Role of the Renin-Angiotensin System in Cardiac Hypertrophy and Renal Glomerular Sclerosis in Transgenic Hypertensive Mice Carrying Both Human Renin and Angiotensinogen Genes

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Tsukuba hypertensive mice (THMs) are transgenic mice carrying human renin and angiotensinogen genes. The aim of this study was to evaluate the role of the renin-angiotensin system (RAS) in cardiac hypertrophy and renal disorders in THMs. After a 2-wk control period, 10-wk-old THMs were treated with lisinopril (ACEI group) or hydralazine (hydralazine group) or left untreated (control group) for 8 wk. C57BL/6 mice of similar age (wild group) were used as normal controls. Systolic blood pressure and urinary albumin excretion were measured once a week. All mice were sacrificed at 20 wk of age, and heart to body weight ratio, cardiac myocyte diameter, renal glomerular sclerosis index, and glomerular size were measured. Fibronectin expression was also evaluated. At 20 wk of age, systolic blood pressure and urinary albumin excretion in the control group were significantly higher than those in the wild group and significantly lower than those in the ACEI and hydralazine groups. Heart to body weight ratio and cardiac myocyte diameter were significantly higher in the hydralazine and control groups than in the other groups. Renal glomerular sclerosis index and glomerular size were also significantly higher in the control group than in the other groups, and there were significant differences between the ACEI and hydralazine groups in these variables. Fibronectin expression was marked in the control and hydralazine groups. These findings suggest that the RAS plays an important role in cardiac hypertrophy in THMs, but that both the RAS and elevation of blood pressure contribute to the pathogenesis of renal glomerular sclerosis. (*Hypertens Res* 1998; 21: 39-46)

Key Words: **cardiac hypertrophy, glomerular sclerosis, renin-angiotensin system**

Hypertension is a multifactorial disease. The renin-angiotensin system (RAS) is one of the most important factors. Recently, the RAS has been reported to exist not only in circulating blood but also in local tissues, such as the brain, heart, and vascular endothelium. The relationship between the tissue-localized RAS and many diseases, including hypertension, has therefore become a focus of study. Angiotensin II (Ang II), the final physiological activator of the RAS, is known to accelerate hypertrophy of cardiac muscles and renal glomerular sclerosis when administrated alone (*1-3*). Mechanical stress such as high blood pressure has also been reported to produce the same effects (*4-6*).

Tsukuba hypertensive mice (THMs), into which both a human renin gene and a human angiotensinogen gene have been introduced, are a model of hypertension (*7*). THMs were established by cross-breeding a mouse into which a 15-kb full length human renin gene, including its 3-kb native promoter, had been introduced with a mouse into which a 14-kb human angiotensinogen gene, including its 1.3-kb native promoter, had been introduced (Fig. 1). The increase in blood pressure in this model is caused only by increased RAS activity. The Ang II concentrations in this mouse are about four to five times higher in serum, heart, and kidneys (*8*) than those in a wild mouse, and its blood pressure is already approximately 30 to 40 mmHg higher than that of a wild mouse at 6 wk of age, when blood pressure first becomes measurable. As compared with hypertension, more serious cardiac hypertrophy and renal glomerular sclerosis are observed in THMs. These findings suggest that cardiac hypertrophy and renal glomerular sclerosis are due to increased RAS activity rather than increased blood pressure. In this study, THMs were treated with lisinopril and hydralazine. Lisinopril is an angiotensin converting enzyme inhibitor (ACEI) that blocks the RAS and decreases blood pressure, while hydralazine decreases blood pressure but does not affect the RAS. By comparing the severity of cardiac hypertrophy and renal dysfunction in our samples, we examined the role of the RAS in the pathogenesis of these two types of disorders.

