Tissue gene expression of renin-angiotensin system in human type 2 diabetic nephropathy

Short running title: Tissue RAS in human diabetic nephropathy

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Abstract

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 non-diabetic and diabetic renal tissues with accurate quantitative method.

Research Design and Methods Subjects were 66 non-diabetic and 8 diabetic patients with biopsy-proven renal diseases. The 8 diabetic subjects suffered from type 2 diabetes mellitus with overt proteinuria. Renal histology revealed typical diffuse or nodular lesions with linear IgG deposit on immuno-fluorescent 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, angiotensin converting enzyme, angiotensin converting enzyme 2, angiotensin II type 1 receptor, angiotensin II type 2 receptor and measured.

Results Among these components, a significant up-regulation was observed in angiotensin converting enzyme gene in diabetic renal tissue.

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

Key words: Angiotensin-Converting Enzyme, ACE, Angiotensinogen, Renin-Angiotensin-Aldosterone System, RAAS , Nephropathy

Recently proposed mechanisms for the development of diabetic nephropathy (DN) include glomerular hyperfiltration (1), disorientation of intracellular signal transduction (2) and involvement of advanced glycation endproducts (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 DN. Recently a number of large-scale prospective studies have proven that blockade of the system with angiotensin converting enzyme (ACEI) and angiotensin II receptor blocker (ARB) retards the progression of DN (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 semi-quantitative 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 angiotensin converting enzyme 2 (ACE2) (17) have emerged recently.

The purpose of this study is to determine whether expression levels of RAS

including RER and ACE2 differ between non-diabetic 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 specimens' quantity.

Materials and Methods

Subjects were 66 non-diabetic and 8 diabetic patients with biopsy-proven renal diseases. The study was approved at the ethic committee of Fukui University (No.17-12) and consent was obtained from all individuals for inclusion onto the study. Salt-intake was standardized to 10 g daily in hospitalization. The non-diabetic 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 were listed in table 1. Significant difference was observed in age, systolic blood pressure (SBP) and serum creatinine concentration (s-Cr) between 2 groups. The total patients' number of administered depressors at renal biopsy were as follows: calcium channel blocker (CCB); 5 in non-diabetics and 4 in diabetics, α-blocker; 0 in non-diabetics and 1 in diabetics, diuretics; 8 in non-diabetics and 1 in diabetics, ACEI; 1 in non-diabetics and 0 in diabetics, ARB; 0 in non-diabetics and 0 in diabetics. Administered ACEI and ARB were replaced by CCB or α-blocker prior to biopsy. Creatinine clearance (Ccr) was determined with s-Cr and urinary creatinine concentration (u-Cr) and ml of daily urine volumes (UV) by a standard formula, Ccr = u-Cr x UV/s-Cr/1440 (ml/min.). Plasma renin activity (PRA) of diabetic patients tended to be lower than that of the non diabetic subjects (p=0.11). The diabetic patients consisted of 6 males and 2 females suffering from type 2 diabetes mellitus with proteinuria,

aged from 32 to 74 years. Three of them were treated with oral administration of glibenclamide and 3 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 immuno-fluorescent 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 (about 2 mm) by echographic-guided percutaneous renal biopsy with 18G needle. Each specimen corresponds to a size and site presumed to contain about 20-30 glomeruli. Immediately after obtaining the biopsy specimen, total RNA was extracted using RNA-Bee (TEL-TEST, INC., USA) 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 Inc., Japan) and M-MLV reverse transcriptase (TOYOBO CO. LTD., Japan). The resultant cDNA was amplified for renin (REN), RER, angiotensinogen (AGT), angiotensin converting enzyme (ACE), ACE2, angiotensin II type 1 receptor (AT1), angiotensin II type 2 receptor (AT2) as target genes and GAPDH as a house-keeping gene. The sequences for primers were as follows; REN: 5'-GTGTCTGTGGGGTCATCCACCTTG-3' (sense) and 5'-GGATTCCTGAAATACATAGTCCGT-3' (anti-sense); RER: 5'-TTCTCAGTTCACTCCCCCTCAA-3' (sense) and 5'-

TAACGCTTCCCAATTTCATCCA-3’ (anti-sense); angiotensinogen: 5’-CTGCAAGGATCTTATGACCTGC-3’ (sense) and 5’-TACACAGCAAACAGGAATGGGC-3’ (anti-sense); ACE: 5’-CCGAAATACGTGGAACTCATCAA-3’ (sense) and 5’-CACGAGTCCCCTGCATCTACA-3’ (anti-sense); ACE2: 5’-CATTGGAGCAAGTGTTGGATCTT-3’ (sense) and 5’-GAGCTAATGCATGCCATTCTCA-3’ (anti-sense); AT1: 5’-AGGGCAGTAAAGTTTTCGTG-3’ (sense) and 5’-CGGGCATTGTTTTGGCAGTG-3’ (anti-sense); AT2: 5’-GGCCTGTTTGTCCTCATTGC-3’ (sense) and 5’-CACGGGTTATCCTGTTCTTC-3’ (anti-sense); GAPDH: 5’-CCCATCACCATCTTCCAGGAG-3’ (sense) and 5’-GTTGTCATGGATGACCTTGGC-3’ (anti-sense). The real time PCR reaction took place with a final volume of 20μl containing 0.5 mM of forward and reverse primer, and 2μl of single strand cDNA template in 2xQuantiTect SYBR Green PCR Master Mix (QIAGEN Inc., Japan). With this method, 6 orders linearity was obtained (Figure 1). Measurement of specific mRNA was carried out using the LightCycler system (Roche Diagnostics Inc., 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, Inc., Japan). All data are expressed as mean ± standard deviation. Data for clinical characteristics were evaluated by ANOVA. Differences of gene expressions were calculated by analysis of covariance (ANCOVA) with 4 covariance (age, SBP and serum creatinine) for all genes and additionally with 4 covariance (age, SBP, serum creatinine and proteinuria) for ACE as ACE up-regulation in the rat kidney with intense proteinuria was reported (18).

