Tissue Gene Expression of Renin-Angiotensin System in Human Type 2 Diabetic Nephropathy

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OBJECTIVE — Recent studies have proved that blockade of the renin-angiotensin system (RAS) retards the progression of diabetic nephropathy, whereas hyporeninemia is known as a typical state in diabetic subjects. The purpose of this study is to determine whether expression levels of RAS differ between nondiabetic and diabetic renal tissues with accurate quantitative method.

RESEARCH DESIGN AND METHODS — Subjects were 66 nondiabetic and 8 diabetic patients with biopsy-proven renal diseases. The eight diabetic subjects suffered from type 2 diabetes with overt proteinuria. Renal histology revealed typical diffuse or nodular lesions with linear IgG deposit on immunofluorescent staining and thickened basement membrane on electronic microscopy. Total RNA from a small part of the renal cortical biopsy specimens was reverse-transcribed, and the resultant cDNA was amplified for new major components of RAS (i.e., renin, renin receptor, angiotensinogen, ACE, ACE2, angiotensin II type 1 receptor, and angiotensin II type 2 receptor) and measured.

RESULTS — Among these components, a significant upregulation was observed in the ACE gene in diabetic renal tissue.

CONCLUSIONS — The results suggest that renal tissue RAS might be activated in the respect that ACE gene expression is upregulated in spite of a tendency to low renin expression in type 2 diabetic nephropathy.

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Recently proposed mechanisms for the development of diabetic nephropathy include glomerular hyperfiltration (1), disorientation of intracellular signal transduction (2), and involvement of advanced glycation end products (3). Activation of the renin-angiotensin system (RAS) by high glucose, mechanical stress, and proteinuria has been implicated in the major changes associated with diabetic nephropathy (4). Thus, renal tissue activation of RAS is thought to contribute to deterioration in renal function of diabetic nephropathy. Recently, a number of large-scale prospective studies have proven that blockade of the system with ACE inhibitors and angiotensin II receptor blockers (ARBs) retards the progression of diabetic nephropathy (5–11). Actually, several studies suggest that the RAS is activated especially at the early stage (12,13). However, from early studies, hyporeninemia has been well known as a typical state of circulatory RAS in diabetic subjects at the late stage (14,15). Although the tissue RAS is thought to be controlled independently of the circulatory RAS, this apparent paradox is still difficult to interpret. It is supposed that the tissue RAS is activated in contrast to the circulatory RAS, and several non- or semiquantitative evaluations were made. However, direct or quantitative evidence in human diabetic nephropathy is very scarce so far. Furthermore, new major components for RAS, renin receptor (RER) (16), and ACE2 (17) have emerged recently.

The purpose of this study is to determine whether expression levels of RAS including RER and ACE2 differ between nondiabetic and diabetic human renal tissues with full quantitative evaluation. For this sake, real-time PCR with a very small part of renal biopsy specimen was applied, making an accurate quantification of mRNA possible, in spite of the inability in similar protein evaluation because of the limitation of specimen quantity.

RESEARCH DESIGN AND METHODS

— Subjects were 66 nondiabetic and 8 diabetic patients with biopsy-proven renal diseases. The study was approved by the ethics committee of Fukui University (number 17-12), and consent was obtained from all individuals for inclusion onto the study. Salt intake was standardized to 10 g daily during hospitalization. The nondiabetic patients consisted of 8 with minor abnormalities, 8 benign nephrosclerosis, 38 primary glomerulonephritis including 4 minimal change nephrotic syndrome, and 12 lupus nephritis. Major clinical characteristics are listed in Table 1. Significant difference was observed in age, systolic blood pressure (sBP), and serum creatinine concentration between the two groups. The total patient numbers of administered depressors at renal biopsy were as follows: calcium channel block-

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Abbreviations: AGT, angiotensinogen; ARB, angiotensin II receptor blocker; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RAS, renin-angiotensin system; RER, renin receptor; sBP, systolic blood pressure.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical characteristics of subjects at renal biopsy*

