Relations Between ACE Gene and ecNOS Gene Polymorphisms and Resistive Index in Type 2 Diabetic Patients With Nephropathy

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OBJECTIVE — ACE and endothelial cell nitric oxide synthase (ecNOS) genotypes have been reported to be related to the incidence of renal diseases and coronary artery diseases. In order to assess the effect of the gene polymorphism of both ACE and ecNOS on renal hemodynamic abnormality, we examined 155 Japanese patients with type 2 diabetes with various stages of nephropathy.

RESEARCH DESIGN AND METHODS — The patients ranged in age from 40 to 72 years (92 men and 63 women). They were divided into four groups: group 1 consisted of patients with urinary albumin excretion (UAE) <30 mg/day ($n = 69$), group 2 had 30 ≤ UAE < 300 mg/day ($n = 44$), group 3 had UAE ≥300 mg/day and serum creatinine <1.5 mg/dl ($n = 22$), and group 4 had serum creatinine >1.5 mg/dl ($n = 20$). Intrarenal hemodynamics were studied by duplex Doppler sonography in patients with type 2 diabetes. The ACE and ecNOS gene polymorphisms were examined by polymerase chain reaction.

RESULTS — There were no significant differences in sex, BMI, and blood glucose level, but there were differences in HbA_{1c} and lipoprotein profiles among the four groups. There were no significant differences in the distribution of ACE genotype or in the frequency of the ecNOS 4a allele among the four groups. Resistive index (RI) values of the interlobar arteries of group 4 were significantly higher than those of groups 1, 2, and 3, whereas the RI values were not significantly different among groups 1, 2, and 3. Multiple regression analysis showed that age, duration of diabetes, systolic and diastolic blood pressure, and creatinine clearance were significantly associated with the increased RI values, but that there was no significant association between RI values and the ecNOS genotype ($R^2 = 0.613$, $P < 0.0001$).

CONCLUSIONS — These results suggest that intrarenal hemodynamic abnormalities are present as a feature of the progression of nephropathy in type 2 diabetes, and that they are associated with age, duration of diabetes, decreased creatinine clearance, and blood pressure, but not with the genetic factors of the ACE and ecNOS gene polymorphism in nephropathy of type 2 diabetes.

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Several studies have demonstrated that diabetic nephropathy occurs in familial clusters, suggesting that a genetic factor(s) may be involved (1). ACE insertion/deletion (I/D) polymorphism of intron 16 was reported to be a genetic marker for coronary artery disease in the general population (2). Accordingly, the I/D polymorphism has been investigated as a strong candidate marker for genetic predisposition to diabetic vascular complications (3,4). However, studies of the association of the ACE genotype with diabetic complications, such as nephropathy, have yielded conflicting results (5–7).

It is now established that endothelial-derived nitric oxide (NO) plays a major role in vascular regulation in normal and disease states (8). NO is synthesized by endothelial cell NO synthase (ecNOS) in the vascular endothelium, and it plays a key role in the regulation of blood flow and pressure. Bank and Aynedjian (9) and Sugimoto et al. (10) showed that NO synthesis, presumably by the vascular endothelium, is increased in animal models of diabetes, and that hemodynamic changes in the kidney caused by overproduction of NO may play a significant role in hyperfiltration. Recently, there have been some reports about an association between the ecNOS gene and essential hypertension (11,12) as well as a positive association between the 4a polymorphism and smoking-dependent risk of coronary artery diseases (13). Morita et al. (14) reported that the ecNOS 4b/a genotype (or ecNOS4a allele) of the ecNOS gene polymorphism may be involved in the progression of IgA nephropathy. In diabetic patients, there have been reports of the association between the 4a allele and diabetic nephropathy (15–17). However, these results are controversial.

Duplex Doppler sonography is a useful method for detecting intrarenal hemodynamic abnormalities, such as those seen in obstructive renal diseases, renal allograft rejection, renovascular hypertension, and diabetic nephropathy (18–20). We also reported that the DD genotype of ACE genes was associated with increased RI in type 1 diabetes (21). We have reported that endothelium-dependent vascular responses of the retinal and intrarenal arteries were impaired

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Abbreviations: ecNOS, endothelial cell nitric oxide synthase; GFR, glomerular filtration rate; PCR, polymerase chain reaction; RI, resistive index; UAE, urinary albumin excretion.
A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

in type 2 diabetic patients with early nephropathy (22).

Our objective in the present study was to assess the effect of ACE and/or ecNOS polymorphisms on the resistive index (RI) of intrarenal arteries, the hemodynamic changes of which are deranged in the progression of nephropathy in type 2 diabetes.