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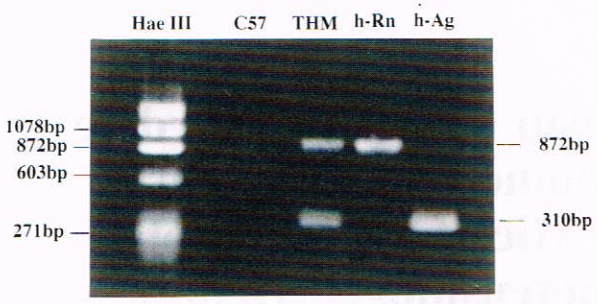

Fig. 1. *Gene introduction of Tsukuba hypertensive mouse. Hae III: Hae III φ 1 digestion, C57: C57BL/6 mouse, THM: Tsukuba hypertensive mouse, h-Rn: human renin gene introduction mouse, h-Ag: human angiotensinogen gene introduction mouse.*
The tail of each mouse was cut off, immediately frozen in liquid nitrogen, and stored at −80°C. DNA was then extracted from the tail, and PCR was performed with the use of human renin PCR primer (sense: CGCGGACTATGTATTTC, antisense: TCCAATGTCTCCATCTGAGG) and human angiotensinogen PCR primer (sense: AGAGAGCCCACAGAGTCTAC, antisense: TTCTACTGCTCACCCATGC). Thirty cycles of the following PCR procedure were performed: denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min.

Materials and Methods

Animals and Administered Drugs
THMs were divided in three groups: a group treated with an ACEI (lisinopril) (20 mg/kg/d), a group treated with hydralazine (30 mg/kg/d), and an untreated group (control group). Each group consisted of 12 THMs. The same number of C57BL/6 mice of the same age (wild group) were used as controls. Data collection began when the mice reached 10 wk of age. The mice were treated with the drugs orally by mixing with drinking water for 8 wk from 12 wk of age and sacrificed at 20 wk of age. The average values of variables at 10 and 11 wk of age were used as pre-administration values. The procedures were in accordance with institutional guidelines, and all mice were killed by anesthesia.

Measurement of Systolic Blood Pressure and Pulse Rate
The ambient temperature was set at 30°C (*9*) for about 10 min, and the systolic blood pressure and pulse rate were measured once a week non-invasively by the tail-cuff method with a programmable sphygmomanometer (BP-98A, Softron Co., Ltd., Tokyo, Japan).

Measurement of Urinary Volume, Water Intake Volume and Urinary Albumin Excretion
Urinary volume and water intake over the course of 24 h were measured once a week with a metabolic cage. Urinary albumin excretion was measured by immunonephelometry using a TIA Micro Alb Kit (Nitto-bo Co., Ltd., Tokyo, Japan).

Measurement of Body Weight and Heart to Body Weight Ratio
The body weights of eight mice in each group were measured once a week. When the mice were killed, these mice were anesthetized by intraperitoneal injection of pentobarbital sodium (Pitman Moore Inc., Mundelein, USA) at a dose of 0.75 mg/g body weight, and the body weight of the mice was measured. The hearts of the mice were excised, and heart weight was measured after the atria were removed. The heart to body weight ratio was then calculated.

Measurement of the Length and Width of Left Ventricular Myocytes
Single isolated myocytes from the left ventricles of four mice in each group were enzymatically dissociated according to the method described by Benndorf *et al.* (*10*). Twenty-five left ventricular myocytes per mouse (a total of 100 myocytes per group), which were Ca^{2+}-tolerant, clearly striated, and rod-shaped without any blebs on the surface, were measured for length and width.

Measurement of the Renal Glomerular Sclerosis Index and Glomerular Size
Kidneys fixed in 10% formalin were embedded in paraffin and 3-μm sections were made with a microtome. The sections were stained with hematoxylin and eosin (HE), periodic acid Schiff (PAS), and Masson-trichrome stains. Two hundred glomeruli were observed in each section, and the renal glomerular sclerosis index was calculated according to the method described by Kohara *et al.* (*11*). In each group, photographs of 100 randomly selected glomeruli, in which vascular poles were observed, were taken. The photographs were scanned into a computer with a scanner, and glomerular size was measured using the computer's image analysis program, NIH Image Ver. 1.57 (Wayne Rasband, National Institutes of Health, USA).

Observation of Fibronectin Expression in the Heart and Kidney
Three-micrometer-thick formalin-fixed paraffin sections of the heart and kidney were prepared after removal of paraffin with xylene and ethanol and treatment with 0.1% trypsin (DAKO Co., Ltd., Glostrup, Denmark). The sections were immunostained with anti-fibronectin polyclonal antibody (DAKO Co.) and a Histofine SAB-PO kit (Nichirei Co., Ltd., Tokyo, Japan). Stained sections were observed with an optical microscope.

