Transforming Growth Factor-β1 in Hypertensive Renal Injury in Dahl Salt-Sensitive Rats

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The kidneys of hypertensive individuals show vascular changes that represent the hallmark of nephro- or angiosclerosis (1). The mechanism through which systemic hypertension can lead to chronic renal insufficiency remains the subject of debate. Two broad hypotheses have been formulated, advocating either glomerular ischemia or glomerular hypertension. Glomerular ischemia is secondary to the progressive hypertensive narrowing of intrarenal blood vessels. The vascular-narrowing changes are characterized by two features: arteriolar hyalinosis with an irregular deposition of homogenous eosinophilic materials in the subendothelial space, and fibroplastic intimal thickening with an acquired inner layer of fibrous or sclerotic tissue (2). Glomerular hypertension is based on the hypothesis that the direct transmission of systemic hypertension to the glomerular capillary bed leads to increased glomerular capillary pressure, with subsequent glomerular endothelial damage and sclerosis (3–5).

Although the molecular mechanisms whereby increased intravascular pressure affects the vascular walls or glomerular capillary walls in vivo are still unknown, the lesion consists of ingrowing endothelial cells, smooth muscle cells, or intraglomerular cells associated with the increased extracellular matrix (ECM). The accumulation of the ECM is a characteristic histological change in hypertensive intimal thickening of either the vascular sclerosis (6) or glomerular sclerosis (7,8). Fibrosis or sclerosis is a complex process requiring the participation of several cell types and ultimately resulting in the pathological deposition of connective tissue (9). 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 (10–12). Our previous study revealed that an experimental model of glomerulonephritis, anti-Thy-1 nephritis in the rats, was associated with the increased production and activity of TGF-β (13). The suppression of this experimental disease achieved with anti-TGF-β antibody treatment indicates the importance of TGF-β in regulating ECM production (14). In the progressive glomerulosclerosis model, the progressive increase in the expression of TGF-β1 was also revealed in the process of glomerulosclerosis and interstitial fibrosis (15).

Dahl salt-sensitive (Dahl-S) rats are used as a model of malignant hypertension in which the renal histological and functional deterioration is raised by severe...
hypertension (16,17). The fibroplastic intimal thickening with an acquired inner layer of sclerotic tissue in the vascular wall and sclerosing glomerul is characteristic lesions in this model. In the study presented here, TGF-β biosynthesis by isolated glomeruli, latent TGF-β localization in a kidney, the mRNA expression of TGF-β1, latent TGF-β binding protein (LTBP), or TGF-β receptors (Types I, II, and III) were compared between the Dahl-S rats fed a high-salt diet and a low-salt diet. The inappropriate expression of TGF-β1 and its receptors may be related to the increased ECM production associated with the hypertensive lesions of the vasculature and glomeruli in this model.

METHOD

Experimental Design

Seven-week-old male Dahl-S rats (Dahl-Iwai salt-sensitive rats; originally obtained from Brookhaven National Laboratories, Upton, NY, and bred by the Eisai Co., Tokyo, Japan), were used in this study. The rats were kept in individual cages and were maintained to tap water and either 0.3% NaCl (low-salt; LS) or 8.0% NaCl (high-salt; HS) diet (both from Oriental Yeast Co., Tokyo, Japan) ad libitum for 4 wk. Before the study, all rats were maintained on 0.3% NaCl diet. The diets were identical in composition except for the NaCl content. Systolic blood pressure was measured by the tail-cuff method. The rats were euthanized after 4 wk on either the HS diet or the LS diet. The kidneys were perfused in situ via the aorta with phosphate-buffered saline (PBS), pH 7.4, and then were excised. The kidney cortexes were saved for preparation in a glomerular conditioned media, a histological analysis by fixation in formalin, as well as for immunofluorescence, an immunohistochemical examination, and RNA extraction by being snap-frozen in liquid nitrogen.