RESEARCH DESIGN AND METHODS

— A total of 155 Japanese patients with type 2 diabetes (age 40–72 years) were enrolled. All type 2 diabetic patients in this study were admitted to Osaka City University Hospital for treatment of diabetes or patient education programs. The diagnosis of type 2 diabetes was established according to the Report of the Expert Committee on Diagnosis and Classification of Diabetes Mellitus (23). Patients fulfilled three additional criteria for inclusion: no episodes of ketoacidosis, no ketonuria, and insulin therapy (if any) initiated after at least 5 years of known disease. After admission to our diabetes ward, medical examinations were performed to exclude other renal diseases. To exclude patients with nondiabetic or obstructive kidney diseases, the patients with microscopic or macroscopic hematuria, abnormal urinary sediment, or a past history of glomerulonephritis or nephro-ureterolithiasis, dilated renal pelvis, or severe atrophied kidney(s) (either uni- or bilateral on real-time ultrasonography) were also excluded from this study. The study design was approved by the hospital committee on ethics. Each subject gave informed consent before entering the study.

For each diabetic patient, 24-h urine collections were performed on 3 consecutive days to determine the level of urinary albumin excretion (UAE) and creatinine clearance. In each patient, the level of 24-h UAE was the mean value from 3 consecutive days. Creatinine clearance was calculated from 24-h urine samples and serum creatinine levels. We used the creatinine clearance corrected per 1.48 m^2 of body surface, representing the mean Japanese body surface area, as the glomerular filtration rate (GFR). The patients were divided into four groups: group 1 consisted of patients with UAE $<$30 mg/day (n = 69), group 2 had 30 $\leq$ UAE $<$ 300 (n = 44), group 3 had UAE $\geq$300 and serum creatinine $<$1.5 mg/dl (n = 22), and group 4 had serum creatinine $>$1.5 mg/dl (n = 20). All of the group 4 patients had UAE $>$300 mg/day.

Blood pressure was recorded three times after a subject had rested in the supine position for at least 15 min. A standard mercury sphygmomanometer with a cuff that adapted to arm circumference was used. The systolic blood pressure was taken as the point of first audibility of Korotokoff sounds, and the diastolic blood pressure was taken as the point of their disappearance. The three measurements were averaged.

Information on smoking habits was obtained by a self-administered questionnaire. Lifelong exposure to smoking was estimated as the product of years of smoking and the number of cigarettes smoked daily at the time of the study (cigarette-years).

Biochemical analysis

Blood was drawn after an overnight fast for the analysis of serum concentrations of glucose, total cholesterol, triglycerides, HDL cholesterol, HbA_{1c}, and serum creatinine by standard laboratory methods. Plasma levels of glucose were measured by the glucose oxidase method, and HbA_{1c} was measured by high-performance liquid chromatography (Hi-Auto A1c; Sekisui Chemical, Osaka, Japan). Serum and urine creatinines were measured using an autoanalyzer. The level of urinary albumin was measured in 24-h urine collections by immunoturbidimetry (TIA MicroAlb Kit; Nittobo, Tokyo, Japan). The UAE rate was expressed in milligrams per 24 h. Patients were considered dyslipidemic if they were taking antihyperlipidemic agents and/or if serum cholesterol was $>$5.69 mmol/l (220 mg/dl), HDL cholesterol was $<$1.03 mmol/l (40 mg/dl), or triglycerides were $>$1.70 mmol/l (150 mg/dl), according to the criteria of the Japan Atherosclerosis Society.

The presence and grade of retinopathy were determined using stereoscopic color fundus photographs and fluorescein angiography. Grading was performed by an experienced ophthalmologist according to the classification of Davis (24).

ecNOS and ACE gene polymorphism

The DNA fragment of the ecNOS gene containing 27-bp repeats in intron 4 was amplified by the polymerase chain reaction (PCR) method using template DNA from peripheral leukocytes, as previously reported (13). The PCR products were separated by electrophoresis in 2% agarose gels. We identified two alleles as ecNOS 4a (with 27-bp repeats, 393 bp total) and ecNOS 4b (with five tandem repeats, 420 bp total).

The ACE I/D polymorphism was determined by PCR amplification of the ACE gene (25). We confirmed the accuracy of the genotyping results by using an insertion-specific primer pair (5′CTGGAGACCACTCCCA TCCTTTCT 3′ and 5′TCGAGACCATCCGGCTAAAAC3′) to avoid mistyping ID as DD. With these primers, the I allele produced a 290-bp product, whereas the D allele was not amplified; therefore, the DD genotype produced no products.

RI of interlobar arteries

The peak systolic flow velocity, the end-diastolic flow velocity, and the time-averaged flow velocity were automatically calculated using the ultrasound apparatus. Flow velocities were determined from signals that were stable for at least five pulse beats, and measurements represented the average of five complete waveforms. The resistance parameter, RI, was determined as follows (18,19): RI = (PSV − EDV), where PSV is peak systolic flow velocity, and EDV is end-diastolic flow velocity.

Three different interlobar arteries from each kidney were randomly selected and examined, and the mean value from the two kidneys was calculated. The coefficient of variance for RI was 3.6% for the patients with type 2 diabetes (18).