Statistical Analyses
Results are given as means ± standard error. Statistical analyses were performed by repeated measure analysis of variance (ANOVA) or the unpaired *t*-test. Values of $p < 0.05$ were considered to indicate statistical significance.

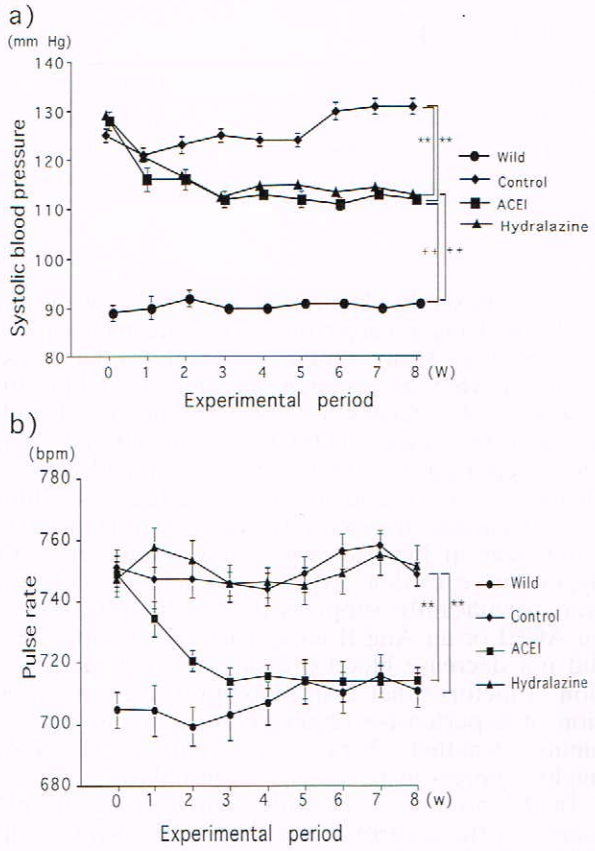

Fig. 2. *Systolic blood pressure and pulse rate.* **p<*0.01 compared with control,* ††p<*0.01 compared with wild. a) systolic blood pressure, b) pulse rate.*

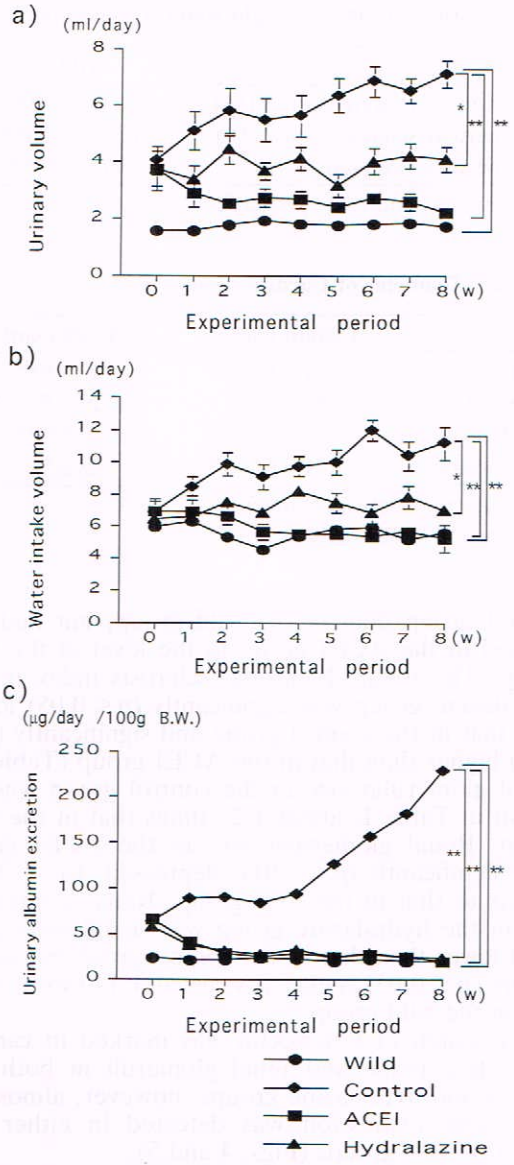

Fig. 3. *Urinary volume, water intake volume, and urinary albumin excretion.* *p<*0.05 compared with control,* **p<*0.01 compared with control. a) urinary volume, b) water intake volume, c) urinary albumin excretion.*