Histological Examination

The kidneys were fixed in neutral buffered formalin and were embedded in paraffin for the light-microscopic study, and sections of 2-μm thickness were stained with periodic acid–Schiff (PAS) stain. A histological examination was made independently by two pathologists without any prior knowledge of the experimental groups. To semiquantitate the glomerular matrix, 50 glomeruli were selected at random, and the degree of glomerular matrix expansion was determined using a published method (18). 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 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: \( \frac{1}{50} \times n_0 + \frac{2}{50} \times n_1 + \frac{3}{50} \times n_2 + \frac{4}{50} \times n_3 + \frac{5}{50} \times n_4 \). To estimate the relative interstitial volume of the kidney, tissue sections were examined with a 121-point (100 square) eyepiece micrometer (19). 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. To semiquantitate the arteriosclerotic change, 50 arterioles were selected at random, and the degree of arteriolosclerosis was determined as described by Bader et al. (20) according to a semiquantitative score of 1 to 4 as follows: 1+, no change; 2+, hyalinosis of the arteriolar wall up to 50% of its circumference; 3+, hyalinosis of the wall between 50% and 100% of its circumference but without any narrowing of the lumen; 4+, complete hyalinosis of the wall with luminal encroachment. The number of arteriole showing a lesion of 1+ was n1, 2+ n2, 3+ n3, 4+ n4, respectively. Fifty arterioles were examined independently, and then the arteriosclerosis index was obtained by the following formula: \( \frac{1}{50} \times n_1 + \frac{2}{50} \times n_2 + \frac{3}{50} \times n_3 + \frac{4}{50} \times n_4 / 50 \).

Immunofluorescence Study

To examine the distribution of fibronectin, collagen Type I, plasminogen activator inhibitor-1 (PAI-1), and LTBP, the indirect immunofluorescence study was performed. The first antibodies used in this study were polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA), polyclonal rabbit anti-rat collagen Type I antibody (Chemicon), polyclonal rabbit anti-rat PAI-1 antibody (American Diagnostica, Greenwich, CT), and anti-human latent TGF-β binding protein (LTBP) antibody (a gift from Dr. K. Miyazono, Ludwig Institute for Cancer Research, Uppsala, Sweden) (21,22). Frozen kidney tissues were sectioned at 2-μm thickness using a cryostat. The air-dried sections were fixed in cold acetone, incubated with the first antibody, and then reacted with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin (IgG) (Organon Teknika Co., West Chester, PA). For the control experiments, tissue sections were incubated with normal rabbit sera, followed by either FITC-labeled goat anti-rabbit IgG or secondary antibody alone.

Immunohistochemical Examination

Rabbit polyclonal antibody, Ab96 (a gift from Dr. K. Miyazono), directed against the latency-associated peptide (LAP) portion of the TGF-β1 precursor was made using a synthetic peptide corresponding to the specific amino acid sequences as previously described (21,23). This antibody is specific for human TGF-β1 LAP (24,25), and also reacts with rat TGF-β1 LAP (26). Frozen specimens were sectioned at 5-μm thickness using a cryostat. The air-dried sections were then fixed in cold acetone. The sections were incubated in PBS containing 5% goat serum, and then were incubated with Ab96 at 4°C overnight. The endogenous peroxidase was inactivated by immersing the sections in methanol containing 0.3% H2O2. The sections were incubated with secondary antibody, biotinated anti-rabbit IgG (Vector Laboratories, Burlingame, CA). ABC complex (Vector Laboratories) was applied and samples were incubated. The sections were developed in 0.05 M Tris-HCl (pH 7.6), 0.01 M NaNO3, 0.04% diaminobenzidine (DAB; Dajin, Kumamoto, Japan), and 0.05% H2O2 for 5 min. Counterstaining was performed with hematoxylin. Non-specific binding of secondary antibody or detection complex was excluded by omitting the primary antibody.

Glomerular Cell Culture

The preparation of the glomerular conditioned media was performed according to the method described in our previous studies (13,15). 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, CA). The tissue that emerged through the 74-μm sieve were collected, washed three times in PBS, and resuspended at 5 x 105 glomeruli per millilitre in serum-free RPMI-1640 (GIBCO, Grand Island, NY) in six-well
multwell plates. All glomerular preparations used consisted of more than 95% glomeruli with minimal tubular contamination. After 24 h of incubation, these conditioned media were harvested and centrifuged for 5 min at 4°C. The pellet of more than 95% glomeruli with minimal tubular contamination was harvested and centrifuged for 5 min at 4°C. The pellet was discarded and the supernatant was collected, aliquoted, and stored frozen at -20°C until the TGF-β bioassay. In a separate experiment, the glomerular cultures were biosynthetically labeled by the addition of 200 μCi/mL of 35S-methionine for 24 h. All isotopes were obtained from the American Radiolabeled Chemicals, Inc. (St. Louis, MO). The culture media were harvested, phenylmethylsulfonyl fluoride, and pepstatin, and aprotonin (Sigma, St. Louis, MO) were added as protease inhibitors, and the mixtures were centrifuged for 5 min to remove any cell debris. The samples were then stored frozen at -20°C.