Statistical analysis

Data are expressed as means ± SD. Values for clinical parameters were compared using a one-way analysis of variance with Scheffe's F test. Univariate χ^2 analyses were used to evaluate the incidence of retinopathy and therapy in type 2 diabetes among the four groups. Multiple regression analysis was performed to evaluate the possible association between ACE gene and/or ecNOS gene polymorphisms and RI of renal arteries in nephropathy in patients with type 2 diabetes. Multiple regression analyses were performed in each model of a 4-model system to clarify whether the ACE genotypes and/or ecNOS polymorphism may be independent affecting factors for the increased RI values in patients with type 2 diabetes. The following independent variables were included in the models: age, BMI,

Table 1—*Clinical characteristics of patients with type 2 diabetes*

	Group 1	Group 2	Group 3	Group 4
Age (years)	60.1 ± 9.8	60.5 ± 8.5	59.0 ± 10.5	64.2 ± 7.8
Sex (male/female)	41/28	26/18	15/7	10/10
BMI (kg/m^2)	23.1 ± 2.6	23.2 ± 2.5	22.9 ± 2.5	23.9 ± 2.1
Cigarette-years	277.3 ± 375.0	352.8 ± 426.6	277.7 ± 296.5	556.8 ± 623.3
Duration of diabetes (years)	7.4 ± 4.5	10.9 ± 7.4	12.8 ± 6.5	19.1 ± 9.7
FPG (mmol/l)	9.84 ± 3.74*	10.22 ± 3.30*	9.61 ± 3.11	7.35 ± 2.43
HbA_{1c}(%)	8.9 ± 2.6*	8.8 ± 1.7*	8.5 ± 2.2	7.0 ± 1.4
TC (mmol/l)	5.43 ± 0.97	5.22 ± 0.93	5.50 ± 1.73	5.96 ± 2.49
TG (mmol/l)	1.55 ± 1.10	1.25 ± 0.30	1.09 ± 0.32	1.13 ± 0.24
HDL-C (mmol/l)	1.30 ± 0.47	1.25 ± 0.30	1.09 ± 0.32	1.13 ± 0.24
sBP (mmHg)	130.8 ± 19.3*	134.6 ± 20.0*	141.6 ± 17.5*	158.6 ± 17.4
dBP (mmHg)	73.1 ± 11.5†	73.5 ± 9.0†	81.8 ± 9.6	77.9 ± 13.5
Creatinine (μmol/l)	58.70 ± 14.76*	56.05 ± 14.41*	75.14 ± 20.04*	281.55 ± 180.91
Creatinine clearance (ml · min · 1.48 m^2)	89.9 ± 31.0*	86.7 ± 27.8*	79.5 ± 19.2*	25.9 ± 19.4
Diabetic retinopathy (NDR/SDR/PPDR/PDR)	19/23/14/13*	11/15/11/7*	2/1/10/9*	0/7/8/5
Therapy (diet/SU/insulin)	22/35/11*	9/26/9*	7/9/6*	8/0/12
ACE genes (II/ID/DD)	31/26/12	16/19/9	8/12/2	8/9/3
ecNOS (with 4a/without 4a)	19/50	12/32	5/17	6/14

Data are means ± SEM or *n*. TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; sBP, systolic blood pressure; dBP, diastolic blood pressure; NDR, no diabetic retinopathy; SDR, simple diabetic retinopathy; PPDR, pre-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; SU, sulfonylurea; NDR, non-diabetic retinopathy; SDR, simple diabetic retinopathy; PPDR, preproliferative diabetic retinopathy; SU, sulfonylurea. *$P < 0.005$ vs. group 4, †$P < 0.05$ vs. group 3.

duration of diabetes, cigarette-smoking, HbA_{1c}, total and HDL cholesterol, triglycerides, systolic and diastolic blood pressure, and RI. As categorical variables, the following were included: sex (female = 0, male = 1), ACE genotypes (II = 0, ID or DD = 1), and ecNOS polymorphism (with 4a allele = 1, without 4a allele = 0). All statistical analyses were carried out with StatView V software on a Macintosh computer. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Clinical characteristics

Table 1 shows the clinical characteristics of the four groups. There were no significant differences in age, BMI, or sex among the four groups. Fasting plasma glucose, HbA_{1c}, systolic blood pressure, and creatinine clearance were higher in patients of groups 1, 2, and 3 than in those of group 4. Duration of diabetes was longer in patients of group 4 than in those of groups 1, 2, and 3. There were significant differences in the therapy, prevalence of diabetic retinopathy, prevalence of hypertension, and hyperlipidemia among the four groups. There were, however, no significant differences in the distribution of ACE genotypes or ecNOS polymorphism among the four groups by the χ^2 test.