	Nondiabetic subjects	Diabetic subjects
n	66	8
Sex (M/F)	29/37	6/2
Age (years)	35.4 ± 18.4	61.0 ± 13.1*
sBP (mmHg)	118 ± 20	153 ± 24*
dBP (mmHg)	70 ± 14	82 ± 10
Proteinuria (g/day)	1.36 ± 3.56	2.52 ± 3.22
Urinary sodium (mEq/gCr)	130.7 ± 79	124.1 ± 55.9
Serum creatinine concentration (mg/dl)	0.8 ± 0.5	1.5 ± 0.5*
Creatinine clearance (ml/min)	101 ± 54	82 ± 52
Plasma renin activity ($ng \cdot ml^{-1} \cdot h^{-1}$)	2.2 ± 2.4	0.8 ± 1.2
Plasma aldosterone concentration (pg/ml)	116.1 ± 58.6	96.4 ± 61.5

Data are means ± SD. dBP, diastolic blood pressure. *$P < 0.05$.

ers, five in nondiabetic subjects and four in diabetic subjects; α-blockers, zero in nondiabetic subjects and one in diabetic subjects; diuretics, eight in nondiabetic subjects and one in diabetic subjects; ACE inhibitors, one in nondiabetic subjects and zero in diabetic subjects; and ARBs, zero in nondiabetic subjects and zero in diabetic subjects. Administered ACE inhibitors and ARBs were replaced by calcium channel blockers or α-blockers before biopsy. Creatinine clearance (Ccr) was determined with serum creatinine concentration (s-Cr) and urinary creatinine concentration (u-Cr) and milliliters of daily urine volumes (UV) by a standard formula: Ccr = u-Cr × UV/s-Cr/1,440 (ml/min). Plasma renin activity of diabetic patients tended to be lower than that of the nondiabetic subjects ($P = 0.11$). The diabetic patients consisted of six men and two women suffering from type 2 diabetes with proteinuria, aged 32–74 years. Three of them were treated with oral administration of glibenclamide, and three other patients were treated with insulin injection. Glycosylated hemoglobin ranged from 4.0 to 8.7% at renal biopsy. Renal histology revealed typical diffuse or nodular lesions with linear IgG deposit on immunofluorescent staining and thickened basement membrane on electronic microscopy (Table 2).

Renal RNA was extracted from a small part of the renal cortex of the subjects (~2 mm) by echographic-guided percutaneous renal biopsy with an 18-G needle. Each specimen corresponds to a size and site presumed to contain ~20–30 glomeruli. Immediately after obtaining the biopsy specimen, total RNA was extracted using RNA-Bee (Tel-Test) according to the protocol recommended by the manufacturer. Single-strand cDNA was synthesized by a reverse-transcriptase reaction with 500 ng/μl Oligo-dT (Toyobo, Tokyo, Japan) and M-MLV reverse transcriptase (Toyobo). The resultant cDNA was amplified for renin, RER, angiotensinogen (AGT), ACE, ACE2, angiotensin II type 1 receptor (AT1), and angiotensin II type 2 receptor (AT2) as target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The sequences for primers were as follows: renin, 5′-GTGTCTGTGGGGTCATCCACCTTG-3′ (sense) and 5′-GGATTCCTGAAATA CATAGTCCGT-3′ (anti-sense); RER, 5′-TTCTCAGTTCACTCCCCCTCAA-3′ (sense) and 5′-TAACGCTTCCCAATT TCATCCA-3′ (antisense); AGT, 5′-CTGCAAGGATCTTATGACCTGC-3′ (sense) and 5′-TACACAGCAAACAG GAATGGGC-3′ (antisense); ACE, 5′-CCGAAATACGTGGAACTCATCAA-3′ (sense) and 5′-CACGAGTCCCCTG CATCTACA-3′ (antisense); ACE2, 5′-CATTGGAGCAAGTGTTGGATCTT-3′ (sense) and 5′-GAGCTAATGCATGC CATTCTCA-3′ (antisense); AT1, 5′-AGGGCAGTAAAGTTTTCGTG-3′ (sense) and 5′-CGGGCATTGTTTTG GCAGTG-3′ (antisense); AT2, 5′-GGCCTGTTTGTCCTCATTGC-3′ (sense) and 5′-CACGGGTTATCCTGT TCTTC-3′ (antisense); and GAPDH, 5′-CCCATCACCATCTTCCAGGAG-3′ (sense) and 5′-GTTGTCATGGATGAC CTTGGC-3′ (antisense). The real-time PCR took place with a final volume of 20 μl containing 0.5 mmol/l of forward and reverse primer and 2 μl single-strand cDNA template in 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen, Tokyo, Japan). With this method, six orders linearity was obtained (Fig. 1). Measurement of specific mRNA was carried out using the LightCycler system (Roche Diagnostics, Tokyo, Japan). Each sample was run and analyzed in duplicate. The quantification was absolutely performed using the samples of known concentration in each run. The mRNA levels were expressed as relative values to GAPDH mRNA.