Results

At 12 wk of age, systolic blood pressure and pulse rate were significantly ($p < 0.01$) higher in the control group (125 ± 1 mmHg, 744 ± 12 bpm) than in the wild group (89 ± 2 mmHg, 709 ± 14 bpm). Systolic blood pressure significantly ($p < 0.01$) decreased to the same extent in both the ACEI and hydralazine groups (Fig. 2a). Pulse rate also significantly ($p < 0.01$) decreased in the ACEI group, but not in the hydralazine group (Fig. 2b). No significant differences in body weight were observed among the groups. Urinary volume and water intake volume were significantly ($p < 0.01$) higher in the control group than in the wild group. Urinary volume and water intake volume in the ACEI group decreased to levels similar to those in the wild group. Urinary volume and water intake volume in the hydralazine group were significantly ($p < 0.05$) lower than those in the control group, but significantly ($p < 0.05$) higher than those in the wild group (Fig. 3a, b). At 20 wk of age, urinary albumin excretion in the control group had increased significantly ($p < 0.01$) to a level about 10 times that in the wild group. Urinary albumin excretion in both the ACEI and hydralazine groups had decreased significantly ($p < 0.01$) to the level observed in the wild group at 20 wk of age (Fig. 3c).

The heart to body weight ratio in the control group was approximately 1.5 times higher than that in the wild group at 20 wk of age. This ratio significantly ($p < 0.01$) decreased in the ACEI group to the level in the wild group, but did not decrease in the hydralazine group (Table 1). The length and width of cardiac myocytes were significantly ($p < 0.01$) greater in the control group than in the wild group. The length and width of cardiac myocytes in the ACEI group was significantly ($p < 0.01$) suppressed similarly to the wild group, whereas neither the length nor the width was suppressed in the hydralazine group (Table 2). The renal glomerular sclerosis index was significantly ($p < 0.01$) higher in the

Table 1. Heart to Body Weight Ratio, Glomerular Sclerosis Index, and Glomerular Size.

	Wild	Control	ACEI	Hydralazine
Heart to body weight ratio (%)	0.51±0.03**	0.79±0.06	0.49±0.01**	0.76±0.05
Glomerular sclerosis index (%)	0.19±0.26**	6.19±1.39	0.25±0.29**	1.19±0.59*
Glomerular size (μm^2)	4,550±133**	5,529±164	4,551±133**	5,179±174*

Difference *vs.* control, $*p<0.05$, $**p<0.01$.

Table 2. Diameter of Cardiac Myocytes.

	Length (μm)	Width (μm)
Wild	101±16**,††	23±6**,††
Control	132±19	29±5
ACEI	105±15**,††	23±5**,††
Hydralazine	131±18	28±5

Difference *vs.* control, $**p<0.01$.
Difference *vs.* hydralazine, $††p<0.01$.

control group than in the wild group, but had decreased in the ACEI group to the level of the wild group. The renal glomerular sclerosis index in the hydralazine group was significantly ($p<0.05$) lower than that in the control group and significantly ($p<0.05$) higher than that in the ACEI group (Table 1). Renal glomerular size in the control group was, as shown in Table 1, about 1.22 times that in the wild group. Renal glomerular size in the ACEI group was significantly ($p<0.01$) depressed to a level similar to that in the wild group. Renal glomerular size in the hydralazine group was significantly ($p<0.05$) lower than that in the control group but significantly ($p<0.05$) higher (by about 1.14 times) than that in the wild group.

Expression of fibronectin was marked in cardiac connective tissue and renal glomeruli in both the control and hydralazine groups; however, almost no fibronectin expression was detected in either the ACEI or wild groups (Figs. 4 and 5).