The clinical characteristics of the patients according to the ACE gene polymorphism are shown in Table 2. We found 26 patients with D/D, 66 with I/D, and 63 with I/I. The genotype distributions of the ACE genes were consistent

Table 2—*Clinical characteristics of patients with type 2 diabetes by ACE gene polymorphisms*

	II	ID	DD
n	63 (41)	66 (42)	26 (17)
Age (years)	60.4 ± 8.7	59.8 ± 10.3	61.0 ± 9.5
Sex (male/female)	35/28	44/22	13/13
BMI (kg/m^2)	22.9 ± 2.6	23.3 ± 2.4	23.7 ± 2.7
Cigarette-years	380.1 ± 411.3	291.1 ± 356.3	336.5 ± 597.2
Duration of diabetes (years)	9.7 ± 6.9	11.6 ± 7.9	10.8 ± 7.9
FPG (mmol/l)	9.40 ± 3.58	9.76 ± 3.43	9.65 ± 3.43
HbA_{1c} (%)	8.8 ± 2.3	8.4 ± 2.2	8.7 ± 2.5
TC (mmol/l)	5.34 ± 0.90	5.39 ± 1.10	5.54 ± 1.24
TG (mmol/l)	1.46 ± 0.83	1.63 ± 0.96	1.54 ± 1.14
HDL-C (mmol/l)	1.26 ± 0.39	1.21 ± 0.37	1.23 ± 0.44
sBP (mmHg)	135.5 ± 22.3	137.4 ± 19.0	139.9 ± 21.9
dBP (mmHg)	74.3 ± 10.5	75.9 ± 11.0	74.7 ± 13.5
Creatinine (μmol/l)	79.38 ± 69.13	95.47 ± 89.73	95.91 ± 107.41
Creatinine clearance (ml/min/1.48 m^2)	80.2 ± 32.4	78.4 ± 35.9	79.0 ± 35.7
Normoalbuminuria	31 (49)	26 (39)	12 (46)
Microalbuminuria	16 (25)	19 (29)	9 (35)
Overt proteinuria	8 (13)	12 (18)	2 (8)
Chronic renal failure	8 (13)	9 (14)	3 (11)
Hypertension	34 (54)	32 (48)	14 (54)
Hyperlipidemia	25 (40)	35 (53)	10 (38)

Data are *n* (%), *n*, or means ± SEM. FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; sBP, systolic blood pressure; dBP, diastolic blood pressure.

Table 3—*Clinical characteristics of patients with type 2 diabetes by ecNOS gene polymorphisms*

	ecNOS 4b/b	ecNOS 4b/a	ecNOS 4a/a
n	113 (73)	40 (26)	2 (1)
Age (years)	59.5 ± 12.0	59.0 ± 8.6	60.7 ± 9.8
Sex (male/female)	35/28	44/22	1/1
BMI (kg/m^2)	23.3 ± 2.6	23.0 ± 2.5	24.2 ± 1.6
Cigarette-years	338.1 ± 417.6	297.6 ± 394.6	900.0 ± 1272.8
Duration of diabetes (years)	10.4 ± 7.0	11.6 ± 8.8	9.5 ± 12.0
FPG (mmol/l)	9.51 ± 3.16	10.02 ± 4.24	5.97 ± 0.00
HbA_{1c}	8.6 ± 2.2	8.9 ± 2.2	5.5 ± 2.5
TC (mmol/l)	5.33 ± 1.05	5.57 ± 1.04	5.74 ± 1.10
TG (mmol/l)	1.53 ± 0.91	1.61 ± 1.06	1.37 ± 0.04
HDL-C (mmol/l)	1.27 ± 0.18	1.28 ± 0.36	1.27 ± 0.18
sBP (mmHg)	137.5 ± 19.8	136.6 ± 23.6	117.0 ± 9.9
dBP (mmHg)	75.5 ± 9.7	74.1 ± 14.9	68.0 ± 2.8
Creatinine (μmol/l)	82.48 ± 83.98	94.15 ± 91.32	57.46 ± 6.28
Creatinine clearance (ml/min/1.48 m^2)	76.64 ± 33.62	86.45 ± 35.93	83.00 ± 32.53
Normoalbuminuria	50 (44)	19 (48)	0 (0)
Microalbuminuria	32 (28)	10 (25)	2 (100)
Overt proteinuria	17 (15)	5 (12)	0 (0)
Chronic renal failure	14 (12)	6 (15)	0 (0)
Hypertension	57 (50)	23 (58)	0 (0)
Hyperlipidemia	47 (42)	22 (55)	1 (50)

Data are *n* (%) or means ± SEM. FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; sBP, systolic blood pressure; dBP, diastolic blood pressure.

with other published reports about Japanese subjects (7,25). The three groups were well matched with regard to sex, age, and BMI. There were no significant intergroup differences in the prevalence of retinopathy, hypertension, hyperlipidemia, fasting plasma glucose, total and HDL cholesterol, triglycerides, GFR, systolic and diastolic blood pressure, or the prevalence of the stages of diabetic nephropathy. Of the patients studied, 33 had Ca+ antagonist treatment, 25 had ACE inhibitors, 12 had combined Ca+ antagonist and ACE inhibitor treatment , and 10 had β-blockers and/or diuretic agents.