Electrophoretic Technique

The samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were mixed with a sample buffer containing 3% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10% β-mercaptoethanol and heated for 5 min at 100°C (28). Aliquots (15 μL) were equally applied to 4 to 20% gradient gels (Daichi Pure Chemicals Co. Tokyo, Japan). The molecular size markers were from R&D Systems (Minneapolis, MN). Fluorography was performed by incubating the gels in Enlightening (New England Nuclear, Boston, MA). The immunoprecipitation of fibronectin or PAI-1 was performed by adding 100 μL of polyclonal rabbit anti-rat fibronectin antibody (Chemicon) or anti-rat PAI-1 antibody (American Diagnostica) to 500 μL of conditioned medium as previously described (27,29).

TGF-β Bioassay

Mink lung epithelial cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco) with 10% fetal calf serum. Subconfluent cells were used in the TGF-β growth inhibition assay as described by Danielpour et al. (31) with a few modifications (15). The cells were trypsinized and suspended in DMEM supplemented with 5% fetal calf serum, 10 mM N-hydroxylpiperazine-N'-2-ethanesulfonic acid (pH 7.4), penicillin (25 U/mL), and streptomycin (25 μg/mL). The cells were seeded at 2 x 10^4 cells per 200 mL in each well of 96-well dishes. After 1 h, the conditioned media were added in dilutions of 1:10. After 22 h of incubation, the cells were pulsed with 1.0 μCi 3H-thymidine per well for 2 h at 37°C. The cells were then washed twice and trypsinized and harvested using a microculture harvesting device and counted in liquid scintillation counter to measure 3H-thymidine incorporation. To neutralize the TGF-β activity, a monoclonal anti-TGF-β antibody (Genzyme, Cambridge, MA) was added at a concentration of 10 μg/mL. The TGF-β activity was expressed as the percentage inhibition of the thymidine incorporation that was reversible by anti-TGF-β antibody. To measure the 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 min, the transiently acidified media was brought to pH 7.4 with 1 N NaOH, and then was used in the mink lung epithelial cell assay.

RNA Extraction and Northern Blot Analysis

Cortical tissue samples were isolated and purified as described above. Total RNA was isolated from cells by using guanidine thiocyanate, according to the method of Chung-wen et al. (32). Ten micrograms of poly (A)+ RNA from the cortex were subjected to electrophoresis in a 2.2 M formaldehyde-1% agarose gel, transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL), and then fixed by baking at 80°C for 2 h.

The cDNA probes used were for rat TGF-β1 and rat LTBP (provided by Dr. T. Nakamura, Kyushu University, Fukuoka, Japan) (33), human TGF-β Type I receptor (provided by Dr. K. Miyazono) (34), human TGF-β Type II receptor, and rat TGF-β Type III receptor (provided by Dr. R.A. Weinberg, Massachusetts Institute of Technology, Cambridge, MA) (35,36), rat fibronectin (provided by Dr. R.O. Hynes, Massachusetts Institute of Technology, Cambridge, MA) (37), mouse procollagen α2(I) (provided by Dr. G. Liu, J.H. Holland Laboratory, Rockville, MD) (38), rat plasminogen activator inhibitor-1 (provided by Dr. T.D. Gelehrter, University of Michigan Medical School, Ann Arbor, MI) (39), and chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as an internal control probe.

The membranes were prehybridized at least for 2 h at 37°C in hybridization solution (5 x standard saline citrate [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 were hybridized in the hybridization solution at 42°C overnight. The membranes were washed twice in 2 x SSC, 0.1% SDS, and twice in 1 x SSC, 0.1% SDS at 42°C for 15 min. Autoradiography was performed by the standard methods. The densitometric values of each mRNA were corrected against the value of GAPDH mRNA. The ratio of each mRNA/GAPDH mRNA from three rats in both group was averaged and compared to the LS rats.

Statistical Method

The values are expressed as the mean ± SE. The parametric data from each group were compared with an analysis of variance followed by the t test with modification by Bonferroni’s method. Significance was defined as P < 0.05.