Discussion

Hypertensive cardiac hypertrophy is considered a compensatory mechanism for afterload. Nevertheless, cardiac hypertrophy has been established as one of the serious risk factors for cardiovascular diseases. In addition to the increase in blood pressure, which places direct mechanical stress on cardiac myocytes, the RAS and neurohumonal factors such as catecholamines are related to the pathogenesis of hypertensive cardiac hypertrophy. Reducing blood pressure has become relatively easy owing to the availability of various depressors, but both prevention or improvement of cardiac hypertrophy and other types of organ failure as well as normalization of blood pressure are necessary for comprehensive treatment of hypertension. Much research has been done on the effectiveness of various depressors in controlling hypertensive cardiac hypertrophy.

In research on humans, a metaanalysis of 104 reports by Cruickshank *et al.* (*12*) and a metaanalysis of 109 reports by Dahlöf *et al.* (*13*) showed that ACEI had the greatest inhibitory effect on cardiac hypertrophy. Many similar observations have also been reported in research on animals. Clinically, however, the results of the "treatment of mild hypertension study (TOMHS)" (*14*) differed from those reported by Dahlöf *et al.* (*13*) and Cruickshank *et al.* (*12*), and it was concluded that inhibition of cardiac hypertrophy was not influenced by differences in hypertensive agents. However, since hypertensive cardiac hypertrophy and renal failure were significantly suppressed by administration of an ACEI or an Ang II antagonist even at doses that did not decrease blood pressure (*15, 16*), participation of factors other than blood pressure in suppression of hypertensive organ failure is likely. We examined whether changes in activity of the RAS might suppress hypertensive organ failure.

In the present study, pulse rate was significantly higher in the control group than in the wild group. Although the pulse rate was not decreased in the hydralazine group, that in the ACEI group was significantly decreased to a level similar to that in the wild group. Ang II accelerates the secretion of endothelin I (*17*), norepinephrine (*18*), and aldosterone (*19*). Moreover, urinary excretion of aldosterone and vasopressin is significantly higher in THMs than in C57BL/6 mice (*20*). These findings suggest that enhancement of RAS activity might have increased the pulse rate *via* autocrine or paracrine effects.

Brooksby *et al.* (*21*) examined isolated cardiac myocytes of SHR and Wistar-Kyoto rats (WKY) and reported that both the length and width of cardiac myocytes in SHR were significantly larger than those in WKY. Although systolic blood pressure was about 65 mmHg higher in SHR than WKY in that study, the systolic blood pressure of THMs in the present study was only about 36 mmHg higher than that of C57BL/6 mice. Therefore, the systolic blood pressure in the present study was not very high as compared with the diagnostic standards for human hypertension. Despite that, cardiac myocytes in the control group had a significantly greater length and width than those in the wild group, and length and width in the ACEI group were significantly suppressed similarly to those in the wild group. No suppression of length or width was found in the hydralazine group. These findings suggest that hypertrophy of cardiac muscle is induced by enhanced RAS activity even when elevation of blood pressure is not marked. This finding suggests that Ang II accelerates cardiac myocyte enlargement. Furthermore, we previously reported

