Relationships Between Angiotensin I Converting Enzyme Gene Polymorphism, Plasma Levels, and Diabetic Retinal and Renal Complications

MICHEL MARRE, PAULINE BERNADET, YVES GALLOIS, FREDERIQUE SAVAGNER, THAM-TAM GUYENE, MAGID HALLAB, FRANÇOIS CAMBIEN, PHILIPPE PASSA, AND FRANÇOIS ALHENC-GELAS

Insulin-dependent diabetes mellitus (IDDM), cardiovascular morbidity, and vital prognosis are linked to diabetic nephropathy, which is probably determined by renal hemodynamic abnormalities and by a genetic predisposition. Angiotensin I converting enzyme (ACE) regulates systemic and renal circulations through angiotensin II formation and kinins metabolism. Plasma and cellular ACE levels are genetically determined; an insertion/deletion polymorphism of the ACE gene is strongly associated with ACE levels, subjects homozygote for insertion (genotype II) having the lowest plasma values. We studied the relationship between the ACE gene polymorphism or plasma levels and microcirculatory disorders of IDDM through two independent studies: one involved 57 subjects with or without diabetic retinopathy, and the other compared 62 IDDM subjects with diabetic nephropathy to 62 diabetic control subjects with the same characteristics (including retinopathy severity) but with normal kidney function. The ACE genotype distribution was not different in diabetic subjects with or without retinopathy and in a healthy population. Conversely, an imbalance of ACE genotype distribution, with a low proportion of II subjects, was observed in IDDM subjects with diabetic nephropathy compared with their control subjects ($P = 0.006$). Plasma ACE levels were mildly elevated in all diabetic groups, independently of retinopathy, but they were higher in subjects with nephropathy than in those without nephropathy ($P = 0.0022$). The II genotype of ACE gene is a marker for reduced risk for diabetic nephropathy. *Diabetes* 43:384–88, 1994

From the Diabetes Unit (M.M., M.H.), Medical Department B, University Hospital, Angers; Biochemistry Laboratory (M.M., Y.G., R.S.), Faculty of Medicine, Angers; INSERM U367 (P.B., T-T.G., F.A-G.) and INSERM SC7 (F.C.), Paris; and Diabetes Department (P.P), Hospital Saint-Louis, Paris, France.

Address correspondence and reprint requests to Dr. Michel Marre, Unité de Diabétologie, Service de Médecine B, Centre Hospitalier Universitaire, 49033 Angers, Cédex 01, France.

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IDDM, insulin-dependent diabetes mellitus; ACE, angiotensin I converting enzyme; I/D, insertion/deletion; DD, homozygote for deletion; II, homozygote for insertion; ID, heterozygote for insertion/deletion; UAE, urinary albumin excretion; GFR, glomerular filtration rate; ANOVA, analysis of variance; BMI, body mass index; WHO, World Health Organization; CI, confidence interval.

In insulin-dependent diabetes mellitus (IDDM) subjects, vital prognosis is affected chiefly by diabetic nephropathy (1), which is associated with high cardiovascular mortality (1,2). However, this complication occurs in <50% of the patients, whereas the risk of diabetic retinopathy, another manifestation of the diabetic microcirculatory disorder, is present in nearly all of them (2). There is probably a genetic basis for diabetic nephropathy (2), and hemodynamic factors play a major role in its development (3). Angiotensin I converting enzyme (ACE) (kininase II), an endothelial ectoenzyme secreted in plasma, has a key role in regulating systemic and renal circulations, activating angiotensin I into the vasoconstrictor peptide angiotensin II, and inactivating the vasodilatory peptide bradykinin (4). Although its intraindividual plasma activity is very stable, its interindividual variability in plasma or cellular levels is quite high and under the influence of a genetic polymorphism (5,6). A large part of the variance of plasma ACE levels is associated with an insertion/deletion (I/D) polymorphism of the ACE gene (7). Subjects homozygote for the deletion (DD) display the highest values and those homozygote for the insertion (II) display the lowest, with heterozygotes displaying intermediate values. An excess of the DD genotype was recently observed in subjects with myocardial infarction compared with unaffected subjects in the general population, which suggests that a genetic polymorphism affecting ACE gene expression could be associated with susceptibility to vascular disease (8).

