetate solution (2.5 mol/l potassium acetate, pH 5.0) were added to the samples to precipitate mRNA. The obtained mRNA was washed with 1 ml of cold 95% ethanol.

RT-PCR analysis for TGF- β 1 mRNA was performed as described previously (11) with each of two specific primers. The 22 mer upstream sense primer (5'-GCCCTGGATACCAACTACTGCT-3') and the 22 mer downstream antisense primer (5'-AGGCTCCAAATGTAGGGGCAGG-3') were derived from the coding region of the rat TGF- β 1 cDNA. Sense primer (5'-CGACGACCCATTTCGAACGTCT-3') and antisense primer (5'-GCTATTGGAGCATGGAATTACCG-3') were used for PCR amplification of 18S ribosomal RNA served as an internal control to yield a 312-base pair (bp) product. A sense primer (5'-CGACGACCCATTTCGAACGTCT-3') and antisense primer (5'-GCTATTGGAGCATGGAATTACCG-3') were used for PCR amplification of 18S ribosomal RNA and served as an internal control to yield a 312-bp product.

To confirm that no genomic DNA was co-amplified by PCR, control RT-PCR experiments were performed with each set of primers but without reverse transcriptase; no product was amplified. For semiquantitative analysis of mRNA, kinetics of the PCR reaction were monitored; the number of cycles at which the PCR products were detectable on the gel was compared between samples (12). Serial 10-fold dilutions of cDNA (100, 10 and 1 ng) were amplified; the PCR products were detectable at earlier cycles with increasing amounts of cDNA. PCR was performed 30 times in an automatic thermocontroller (MJ Research, Watertown, USA) under the following thermal cycling protocol: denaturing at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

Western Blot Analysis of TGF- β 1 Protein in the Renal Cortex

Approximately 50 mg of renal cortex was homogenized completely to achieve uniform suspension in 500 μ l of lysis buffer (50 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, 0.02% sodium azide, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1% Triton X-100). The samples were sonicated for 2 min, centrifuged at 16,000 $\times g$ for 10 min at 4°C, mixed with 400 μ l methanol and 100 μ l chloroform, and sonicated again for 2 min. After centrifugation at 16,000 $\times g$ for 10 min at 4°C, supernatants were evaporated.

Fig. 1. Sections of mid-layer aortic smooth muscle from Wistar-Kyoto rats before (A) and after (B) salt loading and from stroke-prone spontaneously hypertensive rats before (C) and after (D) salt loading. Sections were counterstained with hematoxylin-eosin. Bar = 25 μ m. Original magnification $\times$ 200.

They were then suspended in 100 μ l of phosphate-buffered saline (PBS) and sonicated for 3 min. The protein concentration was determined by method of Lowry *et al.* (13).

Tissue extracts with 30 μ l of sample buffer (63 mmol/l Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol) were subjected to Western blotting. The samples were boiled and subjected to 10% polyacrylamide gel electrophoresis. The proteins were transblotted to a nitrocellulose membrane and incubated with mouse monoclonal antibody specific for TGF- β 1 (R & D Systems, Inc., Minneapolis, USA) or mouse monoclonal antibody specific for α -tubulin as a control (Sigma BioScience, St. Louis, USA) diluted 1:500 in 5% nonfat milk in TBST solution (10 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, and 0.05% Tween 20) for 3 h at room temperature. The membrane was incubated with goat anti-mouse IgG for 1 h at room temperature, then washed with TBST once for 15 min, and four times for 5 min. Immune complexes on the membrane were detected by the enhanced chemiluminescence (Amersham Pharmacia Biotech) method.

Histological Examination

Aortas, hearts and kidneys obtained from WKY rats and SHR-SP before and after salt loading were fixed in 10% neu-

tral buffered formalin and embedded in paraffin for light microscopy study. Samples were sectioned into 2- μ m thick slices and stained with hematoxylin-eosin (H-E).

Immunohistochemical Study of TGF- β 1 in Renal Cortex

Frozen renal cortex tissues were sectioned with cryostat at 3 μ m thickness. Immunofluorescence was achieved by the avidin-biotin peroxidase method as described previously (14). Briefly, frozen tissue cut serially into 3- μ m sections was frozen in cold acetone, air dried, and washed in PBS. The first antibody used in this study was a monoclonal mouse antibody specific for TGF- β 1 (R & D Systems). The fixed sections were immersed in methanol containing 0.3% hydrogen peroxide (v/v) to quench the endogenous peroxidase activity. Non-specific protein binding sites were blocked with 10% normal rabbit serum in PBS. The sections were consecutively incubated with monoclonal antibody for 60 min at room temperature, washed in PBS and incubated with biotin-conjugated F(ab)₂ fragments of rabbit antibody to mouse immunoglobulin G (IgG) (DAKO, Carpinteria, USA) for 30 min at room temperature. After being washed in PBS, the sections were incubated with avidin-biotin peroxidase complex (ABC Vectastatin Elite Kit; Vector, Burlingame, USA), re-washed in PBS, and treated with di-

Fig. 2. Sections of the left ventricle from Wistar-Kyoto rats before (A) and after (B) salt loading and from stroke-prone spontaneously hypertensive rats before (C) and after (D) salt loading. Sections were counterstained with hematoxylin-eosin. Bar = 25 μ m. Original magnification $\times$ 200.