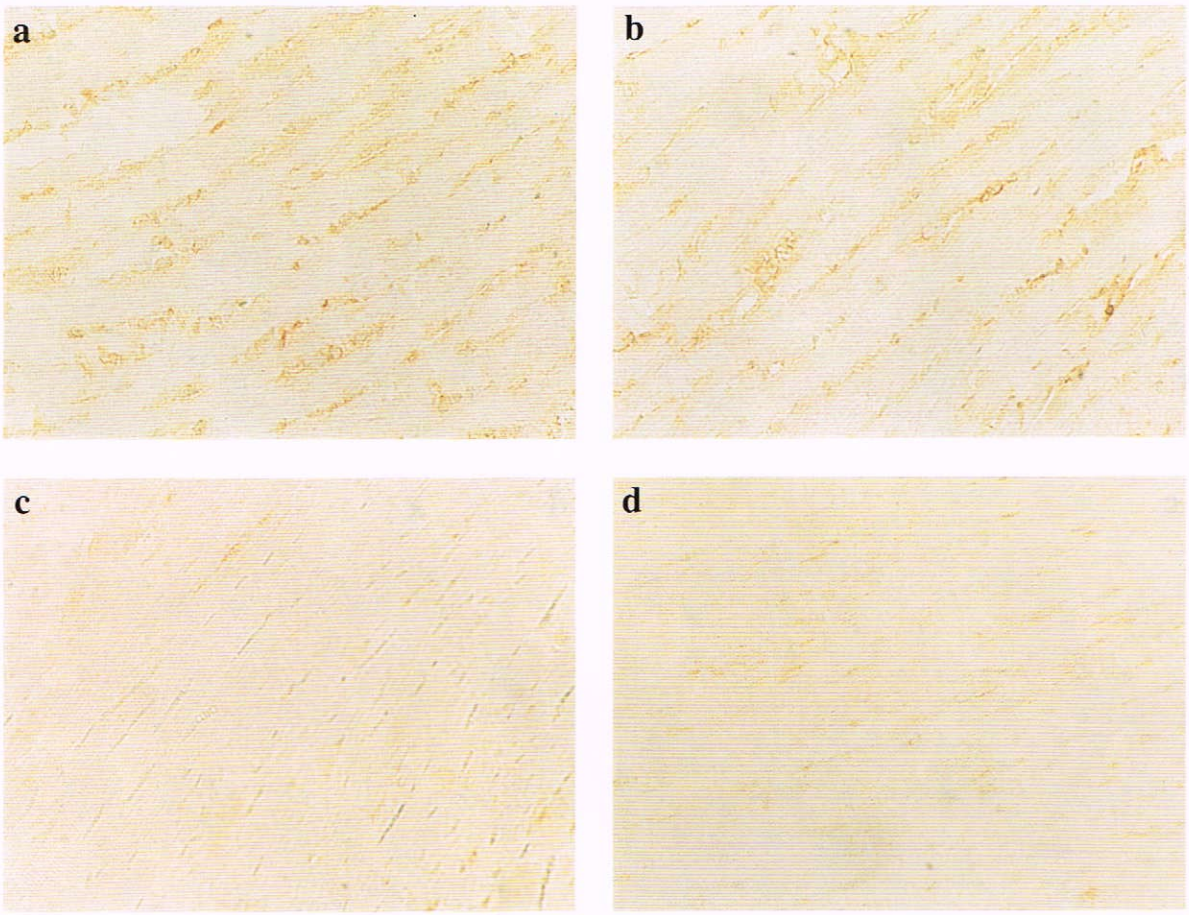

Fig. 4. *Expression of fibronectin in the heart (× 500). a) control, b) hydralazine, c) ACEI, d) wild. The expression of fibronectin was marked in cardiac connective tissue in the control group and hydralazine group, but almost no expression was detected in the ACEI group or wild group.*

that the heart to body weight ratio in SHR was about 1.15 times greater than that in WKY (*22*). In contrast, the heart to body weight ratio in THMs was about 1.5 times greater than that in C57BL/6 mice. Since SHR and THMs belong to different species and their ages at examination differed, a direct comparison of the values between these two groups may not be appropriate. However, these observations suggest that activation of the RAS is more strongly related to the formation of cardiac hypertrophy rather than to the elevation of blood pressure.

In addition, marked expression of fibronectin, a constituent of extracellular matrix (ECM), was found in the control group. Although fibronectin expression was not suppressed in the hydralazine group, similar expression levels were found in the ACEI and wild groups.

As stated above, both cardiac myocyte hypertrophy and expression of fibronectin in the ACEI group were reduced to levels similar to those in the wild group. In contrast, when blood pressure was lowered by administration of hydralazine, with no inhibition of the RAS, neither cardiac myocyte hypertrophy nor fibronectin expression was depressed. Since the cardiac Ang II concentration in THMs is about 4 times higher than that in C57BL/6 mice (*8*) and losartan, one of the Ang II type I receptor antagonists, reduces left ventricular hypertrophy and tissue Ang II contents (*23*), cardiac locally generated, but not circulating, RAS may play the major role in cardiac hypertrophy. These findings suggest that enhancement of RAS activity has two effects: one is to enlarge cardiac myocytes and the other is to accumulate ECM. These activities resulted in cardiac weight gain and enhancement of cardiac hypertrophy.