Reports indicated that plasma ACE activity was elevated in some diabetic subjects (9). We reported recently that serum ACE activity is especially elevated in IDDM subjects with microalbuminuria, i.e., those with incipient

diabetic nephropathy (10). The relationship between ACE gene polymorphism, plasma concentration, and renal or retinal diabetic complications was further assessed by two independent studies in IDDM subjects: one involved subjects with or without severe retinopathy and the other was a case-control study of subjects with diabetic nephropathy. In the subjects, we observed a low proportion of II genotype of ACE in IDDM subjects with diabetic nephropathy compared with those without diabetic nephropathy.

RESEARCH DESIGN AND METHODS

The two studies were conducted according to the Helsinki Declaration's principles, and all participating subjects gave their oral consent after appropriate information concerning study objectives.

Study 1. White patients with IDDM (World Health Organization [WHO] criteria) for >5 years, all born in France (male or female), 18–70 years of age, and attending the diabetic outpatient clinic at Hôpital Saint-Louis, Paris, France were selected consecutively if they had either no diabetic retinopathy or a proliferative one. Exclusion criteria were bronchopulmonary, hepatic, or renal disease (plasma creatinine >500 mM) or the occurrence of stroke or myocardial infarction during the preceding six months. No patient had taken ACE inhibitors or diuretics during the 2 weeks before blood sampling. A total of 57 subjects were recruited over 3 months.

Study 2. A case-control study was conducted independently of study 1 in the Diabetes Unit of Angers Hospital, Angers, France on IDDM subjects with incipient or established diabetic nephropathy and their control subjects. Demographic selection criteria were identical to those of study 1. Patients with urinary tract infection or hematuria were excluded. Incipient diabetic nephropathy was defined by persistent microalbuminuria (urinary albumin excretion [UAE] between 30 and 300 mg/24 h, 2 or 3 times, over 6 months) (11) in the absence of permanent hypertension (WHO criteria) (37 subjects) and established diabetic nephropathy by persistent macroalbuminuria (UAE >300 mg/24 h) associated with preproliferative or proliferative diabetic retinopathy (25 subjects). Subjects with microalbuminuria were matched with normotensive, normoalbuminuric (UAE <30 mg/24 h) IDDM control subjects of the same sex, age (±3 years of age), diabetes duration (±3 years), and diabetic retinopathy severity. Subjects with established diabetic nephropathy were matched with normoalbuminuric, normotensive IDDM subjects with preproliferative or proliferative diabetic retinopathy. All but four subjects on ACE inhibitors stopped their treatment two weeks before blood sampling; all subjects with established diabetic nephropathy continued other hypotensive drugs because these subjects were all hypertensives.

Determinations. Diabetic retinopathy was classified by independent ophthalmologists as zero, background, preproliferative, or proliferative. Blood pressure was measured in the supine position with a mercury sphygmomanometer in study 1 and with an automatic device (Dinamap, Critikon, Tampa, FL) in study 2. UAE was measured by nephelometry on 24-h urine samples. HbA_{1c} levels were determined by high-performance liquid chromatography. In patients with incipient nephropathy and in their control subjects in study 2, plasma prorenin was calculated by subtracting active from total renin, both determined by immunoradiometry (12). Glomerular filtration rate (GFR) was determined in patients in study 2 by plasma disappearance of single shot ^{51}Cr-EDTA.

Plasma ACE levels and activities were determined, respectively, by direct radioimmunoassay (13) in all patients of study 1 and in 47 patients and 51 control subjects of study 2 and by quantifying hippuric acid hydrolyzed from Hip-His-Leu (6) in patients in study 1.

The ACE genotypes were determined by Southern blotting or by polymerase chain reaction amplification of the region of the insertion (14). Subjects were classified, according to presence or absence of a 287 bp insertion in intron 16 of the ACE gene, as II, DD, or heterozygotes for insertion/deletion (ID).

Statistical analysis. Results are given as means ± SD, or median (ranges). Nonparametric tests were used for two group comparisons (Mann-Whitney test), analysis of variance (ANOVA) (Kruskall-Wallis test), and correlation coefficients (Spearman's rank test). Distribution of ACE genotype according to diabetic complications was assessed using the χ^2 test. Odds ratios of diabetic nephropathy associated with elevated plasma ACE were calculated after adjustment for ACE (I/D) genotypes by the Maentel-Haenzel test.