Table 3 shows the clinical characteristics according to the ecNOS gene polymorphism. The distribution of ecNOS genotype was representative for the Japanese population (12). There were no significant differences between the groups in terms of age, sex, BMI, duration of diabetes, metabolic control, or the prevalence of the stages of diabetic nephropathy.

RI of intrarenal arteries in diabetic subjects

In the diabetic patients, the RI values (means ± SEM) were 0.704 ± 0.045, 0.726 ± 0.064, 0.738 ± 0.039, and 0.842 ± 0.048 in groups 1, 2, 3, and 4, respectively. The RI values in group 4 patients were significantly higher than those in group 1, 2, and 3 patients ($P < 0.0001$), whereas there were no significant differences among the RI values in groups 1, 2, and 3 ($P = 0.1603$ between groups 1 and 2, $P = 0.0572$ between groups 1 and 3, and $P = 0.8434$ between groups 2 and 3) (Fig. 1 and 2).

There were no significant differences in RI values according to ACE gene polymorphism in group 1, 2, 3, or 4. The RI values in group 1 were 0.707 ± 0.048, 0.698 ± 0.046, and 0.707 ± 0.036 for the II, ID, and DD genotypes, respectively ($P = 0.724$). Those in group 2 had values of 0.736 ± 0.058, 0.723 ± 0.066, and 0.716 ± 0.072 for the II, ID, and DD genotype, respectively ($P = 0.7270$). Those in group 3 had values of 0.736 ± 0.058, 0.723 ± 0.066, and 0.716 ± 0.072 for the II, ID, and DD genotype, respectively ($P = 0.7270$). Those in group 4 had values of 0.841 ± 0.056, 0.835 ± 0.045, and 0.869 ± 0.041 for the II, ID, and DD

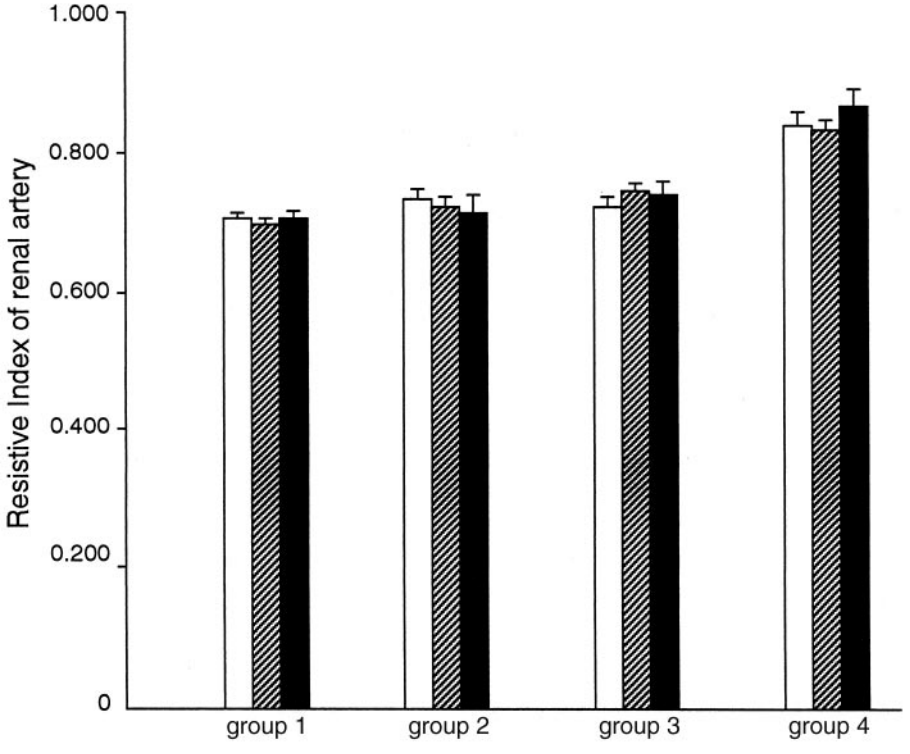

Figure 1—*RI of interlobar arteries according to ACE genotype in each group of patients with diabetic nephropathy. □, RI values of patients with the II genotype; ▨, those with the ID genotype; ■ those with the DD genotype. There were no significant differences in RI according to the ACE I/D genotype in groups 1, 2, 3, or 4.*