Although the mechanism by which hypertension causes renal failure is still unclear, two theories have been proposed: the hyperfiltration theory (*24*) and the glomerular hypertrophy theory (*25*). Both theories emphasize the role of the intrarenal RAS (*26*), and many studies of the relationship between renal failure and the RAS have been reported. Ang II increases renal efferent arteriole resistance above renal afferent arteriole resistance. Consequently, glomerular hypertension, together with the systemic vasopressor effect, is reinforced. Ang II also has direct (*3*) or TGF-β-mediated (*27*) effects on the proliferation of mesangial cells and increases in

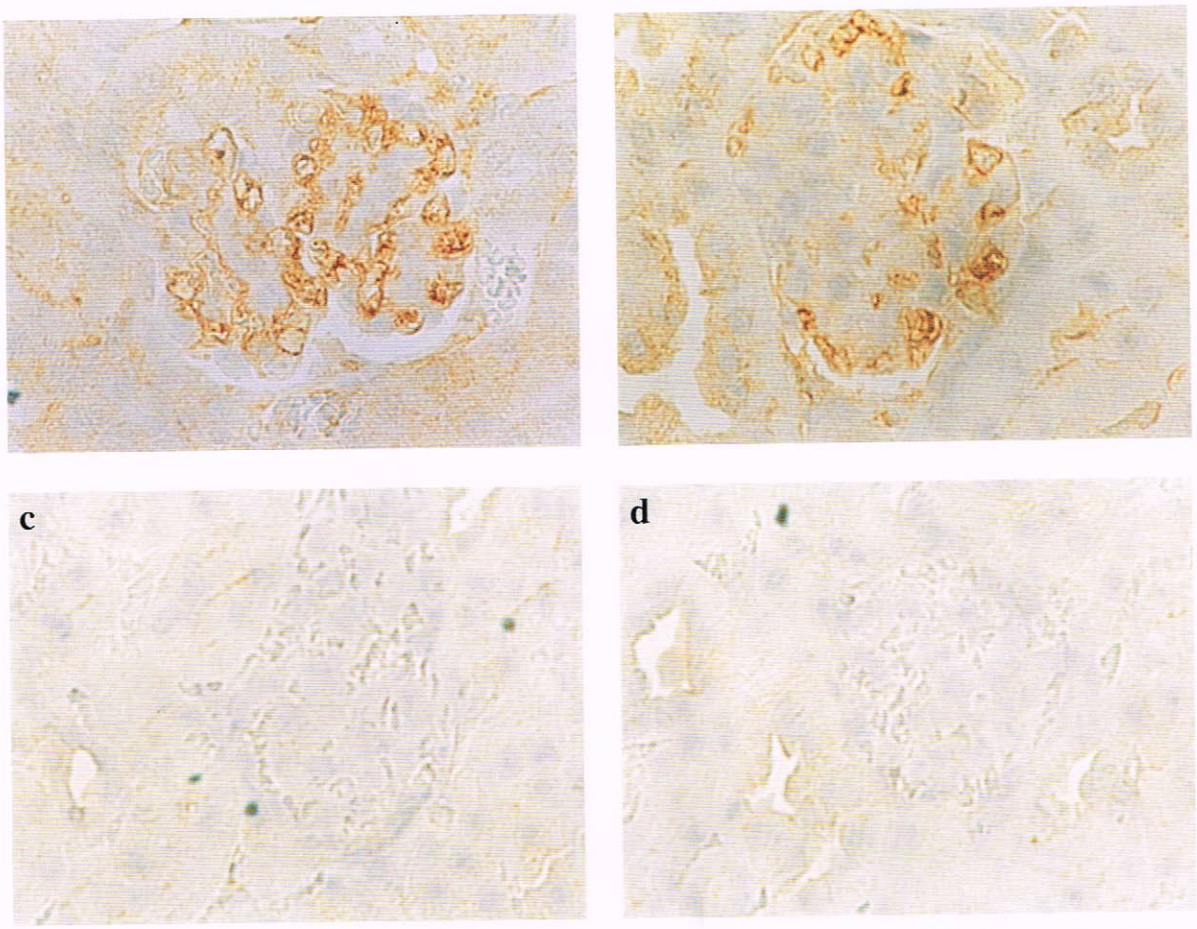

Fig. 5. *Expression of fibronectin in the kidney (×800). a) control, b) hydralazine, c) ACEI, d) wild. The expression of fibronectin was marked in renal glomeruli in the control group and hydralazine group, but almost no expression was detected in the ACEI group or wild group.*