RESULTS

Experimental Hypertensive Renal damage in Dahl-Salt Sensitive Rats

The data on the body weights and blood pressure responses in this model are given in Table 1. There was no significant difference in the body weight between the LS rats and the HS rats. However, a marked elevation of blood pressure was observed in the HS rats. A light-microscopic examination revealed that, in the LS rats, the glomeruli, arterioles, and interstitium...
were kept mostly intact (Figure 1A). In the HS rats, small artery or arterioles showed intimal thickening and onion-skin like lesions with deposition of fibrinoid material in the intima, accompanied with ischemic glomerulus (Figure 1, B and C). Focal segmental or global glomerulosclerosis was often observed (Figure 1D). Some glomeruli showed an increase in size and increased PAS-positive material in the glomerular capillary walls or in Bowman’s space (Figure 1E). Tubulointerstitium showed patchy changes with round cell infiltration and fibrosis around the sclerosing glomeruli and arterioles (Figure 1F). Some tubules are dilated with tubular casts and atrophy. Figure 2 shows an increase in the degree of glomerular sclerosis, arteriosclerosis, and interstitial change in the HS rats.

TGF-β Bioassay

The total or mature TGF-β activity in the glomerular culture supernatants from the LS rats and the HS rats was measured by the inhibition of thymidine incorporation of mink lung epithelial cells according to the previous studies (15, 40). The specificity of this inhibition was determined by comparing it to the cortical conditioned medium in the presence of anti-TGF-β antibody. The results were expressed as the percentage inhibition of thymidine incorporation that was reversible by anti-TGF-β antibody.

The mature TGF-β activity, which is revealed by a non-acidified medium, was not detected in the glomerular conditioned media from the LS or the HS rats (data not shown). The total (latent + active) TGF-β activity, which is found after the transient acidification of the conditioned medium, was detected in the glomerular conditioned medium both in the LS rats and in the HS rats (Figure 3). These results indicated that the secreted TGF-β from the glomeruli of the LS rats and the HS rats mostly existed as a latent form. The latent TGF-β secretion from the glomeruli of the HS rats was much more than that of the LS rats.

Gene Expression of TGF-β1, Latent TGF-β Binding Protein, and TGF-β Type I, II, and Type III Receptors

Figures 4 and 5 summarize data from a Northern blot analysis of TGF-β1, LTBP, and TGF-β Type I, II, and Type III receptors from the cortex of the HS and LS rats. TGF-β is secreted as a latent form, which is composed of TGF-β precursor (mature TGF-β + LAP) and LTBP (41, 42). Therefore, to determine whether the increase in glomerular TGF-β production could be a result of the increase in steady-state mRNA, a Northern blot analysis of TGF-β1 and LTBP in the kidney cortex was performed. In the HS rats, a significant upregulation of TGF-β1 mRNA was observed, compared with the LS rats. LTBP mRNA expression of the HS rats also increased compared with the LS rats. These results suggested that the increased secretion of latent TGF-β from the glomeruli of the HS rats was a result of the increase in the steady-state mRNA levels.

The mRNA levels for TGF-β Type I, Type II, and Type III receptors in the cortex were also examined. The TGF-β Type I, Type II, and Type III receptor mRNA of the HS diet were significantly higher than those of the LS rats.

Gene Expression of Fibronectin, Collagen Type I, and Plasminogen Activator Inhibitor-1

TGF-β stimulates the synthesis of ECM components such as collagens and fibronectin, and suppresses matrix degradation by inducing protease inhibitors such as plasminogen activator inhibitor (PAI). To investigate the effect of TGF-β on the renal injury in this model, a Northern blot analysis of fibronectin, collagen Type I, and PAI-1 was examined in the cortex. The results are shown in Figures 6 and 7. These results indicated that the HS treatment of Dahl-S rats for 4 wk led to an increase in the gene expression of fibronectin, collagen Type I, and PAI-1, compared with the LS treatment.

Glomerular Fibronectin and Plasminogen Activator Inhibitor-1 Synthesis

An immunoprecipitation analysis for fibronectin and PAI-1 was performed on isolated glomerular tissue samples to determine the relationship between changes in mRNA and protein. The isolated glomeruli were placed in culture and biosynthetically labeled to identify any newly synthesized fibronectin and PAI-1. There was more fibronectin synthesis in the glomeruli in the HS rats than in the LS rats (Figure 8A). The glomerular PAI-1 synthesis of the HS rats was also augmented compared with that of the LS rats (Figure 8B). These findings correlated with the data from the Northern blot analysis.