Statistical analyses were performed with the use of SPSS Version 11.0J (SPSS Japan). All data are expressed as means ± SD. Data for clinical characteristics were evaluated by ANOVA. Differences of gene expressions were calculated by ANCOVA with three covariance (age, sBP, and serum creatinine) for all genes and additionally with four covariance (age, sBP, serum creatinine, and proteinuria) for ACE, since ACE upregulation in the rat

Table 2—*Clinical characteristics of diabetic subjects at renal biopsy*

Case	Sex	Age (years)	Type of diabetes	Duration of diabetes (years)	Treatment	A1C (%)	Renal histology
1	F	70	Type 2	7	Glibenclamide	7.3	Nodular
2	M	67	Type 2	23	Glibenclamide	8.2	Nodular
3	F	74	Type 2	19	Insulin	5.1	Nodular
4	M	32	Type 2	6	Diet therapy only	8.7	Nodular
5	M	64	Type 2	26	Insulin	7.2	Nodular
6	M	59	Type 2	25	Glibenclamide	7.4	Nodular
7	M	61	Type 2	6	Insulin	4.0	Nodular
8	M	55	Type 2	2	Diet therapy only	6.3	Diffuse

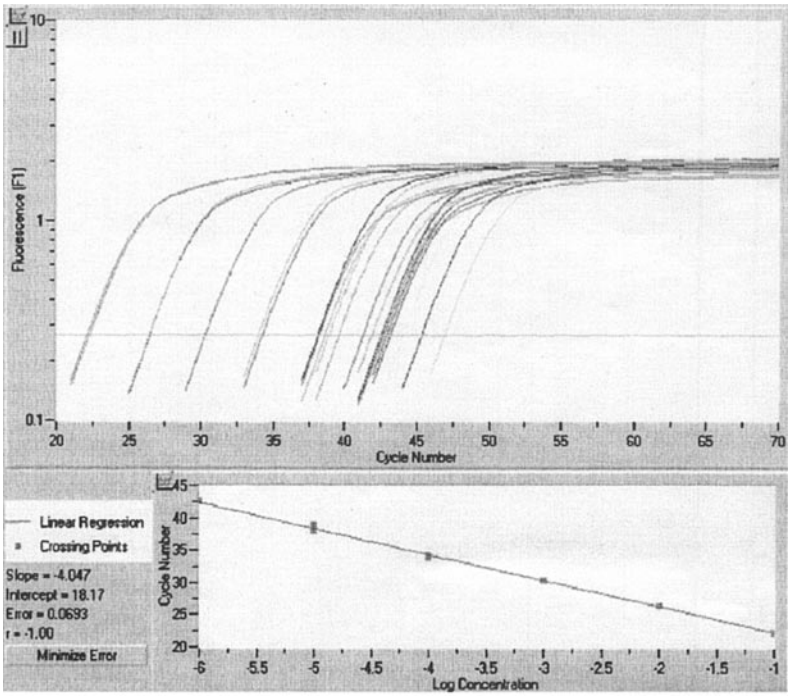

Figure 1—*Measurement of renal mRNA by the real-time PCR method. One example is demonstrated for measurement of renal mRNA by using the LightCycler system. Six orders linearity was obtained as shown.*

kidney with intense proteinuria was reported (18).