matrix production, and thereby increases mesangial area (*28*), decreases the glomerular arteriole surface area available for filtration, and decreases ultrafiltration activity of the kidney. Ang II also contracts mesangial cells (*29*) and decreases ultrafiltration and the glomerular filtration rate of each nephron. Moreover, Ang II promotes the accumulation of type I and III collagens, which accelerate sclerosis in the mesangial matrix. Taken together with the fact that the local angiotensin concentration in glomeruli is about 10^3 times higher than that in peripheral blood (*30*), Ang II might promote glomerular sclerosis. In contrast, Johnson *et al.* (*31*) reported that long-term administration of Ang II elevated blood pressure and produced renal failure but did not induce significant changes in glomeruli. However, it is possible that the local angiotensin concentration in the glomeruli in their study was not high enough to cause any changes in glomeruli after the administration of Ang II in peripheral blood. When a human renin gene and a human angiotensinogen gene were administered to rats *via* the left renal artery, renin proteins were overexpressed in the glomeruli of the left kidney and a high local Ang II concentration in the glomeruli was produced; in addition, accumulation of ECM in the glomeruli and transformation of mesangial cells were observed (*32*).

ACEI protects the kidney by decreasing production of Ang II and increasing production of bradykinin. In various animal models used to study renal failure, ACEI has been shown to improve glomerular hypertension and glomerular hypertrophy, depress urinary protein excretion, and prevent the development of glomerular sclerosis (*33*). In a comparative study of the renal protective effects of various depressors, ACEI was reported to be most effective (*34*). In the present study, the administration of ACEI decreased water intake volume, urinary volume, and urinary albumin excretion volume, and prevented glomerular hypertrophy and glomerular sclerosis. Thus, the renal protective effect of ACEI was confirmed. The ability of ACEI to prevent glomerular failure has been attributed to correction of glomerular hypertension through the elimination of efferent arteriole contraction (*35*). Recently, ACEI has been found to have positive effects on the glomeruli themselves, so that ACEI may inhibit glomerular failure (*36*).

In the present study, administration of hydrala-

zine decreased urinary albumin excretion to the same level as that when ACEI was administered. Since proteinuria is caused by an increase in secondary non-selective pores due to glomerular hypertension (*37*), it can be inferred that proteinuria in the hydralazine group was quickly improved by correction of glomerular hypertension. However, it is unclear whether the proteinuria in the hydralazine group was improved. ACEI has been reported to increase the size-selectivity of glomerular basement membranes and to depress the permeability of proteins (*38*), whereas similar effects have not been reported for hydralazine.

Activation of fibronectin protein expression was observed in both the control and hydralazine groups. Glomerular sclerosis is a common lesion that is detected in various progressive glomerular diseases. It is caused by proliferation of mesangial cells and accumulation of ECM, primarily laminin, type IV collagen, and fibronectin (*39*). In our study, glomerular sclerosis was prevented in the ACEI group but not in the hydralazine group. A possible explanation for this is that the Ang II level was depressed by ACEI, inhibiting the increase in ECM, including fibronectin. Glomerular sclerosis in the ACEI group was prevented to the same extent as in the wild group. This indicates that Ang II strongly promotes glomerular sclerosis, and since the renal Ang II concentration in THM is about five times higher than that in C57BL/6 mice (*8*), the RAS locally generated in the kidney may have been an important factor for renal glomerular sclerosis. Moreover, the presence of differences in the glomerular sclerosis index among the ACEI, hydralazine, and control groups suggests that mechanical stress, such as that caused by increased blood pressure, plays an important role in the pathogenesis of glomerular sclerosis.

The findings of the present study indicate that RAS, especially locally generated RAS, activity is principally responsible for cardiac hypertrophy in THMs, while both the RAS and elevation of blood pressure contribute to the pathogenesis of renal glomerular sclerosis; lisinopril may suppress the tissue locally generated RAS to induce regression of cardiac hypertrophy and renal glomerular sclerosis.

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