Distribution of Latent TGF-β Binding Protein, Collagen Type I, Fibronectin, and PAI-1

To determine the tissue localization of LTBP, fibronectin, collagen Type I, and PAI-1 in the kidney tissue, an immunofluorescence study was performed. Our previous study (43), as well as other studies (26, 44), have shown that the immunodetection of LTBP is thought to be an extracellular marker of latent TGF-β with LTBP. Representative sections are also shown in Figure 9. In the LS rats, fibronectin was present in the glomeruli, vessel walls, and interstitium. Collagen Type I was observed in the vessel walls and faintly in the interstitium. LTBP and PAI-1 were observed in the glomerular mesangium and the vascular walls in the LS group. In the HS rats, the ECM proteins, PAI-1, and LTBP were found in the sclerosing mesangial area of the enlarged glomeruli, along the thickened vascular walls, and in the fibrous interstitium.
Figure 1. Photomicrographs show renal tissue specimens in Dahl-S rats receiving the low-salt (LS) diet (a) or the high-salt (HS) diet (b, c, d, e, and f) for 4 wk. Panel a shows the kidney sections from the LS rat with intact glomeruli, arterioles (arrow), and interstitium; b, a small artery showing an onion-skin-like lesion with fibrinoid material in the kidney sections of the HS rat; c, severe arteriosclerosis with ischemic glomerular damage in the HS rat; d, sclerosing glomeruli detected in the HS group; e, enlarged glomeruli including increased fibrinoid material in glomerular tuft and Bowman's space in the HS rat; f, focal tubular atrophy and dilatation with cast formation, round cell infiltration with interstitial fibrosis, and normal tubules and glomeruli existing next to the area with the damaged lesions (periodic-acid Schiff stain; a, b, c, d, e, original magnification x360; f, original magnification x180).
Figure 2. The degree of glomerular sclerosis (A), arteriosclerosis (B), and interstitial change (C) in the kidney section from the LS rats and HS rats. The values are semiquantitative scores from eight rats and are expressed as the mean ± SE. * P < 0.01 compared with the LS rats.

Figure 3. The TGF-β activity in the glomerular conditioned medium (CM) from the Dahl-S rats fed the LS diet and the HS diet, assayed after transient acidification. Each sample was assayed in either the presence or absence of anti-TGF-β antibody. The results were expressed as the percentage inhibition of thymidine incorporation that was reversible by anti-TGF-β antibody. Each value represents the mean of six samples from three rats. The non-acidified CM from the glomerular tissue culture of the LS rats and the HS rats had no significant inhibitory effects on the mink lung epithelial cells (data not shown). Transiently acidified CM from the glomeruli of both diet groups showed an inhibitory effect on the mink lung cell proliferation. These results revealed that secreted TGF-β thus existed mostly in a latent form. Therefore, the results of transiently acidified CM shown in the figure indicated that latent TGF-β was thus secreted from the glomeruli of the LS rats and the HS rats in this model. The latent TGF-β secretion from the isolated glomeruli of the HS rats increased significantly compared with that of the LS rats. This experiment was performed three times. Representative results are shown in the figure. The values are the mean ± SE. * P < 0.05 compared with the LS rats.

TGF-β1 Synthesizing Cells

To detect TGF-β1-producing cells, the kidney tissue specimens were immunologically stained by Ab96, the affinity-purified antibody to the LAP portion of the TGF-β1 precursor (23). This antibody recognizes human TGF-β1 LAP (24,25) and also rat TGF-β1 LAP (26), and reacts with the cells that are thought to be synthesizing TGF-β1. In the LS rats, immunostaining for TGF-β1 was found in the glomerular cells (Figure 10A) and in the vascular cells (Figure 10B). In the HS rats, the TGF-β1-positive cells increased in the glomeruli (Figure 10C) and along the thickened vascular walls (Figure 10D). The TGF-β1-positive cells were also detected in the interstitial area. These results indicated that the glomerular cells, vascular cells, and...
Low Salt High Salt

TGF-β1

LTBP

TGF-β type I
Receptor

TGF-β type II
Receptor

TGF-β type III
Receptor

GAPDH

Figure 4. Northern blot hybridization with cDNA probes for TGF-β1, LTBP, and TGF-β type I, type II, and type III receptors in the cortex. Each lane contains 10 μg of cortical poly (A)⁺ RNA from a single sample from one animal of the LS rats or the HS rats. The blots were rehybridized with GAPDH cDNA to confirm that approximately equal amounts of RNA were loaded into each lane. The same results were obtained in three different experiments. The arrows indicate the sizes of the major transcripts for TGF-β1 (2.5 kilobase (kb) and 1.9 kb), LTBP (6.2 kb and 5.3 kb), TGF-β type I receptor (5.5 kb), TGF-β type II receptor (5.5 kb), TGF-β type III receptor (6.0 kb), and GAPDH (1.3 kb).