RESULTS — All the results are shown in Table 3.

Renal tissue renin mRNA of nondiabetic and diabetic subjects

Renin expression was measured at 10^{-3} order to GAPDH expression. No difference was observed between the expression levels of nondiabetic subjects (0.89 ± 2.12) and diabetic subjects (0.60 ± 0.56) ($P = 0.85$).

Renal tissue RER mRNA of nondiabetic and diabetic subjects

RER expression was measured at 10^{-3} order to GAPDH expression. No difference was observed between the expression levels of nondiabetic subjects (2.32 ± 2.53) and diabetic subjects (2.07 ± 2.42) ($P = 0.49$).

Renal tissue AGT mRNA of nondiabetic and diabetic subjects

AGT expression was measured at 10^{-2} order to GAPDH expression. AGT expression of nondiabetic subjects (6.00 ± 10.7) tended to be higher than that of diabetic subjects (2.82 ± 2.57) with no statistical significance ($P = 0.27$).

Renal tissue ACE mRNA of nondiabetic and diabetic subjects

ACE expression was measured at 10^{-3} order to GAPDH expression. A significant difference was observed between ACE expression of nondiabetic subjects (2.66 ± 5.44) and diabetic subjects (8.98 ± 14.7) ($P = 0.026$).

Renal tissue ACE2 mRNA of nondiabetic and diabetic subjects

ACE2 expression was measured at 10^{-2} order to GAPDH expression. No difference was observed between the expression levels of nondiabetic subjects (1.94 ± 2.83) and diabetic subjects (2.99 ± 2.36) ($P = 0.75$).

Renal tissue AT1 mRNA of nondiabetic and diabetic subjects

AT1 expression was measured at 10^{-2} order to GAPDH expression. AT1 expression of nondiabetic subjects (3.54 ± 4.03) tended to be higher than that of diabetic subjects (2.50 ± 2.11) with no statistical significance ($P = 0.08$).

Renal tissue AT2 mRNA of nondiabetic and diabetic subjects

AT2 expression was measured at 10^{-4} order to GAPDH expression. No difference was observed between the expression levels of nondiabetic subjects (2.75 ± 4.12) and diabetic subjects (2.50 ± 3.42) ($P = 0.34$).

Table 3—*Renal tissue mRNA levels of RAS*

	Nondiabetic subjects	Diabetic subjects
Gene		
REN (10^{-3})	0.89 ± 2.12	0.60 ± 0.56
RER (10^{-3})	2.32 ± 2.53	2.07 ± 2.42
AGT (10^{-2})	6.00 ± 10.7	2.82 ± 2.57
ACE (10^{-3})	2.66 ± 5.44	8.98 ± 14.7*
ACE2 (10^{-2})	1.94 ± 2.83	2.99 ± 2.36
AT1 (10^{-2})	3.54 ± 4.03	2.50 ± 2.11
AT2 (10^{-4})	2.75 ± 4.12	2.50 ± 3.42

Data are means ± SD. *$P < 0.05$.

CONCLUSIONS — The results of the study suggest the upregulation of the ACE gene in renal tissue of human diabetic nephropathy. For animal models, a considerable number of data have been accumulated, especially for the streptozotocin diabetes model. First, renal tissue angiotensin II concentration has been variously reported to be increased (19,20), to be comparable (21), and to be decreased (22) compared with nondiabetic kidney. With respect to the gene expressions of RAS in the animal model kidney, renin expression is reportedly increased at the beginning of the disease (19,23,24) but decreased at the late stage (20,23). Renal tissue AGT expression was reported to be comparable (20,23,25). Renal tissue ACE was reported to be comparable (20,23,25) and to be decreased (26). Renal tissue ACE2 was reported to be decreased (26). With regard to receptors, it was reported that nonglycosylated AT1 receptor protein expression was increased in isolated glomeruli in streptozotocin-induced diabetic rats with no change in mRNA (27), while reduced expression of the AT1 receptor in diabetic spontaneously hypertensive rats and no such reduction in AT1 expression was observed

in diabetic Wistar Kyoto rats (28). Because the streptozotocin-induced diabetic animal is a model of type 1 diabetes, it is possible that the expression of genes differ from that in type 2 diabetes.