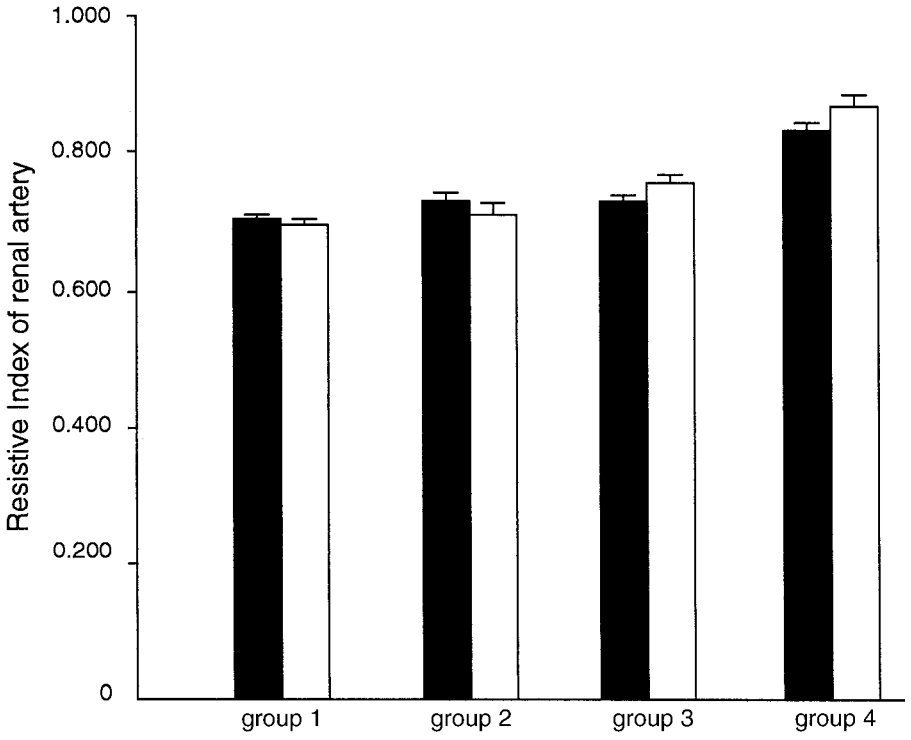

Figure 2—*RI of interlobar arteries according to the ecNOS genotype (with or without the 4a allele) in each group of patients with diabetic nephropathy.* □*, RI values of patients with the 4a allele;* ■*, those without the 4a allele. There were no significant differences in RI between genotypes with and without the 4a allele of the ecNOS gene in groups 1, 2, 3, or 4.*

genotype, respectively ($P = 0.5888$) (Fig. 1).

There were no significant differences in the RI values between those patients with and without the 4a allele of the ecNOS gene in groups 1, 2, 3, or 4. The RI values in group 1 were 0.698 ± 0.041 and 0.706 ± 0.046 in patients with and without 4a, respectively ($P = 0.5468$). RI values in group 2 were 0.711 ± 0.062 and 0.732 ± 0.064 in patients with and without 4a, respectively ($P = 0.3313$). RI values in group 3 were 0.757 ± 0.029 and 0.732 ± 0.041 in patients with and without 4a, respectively ($P = 0.2173$). RI values in group 4 were 0.867 ± 0.042 and 0.832 ± 0.048 in patients with and without 4a, respectively ($P = 0.1367$) (Fig. 2).

Correlation between the RI of intrarenal arteries and clinical parameters

The relationship between RI values and creatinine clearance in diabetic patients showed a negative correlation coefficient of $r = -0.698$ ($P < 0.001$). The relationship between RI values and age in patients showed a positive correlation coefficient of $r = 0.458$ ($P < 0.001$). There was also a significant correlation between creatinine clearance and age in diabetic patients ($r = -0.781$, $P < 0.0001$).

Factors associated with RI of intrarenal artery in diabetic patients

Table 4 summarizes the results of multiple regression analyses examining the possible risk factors that independently affect the RI of intrarenal arteries in diabetic nephropathy. In models 1–4, age, sex, BMI, duration of diabetes, cigarette-years, HbA_{1c}, total cholesterol, triglycerides, HDL cholesterol, systolic or diastolic blood pressure, and creatinine clearance were included as independent variables.

Table 4—*Independent factor(s) related to RI of intrarenal artery in multiple regression analyses*