Interstitial cells are thus the likely sources of TGF-β1 in this hypertensive model.

DISCUSSION

A marked elevation of blood pressure associated with renal destructive changes was found after a 4-wk observation of the inbred Dahl-S rats that were fed a HS diet. The histological study revealed both ischemic glomerular damages secondary to the vascular narrowing and direct pressure-induced glomerular damages in this hypertensive model. The characteristic lesions of the arterioles and small arteries included a marked intimal thickening associated with an inner layer of fibroplastic tissue and sclerotic tissue, result-

Figure 5. A quantitative analysis of the gene expression for TGF-β1, LTBP, and TGF-β type I, type II, and type III receptors, by scanning the autographies of Northern blotting. The values represent the mean ± SE and are expressed as arbitrary units normalized to GAPDH. * P < 0.05 compared with the LS rats. ** P < 0.01 compared with the LS rats.

Figure 6. Northern blot hybridization with cDNA probes for fibronectin, collagen type I, and plasminogen activator inhibitor-1 (PAI-1) in the cortex. Each lane contains 10 μg of cortical poly (A)⁺ RNA from a single sample from one animal of the LS rats or the HS rats. The blots were rehybridized with GAPDH cDNA. The same results were obtained in three different experiments. The arrows indicate the sizes of the transcripts for fibronectin (8.0 kb), collagen type I (5.7 and 4.7 kb), PAI-1 (3.0 kb), and GAPDH (1.3 kb).
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Figure 7. A quantitative analysis of the gene expression for fibronectin, collagen type I, and PAI-1, by scanning the autoradiographs of Northern blotting. The values are expressed as arbitrary units normalized to GAPDH. * P < 0.05 compared to the LS rats.

ing in the collapse of a part of the glomeruli. In contrast, some sclerosing glomeruli showed capillary tuft necrosis and a fibrin crescent in Bowman’s space, which indicates direct pressure damage to the capillaries. Thus, hemodynamic factors are considered to be important in the localization and initiation of vascular or glomerular sclerosis. The molecular mechanisms whereby increased intravascular pressure affects the vascular or glomerular wall in vivo are unknown. The lesions consist of ingrowing endothelial cells, smooth cells, or intraglomerular cells associated with the increased ECM. An extensive deposition of fibronectin and collagen Type I was revealed in the vascular walls and the sclerosing glomeruli in this model. Both matrix proteins are key molecular components found in ECM of fibrous tissues. Fibronectin first appears before other matrix protein scarring tissues and provides a scaffold for the deposition and fibrogenesis of interstitial collagens (45). The study presented here also demonstrated that an increase in fibronectin synthesis in glomerular culture was correlated with the glomerulosclerosis in this model.

Although many cytokines may be implicated in the sclerotic response of vasculatures and glomeruli, TGF-β seems to be a main factor in regulating the fibrosis and sclerosis, because of its widespread effects on ECM. TGF-β actions on ECM are mediated through regulatory actions on (1) ECM synthesis (10–12), (2) enzymes that degrade ECM (46,47), and (3) the expression of ECM receptor on cells (48). In fact, TGF-β1 has a dramatic effect on the production of ECM in the cultured vascular smooth muscle cells (49,50) and glomerular mesangial cells (29). In the study presented here, a TGF-β bioassay using mink lung epithelial cells revealed the increase in latent TGF-β synthesis by the sclerosing glomeruli. The increased expression of TGF-β1 mRNA, LTBP mRNA, and TGF-β receptors mRNA was observed in the renal cortex from the HS rats. Immunohistologically, LTBP, an extracellular parameter of latent TGF-β (26,43,44), was identified in the damaged vascular wall and glo-