Compared with animal data, only a small number of studies have been conducted about the expression of renal tissue RAS on human specimens. At first, elevated angiotensin II immunohistostaining was observed in tubular and infiltrating cells in diabetic human kidney (29). With regard to ACE, the immunostain was elevated in tubular cells and appeared in interstitial cells (29). Another immunohistochemical study indicated that ACE staining was significantly enhanced in glomeruli in diabetic patients (30). The former study also reported a downregulation of AT1 and upregulation of AT2 receptors (29). These assessments were based on non- or semiquantitative histochemical methods, making precise comparisons difficult. Only one quantitative assay was made for AT1 expression with competitive RT-PCR method, and the authors reported that AT1 receptor mRNA levels were significantly lower in eight samples from patients with diabetic nephropathy (31).

As described above, systematic quantitative assessment of gene expression of RAS in human diabetic nephropathy has not been performed. Therefore, we examined this issue for the first time and revealed the upregulation of the ACE gene in renal tissue of human diabetic nephropathy among the classic and new major components of RAS. The previous reports of semiquantitative immunohistological study on ACE were in accordance with our study (29,30). Before concluding the diabetes-specific upregulation of ACE, we should exclude the effect of proteinuria in our set because ACE upregulation in the rat kidney with intense proteinuria was reported (18). First, no correlation was found between the amount of proteinuria and the ACE expression ($n = 74$, $P = 0.91$, $r = 0.01$). Prevalence of the subjects with nephrotic range proteinuria was not different between the two groups (10/66 in nondiabetic subjects and 2/8 in diabetic subjects, $P = 0.48$, $\chi^2 = 0.51$). Lastly, the difference of ACE gene expressions was calculated by ANCOVA with four covariance (age, sBP, serum creatinine, and proteinuria). And a significant difference was confirmed between ACE expressions ($P = 0.028$).

Because the effects or biases of age, blood pressure, sodium intake, renal function, and proteinuria were almost excluded, the explanation for mechanism of the upregulation is unknown. One remaining possibility is the effect of hyperglycemia itself. A nonsignificant tendency for correlation was found between HbA_{1c} and the ACE expression among diabetic subjects ($n = 8$, $P = 0.24$, $r = 0.47$), and a significant correlation was observed among subjects, including a limited number of nondiabetic subjects ($n = 29$, $P = 0.03$, $r = 0.41$). A glucose response element was located on the AGT gene promoter (32), but no similar element has been recognized on the ACE gene so far. Accordingly, it is uncertain if the effect of hyperglycemia on renal ACE expression might be direct or indirect.

Thus, these results indicate the upregulation of the ACE gene in renal tissue of human diabetic nephropathy (i.e., in spite of the hyporeninemic state of the circulatory system, tissue RAS is activated). Accordingly, ACE inhibitors and ARBs might counteract this activation, thereby contributing to the favorable effects described in large-scale prospective studies (5–11). Alternatively, in the view of personal oriented medicine, our assessment might provide a new therapeutic approach based on renal tissue gene expression on renal diseases.

In summary, the gene expression of RAS, i.e., renin, renin receptor, AGT, ACE, ACE2, AT1, and AT2, was assayed with a very small quantity of human renal tissues of nondiabetic and diabetic subjects by quantitative methods. The results suggest that renal tissue RAS might be activated in the respect that ACE gene expression is upregulated in spite of a tendency to low renin expression in type 2 diabetic nephropathy. Further investigations including assessment of disease stage and severity might provide further insight into the role of RAS in human diabetic nephropathy.

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