	Model 1	Model 2	Model 3	Model 4
Age (years)	−0.182 (0.0452)*	−0.190 (0.0361)*	−0.188 (0.0391)*	−0.195 (0.0310)*
Sex (male/female)	0.033 (0.5551)	0.035 (0.5361)	0.037 (0.5129)	0.038 (0.4970)
BMI (kg/m^2)	−0.072 (0.1869)	−0.075 (0.1764)	−0.070 (0.1971)	−0.072 (0.1895)
Cigarette-years	0.051 (0.3664)	0.053 (0.3566)	0.050 (0.3719)	0.047 (0.4009)
Duration of diabetes (years)	0.147 (0.0140)*	0.150 (0.0127)*	0.136 (0.0232)*	0.141 (0.0203)*
HbA_{1c} (%)	0.067 (0.2642)	0.070 (0.2445)	0.071 (0.2281)	0.074 (0.2206)
TC (mmol/l)	0.087 (0.1755)	0.095 (0.1484)	0.085 (0.1867)	0.089 (0.1799)
TG (mmol/l)	−0.075 (0.2419)	−0.082 (0.2158)	−0.079 (0.2162)	−0.083 (0.2063)
HDL-C (mmol/l)	−0.084 (0.1639)	−0.088 (0.1460)	−0.090 (0.1346)	−0.093 (0.1255)
sBP (mmHg)	0.203 (0.0020)*	0.201 (0.0022)*	0.201 (0.0021)*	0.200 (0.0023)*
dBP (mmHg)	−0.211 (0.0006)*	−0.201 (0.0013)*	−0.207 (0.0008)*	−0.197 (0.0015)*
Creatinine clearance	−0.757 (0.0001)*	−0.759 (0.0001)*	−0.775 (0.0001)*	−0.776 (0.0001)*
ACE genes	—	−0.030 (0.5810)	—	−0.067 (0.2177)
ecNOS genes	—	—	0.070 (0.1930)	−0.031 (0.5638)
R^2	0.607	0.609	0.612	0.613

Data are β (*P* value). Model 1 through model 4 consisted of all patients. Parameters from model 1 to model 4 includes age, sex, cigarette-years, duration of diabetes, HbA_{1c} total cholesterol, triglyceride, HDL cholesterol, systolic and diastolic blood pressure, and creatinine clearance. In model 2, D allele of, ACE gene (D– = 0, D+ = 1) was added to the parameters of the model 1; in model 3, ecNOS gene (with 4a = 1, without 4a = 0) was added; and in model 4, both the ACE genes and ecNOS were added. R^2, multiple coefficient of determination; β, standard correlation. In each model, all patients were included.

In model 1, age, duration of diabetes, systolic and diastolic blood pressure, and creatinine clearance were significant important factors for the RI values in type 2 diabetic patients ($R^2 = 0.607$). When the effect of the ecNOS gene polymorphism, the polymorphism of the D alleles of the ACE gene, and both of the gene polymorphisms combined were included as independent variables in models 2, 3, and 4, respectively, there was no significant association between the RI value and either of the two gene polymorphisms in any model.

CONCLUSIONS — In the present study using duplex Doppler sonography, we demonstrated that the intrarenal hemodynamic abnormalities were affected by age, duration of diabetes, blood pressure, and creatinine clearance in patients with type 2 diabetes with nephropathy, as defined by the presence of increased albuminuria and renal insufficiency. However, we demonstrated no significant association between the ACE gene polymorphism and RI or between the ecNOS gene polymorphism in intron 4 and RI in patients with type 2 diabetes.

Changes in the RI have been observed in diabetic nephropathy, usually at an advanced stage (18,19). In this disease, RI changes generally follow changes in serum creatinine and creatinine clearance (18,19). The RI does not appear to become significantly elevated in the early course of diabetic nephropathy, inasmuch as many patients with normal RI values have proteinuria and clinical renal disease for years. This may be because the renal damage at an early stage is located primarily in the glomeruli, in which case a normal RI would be expected (26), and because in advanced diabetic nephropathy, glomeruli become sclerotic, tubules become atrophic, and interstitial fibrosis is increased. Increased interstitial fibrosis may have caused elevated RI values in advanced diabetic nephropathy in group 4 in the present study, because elevated RI values have been reported in tubulointerstitial diseases (27). Among patients with nonobstructive (medical) diseases, those with vasculitis/vasculopathy were reported to have extremely high RI values (27). In the present study, the increased RI values in advanced diabetic nephropathy may have been caused by intrarenal vascular abnormalities, i.e., arteriosclerosis.

Recent observations have indicated that the genetic predisposition to hypertension is a candidate factor for the emergence and progression of diabetic nephropathy (1). The D allele has been suggested by some, but not by all studies, to predispose people to several common cardiovascular and renal diseases (2), especially people with diabetes (6). Our previous reports (21) demonstrated that the increased RI values in superficial renal arcuate arteries were significantly increased in type 1 diabetic patients with the DD genotype, but that there was no significant association between ACE I/D gene polymorphism and the RI values in the interlobar arteries. Miller et al. (28) reported that recently diagnosed young type 1 diabetic patients with ACE II genotypes displayed higher GFR and renal plasma flow than those patients with the ID or DD genotype. The results of the present study examining type 2 diabetic patients, however, were somewhat different from those of the previous study examining type 1 diabetic patients. This may be because of the differences in age, the duration of diabetes, the type of diabetes, and the size of the artery examined. We found no significant association between the ACE gene I/D polymorphism and RI values in the interlobar arteries of those type 2 diabetic patients with relatively longer duration of type 2 diabetes and higher age.