Figure 8. (A) Glomerular fibronectin synthesis. An equal number of glomeruli isolated from either the LS rats or HS rats (every two lanes are from a single sample from three animals) were cultured for 24 h and were biosynthetically labeled with 35S-methionine. The conditioned media were immunoprecipitated with anti-fibronectin antibody for an analysis of SDS-PAGE with autoradiography. Fibronectin synthesis of the glomerular conditioned media from the HS rats increased compared to the LS rats. FN, fibronectin. (B) Glomerular PAI-1 synthesis. The increased PAI-1 synthesis was observed in the glomerular conditioned media from the HS rats. These experiments were performed three times.
Figure 9. Immunofluorescence microscopy for fibronectin (a), collagen type I (b), PAI-1 (c), and LTBP (d) in the glomeruli, and LTBP (e) in the vascular walls of the kidney sections from the LS rat, and for fibronectin (f), collagen type I (g), PAI-1 (h), and LTBP (i) in the glomeruli, and LTBP (j) in the vascular walls of the kidney sections from the HS rat. In the HS rat, fibronectin, collagen type I, PAI-1, and LTBP were found in the segmental sclerotic area of the glomeruli. LTBP was observed along the thickened vascular walls. Similar staining patterns were also observed in three different kidney sections (original magnification ×280).

...These findings thus indicated that the increased expression of TGF-β1 may be related to the hypertensive renal injury in this model.

In this model, most of the TGF-β was present in a latent form in the conditioned medium from the glomeruli. TGF-β is known to be synthesized and secreted in a latent form from a wide variety of cells (21,42). The latent TGF-β complex is composed of mature TGF-β, LAP, and LTBP. LAP is necessary for TGF-β latency and the dissociation of LAP from mature TGF-β renders TGF-β biologically active. Although acidification, heating, and protease treatment have also been known to be effective for TGF-β activation in vitro, it is still unclear how this latent TGF-β complex is activated in vivo.

The mRNA expression of LTBP as well as TGF-β1 was enhanced in the kidney of the hypertensive model. This finding indicated that latent TGF-β secreted from the diseased tissue may be a latent complex with LTBP. Our immunohistological studies revealed that LTBP was localized in the connective tissue, and this finding also correlated with previous...
Figure 10. The immunohistochemical localization of anti-TGF-β1 (anti-β1 LAP) antibody on renal sections from a LS rat and a HS rat. In the LS rat, glomerular cells (a, arrowheads) and vascular cells (b) were positive for TGF-β1. There was an increase in the number of TGF-β1-positive cells in the glomeruli (c, arrowheads) and in the thickened vascular walls (d) in the HS rat. Similar staining patterns were also observed in three different kidney sections (original magnification x400).

reports (26,43,44,51). There is a recent report in which latent TGF-β complex was reported to be associated with the ECM via LTBP after its secretion (52). LTBP plays an important role in targeting the latent TGF-β complex to specific localization. It has also been demonstrated that LTBP participates in the activation of latent TGF-β complex, probably because of the concentration of latent growth factor on the cell surface where activation occurs (53). The direct interaction of the latent TGF-β complex and cell-surface molecules is necessary for the activation of the latent complex (54). LTBP may play a functional role to release mature TGF-β from the latent complex by interaction with either a cell-surface or matrix macromolecules (33,53). LTBP may thus contribute to the matrix-association and activation of latent TGF-β.

TGF-β exerts its action via binding to specific cell surface receptors. In most cell types, three different types of TGF-β receptors, i.e., Type I (53 kd), Type II (70 to 80 kd), and Type III (300 kd) can be observed (55). 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-β. TGF-β Type I and Type II receptors have recently been cloned (34,35). TGF-β signals through a heterometric complex between the Type I and Type II receptors (56). For the binding of TGF-β to Type I receptor, the presence of Type II receptor is necessary, although both TGF-β Type I and Type II receptors are needed for TGF-β action (57). In the study presented here, the increased expression of TGF-β receptors in the kidneys also seems to be involved in the hypertensive renal lesions.

In summary, the increase in latent TGF-β secretion from the sclerosing glomeruli, and the localization of latent TGF-β in intrarenal vascular walls and glomeruli, were both revealed in inbred Dahl-S rats fed a HS diet. The mRNA expression of TGF-β1, LTBP, and TGF-β receptors (Types I, II, and III) in kidney tissue was also enhanced in the hypertensive rats. These results suggested that the inappropriate expression of TGF-β1 may be related to the ECM accumulation in both the thickened vascular wall and sclerosing glomeruli of hypertensive individuals.
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