Results

All the results were shown in table 3.

Renal tissue REN mRNA of non-diabetic and diabetic subjects

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

Renal tissue RER mRNA of non-diabetic and diabetic subjects

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

Renal tissue AGT mRNA of non-diabetic and diabetic subjects

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

Renal tissue ACE mRNA of non-diabetic and diabetic subjects

ACE expression was measured at 10^{-3} order to GAPDH expression. A significant difference was observed between ACE expression of non-diabetic

subjects (2.66±5.44) and diabetics (8.98±14.7) (p=0.026).

Renal tissue ACE2 mRNA of non-diabetic and diabetic subjects

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

Renal tissue AT1 mRNA of non-diabetic and diabetic subjects

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

Renal tissue AT2 mRNA of non-diabetic and diabetic subjects

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

Discussion

The results of the study suggest the up-regulation 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 streptozotosin (STZ) DM model. First, renal tissue angiotensin II (Ang II) concentration has been variously reported to be increased (19; 20), to be comparable (21) and to be decreased (22) compared to non-diabetic kidney. About the gene expressions of RAS in animal model kidney, renin expression has been reported to be increased at the beginning of the disease (19; 23; 24) but decreased at the late stage (20; 23). Renal tissue AGT expression has been reported to be comparable (20; 23; 25). Renal tissue ACE has been reported to be comparable (20; 23; 25) and to be decreased (26). Renal tissue ACE2 has been reported to be decreased (26). About receptors, it was reported that nonglycosylated AT1 receptor protein expression was increased in isolated glomeruli in STZ 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 were observed in diabetic Wistar Kyoto rats (28). Since STZ diabetic animal is a model of insulin dependent diabetes mellitus, it is possible that the expression of genes differ from that of non-insulin dependent diabetes mellitus.

Compared to 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). About 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 down-regulation of AT1 and up-regulation of AT2 receptors (29). These assessments were based on non- or semi-quantitative 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 8 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 up-regulation of the ACE gene in renal tissue of human diabetic nephropathy among the classical and new major components of RAS. The previous reports of semi-quantitative immunohistological study on ACE were in good accordance with our study (29; 30). Before concluding the diabetes-specific up-regulation of ACE, we should well exclude the effect of proteinuria in our set as ACE up-regulation in the rat kidney with intense proteinuria was reported (18). First, no correlation was found between 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 two groups (10/66 in non-diabetics and 2/8 in diabetics, p=0.48, χ^2=0.51). At last, the difference of ACE gene expressions was calculated by ANCOVA with 4 covariance (age, SBP, serum creatinine and proteinuria). And a significant difference was confirmed between ACE expressions (p=0.028).

Thus, as the effects or biases of age, blood pressure, sodium intake, renal function and proteinuria were almost excluded, the explanation for mechanism of the up-regulation is unknown. Remained one possibility is the effect of hyperglycemia, itself. A tendency to correlation was found between HbA1c and the ACE expression among diabetics (n=8, p=0.24, r=0.47) and a significant correlation was observed among subjects including limited number of non-diabetics (n=29, p=0.03, r=0.41). A glucose response element has been located on AGT gene promoter (32) but no similar element has been recognized on ACE gene so far. Accordingly, it is not sure the effect of hyperglycemia on renal ACE expression might be direct or indirect.

Thus, these results indicate the up-regulation of the ACE gene in renal tissue of human diabetic nephropathy i.e. that in spite of the hypo-reninemic state of the circulatory system, tissue RAS is activated. Accordingly ACEI and ARB 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 non-diabetic and diabetic subjects by quantitative methods. The results suggest that renal tissue RAS might be activated in the respect that ACE gene expression is up-regulated 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 insights into the roles of RAS in human diabetic nephropathy.

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Conflict-of-Interest Disclosure Statement

None

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Figure legend

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

Table 1. Clinical characteristics of subjects at renal biopsy

Characteristics	Non diabetic	Diabetic
Number	66	8
Gender (Male/Female)	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
u-Na (mEq/gCr)	130.7±79	124.1±55.9
s-Cr (mg/dl)	0.8±0.5	1.5±0.5*
Ccr (ml/min)	101±54	82±52
PRA (ng/ml/hr)	2.2±2.4	0.8±1.2
PAC (pg/ml)	116.1±58.6	96.4±61.5

SBP: systolic blood pressure, DBP: diastolic blood pressure, u-Na: urinary sodium, s-Cr: serum creatinine concentration, Ccr: Creatinine clearance, PRA: plasma renin activity, PAC: plasma aldosterone concentration. *$p<0.05$

Table 2. Clinical characteristics of diabetic subjects at renal biopsy

Case	Gender	Age (years)	Type of DM	Duration of DM (years)	Treatment	HbA1c (%)	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	-	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	-	6.3	diffuse

DM: diabetes mellitus, HbA1c: glycosylated hemoglobin A1c, F: female; M: male, -:diet therapy only

Table 3. Renal tissue mRNA levels of RAS

Gene	Non diabetic	Diabetic
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

Values are expressed as means ± S.D. REN: renin, RER: renin receptor, AGT: angiotensinogen, ACE: angiotensin converting enzyme, ACE2: angiotensin converting enzyme 2, AT1: angiotensin II type 1 receptor, AT2: angiotensin II type 2 receptor. *$p<0.05$