Fig. 3. Sections of the glomeruli from Wistar-Kyoto rats before (A) and after (B) salt loading and from stroke-prone spontaneously hypertensive rats before (C) and after (D) salt loading. Sections were counterstained with hematoxylin-eosin. The arrow indicates glomerular sclerosis lesion. Bar = 50 μ m. Original magnification $\times$ 100.

aminbenzidine. The sections were counterstained with hematoxylin, mounted, and observed under a conventional light microscope. Negative control sections were incubated with

an irrelevant IgG (a mouse anti-human C3bi receptor; DAKO).

Fig. 4. Expression of transforming growth factor (TGF)- β 1 mRNA in aorta, heart and kidney from Wistar-Kyoto (W) rats and from stroke-prone spontaneously hypertensive rats (S) before (A) and after (B) salt loading. Levels of TGF- β 1 and 18S ribosomal RNA mRNAs were determined by reverse transcription and polymerase chain reaction analysis. The ratio of the abundance of TGF- β 1 mRNA to that of 18S mRNA was evaluated by densitometric analysis. Data are the means of experiments carried out in duplicate.

Compounds

Phenylmethylsulfonyl fluoride, aprotinin, Triton X-100, Tween 20, and diaminobenzidine were purchased from Sigma.

Results

Blood Pressure

SBP in WKY rats was 142 ± 6 mmHg before salt loading and 152 ± 8 mmHg after salt loading. SBP in SHR-SP was 255 ± 11 mmHg before salt loading and 272 ± 16 mmHg after salt loading, respectively. The level of SBP in SHR-SP was significantly ($p < 0.01$) higher than that in WKY rats before and after salt loading.

Fig. 5. Expression of transforming growth factor- β 1 protein in renal cortex from Wistar-Kyoto (WKY) rats ($n = 2$) and stroke-prone spontaneously hypertensive rats (SHR-SP) ($n = 2$) before and after salt loading (upper panel). Levels of TGF- β 1 protein were determined by Western blot analysis. The arrows indicate 12.5 and 25 kDa monomer and dimer TGF- β 1 proteins. Abundance of 12.5 kDa TGF- β 1 protein was evaluated by densitometric analysis (lower panel).

Morphological Changes in Cardiovascular Organs

Midlayer sections of aortic smooth muscle taken from SHR-SP and WKY rats before and after salt loading are shown in Fig. 1. Hypertrophic changes were observed in the aortic smooth muscle of SHR-SP but not in the aortic smooth muscle of WKY rats after salt loading. Figure 2 shows sections of the left ventricle taken from SHR-SP and WKY rats before and after salt loading. Hypertrophic changes were observed in the left ventricle of SHR-SP but not in the left ventricle of WKY rats after salt loading. Figure 3 shows sections of renal cortex taken from SHR-SP and WKY rats before and after salt loading. In glomerular sclerosis lesions, slightly atrophic tubules and interstitial fibrosis were observed in SHR-SP but not in WKY rats after salt loading.

Expression of TGF- β 1 mRNA in Cardiovascular Organs

TGF- β 1 mRNA was detected only in the SHR-SP left ventricle before salt loading. After 4 weeks of salt loading, TGF- β 1 mRNA was detected in the mid-layer smooth muscle of the aorta, left ventricle and renal cortex taken from both rat strains. TGF- β 1 mRNA expression did not differ in mid-layer smooth muscle or in left ventricle between SHR-SP and WKY rats. TGF- β 1 mRNA expression was stronger in the renal cortex of SHR-SP than in the renal cortex of WKY rats after salt loading (Fig. 4). We therefore evaluated TGF- β 1 protein expression in the renal cortex taken from SHR-SP

Fig. 6. Immunohistochemical study of transforming growth factor (TGF)- β 1 in renal cortex from Wistar-Kyoto rats (A) and in renal cortex from stroke-prone spontaneously hypertensive rats (SHR-SP) after salt loading (B) (original magnification $\times 100$). The high power field of TGF- β 1 positive capillary arteries in the renal cortex of SHR-SP after salt loading is shown in C (original magnification $\times 200$), and the negative control section of the renal cortex from SHR-SP using an irrelevant monoclonal antibody is shown in D (original magnification $\times 100$). Bar = 25 μ m.

and WKY rats after salt loading by both Western blot and immunohistochemical analysis.

Expression of TGF- β 1 Protein in the Renal Cortex

Western blot analysis of TGF- β 1 protein expression in the renal cortex obtained from SHR-SP and WKY rats before and after salt loading revealed two major bands with molecular weights of 25 and 12.5 kDa. TGF- β 1 protein expression was faint in the renal cortex of both rats strains before salt loading. After salt loading, TGF- β 1 protein expression was increased in the renal cortex of both strains and stronger in the renal cortex of SHR-SP than in the renal cortex of WKY rats (Fig. 5).