The reduced production of NO by ecNOS in experimental animals is reported to be an important risk factor in the progression of glomerular damage via systemic or intraglomerular hypertension (29,30). However, in diabetic animals, excessive production of NO and increased expression of the ecNOS gene are associated with hemodynamic abnormalities (hyperfiltration) and are thus considered to increase the risk of the progression of diabetic nephropathy. It was reported that abnormal renal hemodynamics in a diabetic animal model was associated with the increased expression of ecNOS (9,10). Evidence for a possible relation of ecNOS gene polymorphism and impaired function of ecNOS was revealed by a recent study of NO metabolite (Nox) levels in patients with different genotypes of the ecNOS gene (31). Recently, there have been reports about the contribution of ecNOS gene polymorphisms to diabetic nephropathy in type 1 and type 2 diabetes (15–17). However, these results are controversial because the subjects may be different. Neugebauer et al. (15) found an association between the ecNOS gene polymorphism and the progression of diabetic nephropathy in Japanese type 2 diabetic patients. In our study, there was a lack of relation between the ecNOS gene polymorphism and RI indexes in Japanese type 2 diabetic patients. The discrepancy between the former study and our study may be attributable to the classification of diabetic nephropathy, because the creatinine threshold in our study was lower than that used in the previous study.

There were no significant differences in RI values according to the ACE gene or ecNOS gene polymorphism at any stage of diabetic nephropathy (Figs. 1 and 2). In the present study, we performed multiple regression analyses to determine the combined impact on RI of such clinical variables as age, sex, BMI, cigarette-years, duration of diabetes, HbA_{1c}, total cholesterol, triglycerides, HDL cholesterol, systolic and diastolic blood pressure, creatinine clearance, and ACE gene and ecNOS gene polymorphisms. The analyses revealed that age, creatinine clearance, duration of diabetes, and systolic and diastolic blood pressure significantly and independently contributed to the RI values in patients with type 2 diabetes. The positive and negative correlation coefficients of systolic and diastolic pressure, respectively, may have been noted because RI is calculated from the difference between the intrarenal systolic and diastolic velocity of blood flow, which may be related to systolic and diastolic blood pressure, respectively. In model 1, neither ACE nor ecNOS gene polymorphisms were included. In models 2–4, either ecNOS or ACE gene polymorphisms or both were included. However, neither ecNOS nor ACE gene polymorphism, nor both, were significant independent risk factors for RI in any model. Furthermore, in models 1–4, R^2 (multiple coefficient of determination) was similar from 0.607 to 0.613. These variables jointly accounted for 60.7% in model 1, 60.9% in model 2, 61.2% in model 3, and 61.3% in model 4. This result clearly ruled out the contribution of ecNOS and/or ACE gene polymorphism on RI, because ecNOS and ACE gene polymorphism only added an explanatory power of 0.6%.

In a previous report (19), we demonstrated that the increased RI values at the advanced stage of diabetic nephropathy

were probably caused by the advanced arteriosclerosis in intrarenal arteries. This was shown by significant associations between RI values in intrarenal arteries and intima-medial thickness of the carotid and femoral arteries in patients with type 2 diabetes. The results of the present study were consistent with our previous report on type 2 diabetic patients (18).

In the present study examining different stages of diabetic nephropathy, we found that there were no significant differences in the distribution of ecNOS gene polymorphism according to diabetic nephropathy, and that the RI values were not affected by ecNOS gene polymorphism at any stage of diabetic nephropathy. The absence of association between RI and ACE gene and/or ecNOS gene polymorphisms may be explained as follows. *1*) The correlation between the RI values and the risk factors for macroangiopathy (e.g., age, duration of diabetes, and systolic and diastolic blood pressure) strongly suggests that these factors are much more responsible for the increased RI values than genetic factors, such as ACE and ecNOS gene polymorphisms. *2*) The RI values may be affected not only by glomerular capillary hypertension and glomerular sclerosis, but also by tubulointerstitial lesions that may not be associated with genetic factors. *3*) There was a relatively small number of patients with overt-proteinuria and chronic renal failure. *4*) There was a relatively small number of patients with the ecNOS 4a allele and/or the DD genotype of the ACE gene. *5*) The pharmacological treatments may have interfered with the genetic factors under investigation. 6) Quantitative criteria for structural parameters were adopted. These reasons may have lead to the present results showing that the RI value was affected by age, duration of diabetes, and systolic and diastolic blood pressure rather than genetic factors such as ACE and/or ecNOS gene polymorphisms.

In summary, we demonstrated that intrarenal hemodynamic abnormalities, as assessed by duplex Doppler sonography, were primarily affected by the GFR and the risk factors associated with advanced arteriosclerosis in patients with type 2 diabetes. Neither ACE nor ecNOS gene polymorphisms significantly affected intrarenal hemodynamic abnormalities.

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