Immunohistochemical Study of TGF- β 1 in the Renal Cortex

Figure 6 shows immunohistochemical localization of TGF- β 1 in the renal cortex obtained from SHR-SP and WKY rats. Glomeruli stained positively for TGF- β 1, and staining was greater in the renal cortex of SHR-SP than in the renal cortex of WKY rats (Fig. 6A and B). Strong staining for TGF- β 1 was observed in the capillaries of the renal cortex of SHR-SP after salt loading; such staining was not observed in the renal cortex of WKY rats (Fig. 6C).

Discussion

SHR-SP showed mid-layer aortic smooth muscle hypertrophy, ventricular hypertrophy and glomerulosclerosis with atrophic tubules and interstitial fibrosis in the kidney as a result of the development of hypertension with salt loading. The expression of TGF- β 1 mRNA in both the mid-layer smooth muscle and ventricle was similar between SHR-SP and WKY rats after salt loading, but the expression of TGF- β 1 mRNA was stronger in the glomeruli and the capillaries of the renal cortex obtained from SHR-SP than in those of WKY rats. These findings suggest that the vascular smooth muscle hypertrophy and the ventricular hypertrophy in SHR-SP resulted directly from the severe hypertension, but that the renal damage may be associated not only with the hypertension but also with the local production of TGF- β . In addition, dietary salt has been reported to modulate production of TGF- β in the kidney (15, 16). Ying and Sanders (15) reported that an increase in dietary salt produced sustained increases in expression of TGF- β 1 mRNA in rat kidney, but no changes in serum concentration of TGF- β 1. Tamaki *et al.* (16) also demonstrated marked increases in TGF- β 1 production in the kidneys of Dahl salt sensitive rats. In the present experiment, expressions of TGF- β 1 protein and mRNA were increased in the kidneys of SHR-SP after salt loading. It is

possible that this increase in TGF- β 1 production in the kidneys of SHR-SP was partially associated with salt loading.

TGF- β has been reported³ to be involved in Thy-1 antibody-induced glomerulonephritis (17). The injured glomeruli express much greater levels of TGF- β 1 mRNA, TGF- β 1 protein, and extracellular matrix components such as fibronectin and proteoglycans than the normal glomeruli (17). Injection of a neutralizing antibody against TGF- β 1 into nephritic rats suppresses production of these matrix components in the glomeruli and prevents mesangial proliferation (18). Okuda *et al.* (17) observed that increases in TGF- β production accompanied increases in collagen type I and fibronectin in glomerulosclerosis in Dahl salt-sensitive rats, a model of malignant hypertension. These observations and our present findings indicate that TGF- β is involved in the renal changes seen in glomerulonephritis and glomerulosclerosis through its effect on mesangial cell growth and extracellular matrix formation.

The glomerulosclerosis we observed in SHR-SP was accompanied by capillary arterial hypertrophy and strong TGF- β expression with the development of hypertension. Glomerular hypertension results from systemic hypertension and leads to increased glomerular capillary pressure with subsequent glomerular endothelial damage and sclerosis (19, 20). TGF- β is also thought to contribute to the associated glomerulosclerosis through its induction of arteriosclerosis in the renal cortex.

TGF- β is usually present in tissues in latent form in combination with latency-associated peptide (21). When tissues are damaged and need to be repaired, TGF- β dissociates from the latency-associated peptide to be activated and released from the tissue (22, 23). In the present study, the mature 12.5 kDa TGF- β 1 protein was increasingly expressed in the renal cortex and the sclerotic glomeruli attained positively for TGF- β 1 with the development of hypertension, suggesting that TGF- β is associated with the damage and that it contributes to the glomerulosclerosis in SHR-SP with the development of hypertension.

Mesangial cells from SHR and SHR-SP have been reported to show exaggerated growth compared to mesangial cells from WKY rats (24, 25). Lopes de Faria *et al.* (24) showed an increased growth rate for cultured mesangial cells from prehypertensive SHR without an increase in extracellular matrix formation, suggesting that the faster growth of mesangial cells from SHR is partially independent of hypertension and may be associated with intrinsic abnormalities in the cells themselves. SHR-derived VSMC in culture also show exaggerated growth (1). We have found that VSMC from SHR increasingly produce angiotensin II and growth factors including TGF- β 1 owing to changes to the synthetic VSMC phenotype and abnormal regulation of TGF- β by angiotensin II, suggesting that these abnormalities are associated with the exaggerated growth of VSMC in SHR (7, 26–28). It is therefore possible that the glomerulosclerosis observed in SHR-SP is partially due to increased production of TGF- β in

mesangial cells.

In conclusion, expression of TGF- β 1 was increased in the glomeruli and capillaries of the renal cortex with the development of hypertension in an animal model of the disease, suggesting that the renal damage associated with hypertension involves TGF- β .

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