RESULTS

Study 1. The 57 IDDM subjects consisted of 22 women and 35 men, 44 ± 12 years of age with 18 ± 10 years diabetes duration, and 23.6 ± 2.7 kg/m^2 body mass index (BMI). Mean arterial pressure (diastolic plus one third pulse pressure) was 97 ± 11 mmHg and HbA_{1c} 8.2 ± 1.7%. Proliferative diabetic retinopathy was present in 18 subjects, microalbuminuria in 8 subjects, and macroalbuminuria in 3 subjects. Plasma ACE immunoreactivity was 475 ± 168 μg/L, mildly but significantly elevated compared with the levels found in two independent reference populations studied previously in our laboratory using the same technique: 412 ± 100 μg/L (n = 80, P < 0.01) (7) and 425 ± 119 μg/L (n = 434; P < 0.02) (5). Plasma ACE activity was also elevated; 42 ± 11 vs. 37 ± 7 μmol Hip-His-Leu · min^{-1} · L^{-1} (n = 300, P < 0.01) (5). Plasma ACE immunoreactivity and activity were highly significantly correlated in these diabetic subjects, as was the case in normal subjects (5): r' = 0.86 (P < 0.001). The II, ID, and DD genotypes were present in 15, 22, and 20 subjects, respectively; the population was in Hardy-Weinberg equilibrium, and the frequencies were not different from a control population (7). Plasma immunoreactive ACE levels were related to genotype. The levels in II, ID, and DD were 333 ± 69, 480 ± 118, and 577 ± 194 μg/L, respectively (P < 0.05, ANOVA). Within the three genotypes, ACE levels were elevated compared with a reference population (7). No relationship was found between plasma ACE immunore-

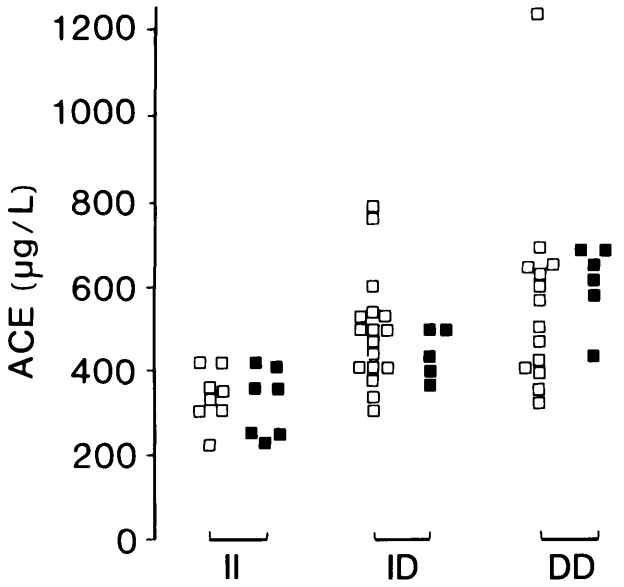

FIG. 1. Individual plasma ACE levels according to ACE genotype in study 1. Fifty-seven IDDM subjects with proliferative diabetic retinopathy (■) or without retinopathy (□). Subjects groups are indicated: II, ID, and DD. See RESULTS for analysis.

activity and age ($r' = 0.001$), diabetes duration ($r' = 0.06$), BMI ($r' = 0.05$), mean arterial pressure ($r' = 0.02$), or HbA_{1c} ($r' = 0.04$). Plasma ACE immunoreactivity was not elevated in subjects with diabetic retinopathy compared with those without diabetic retinopathy (Fig. 1).

Study 2. Table 1 gives the clinical characteristics of 62 IDDM cases with incipient ($n = 37$) or established ($n = 25$) diabetic nephropathy and of their 62 control subjects. The ACE genotype distribution was different in IDDM cases than in control subjects: χ^2 test, 7.53 ($P = 0.0232$). The II genotype prevalence was lower in IDDM cases than in control subjects: 4 IDDM cases versus 15 control subjects, χ^2 test, 7.52 ($P = 0.006$), odds ratio 0.216 (95% confidence interval [CI] 0.067–0.695) (Table 2). The genotype distribution fitted with the Hardy-Weinberg equilibrium in control subjects and was not different from the genotype distribution observed in study 1 or in a healthy population (7). Conversely, a moderate deviation from Hardy-Weinberg equilibrium was observed for genotype distribution in cases with a higher D allele frequency in IDDM cases (0.65) than in control subjects (0.53; χ^2test, 3.76 ($P = 0.0525$).

TABLE 2
ACE genotypes of 62 IDDM subjects with nephropathy (cases) and of 62 IDDM normoalbuminuric, normotensive control subjects

Genotype	IDDM cases	IDDM control subjects
DD	23	19
ID	35	28
II	4	15

χ^2 test (7.53; $P = 0.0232$) comparing DD, ID, and II genotypes. χ^2 test comparing DD + ID and II genotypes (7.52; $P = 0.006$).

As indicated in Fig. 2, plasma ACE immunoreactivity was higher in IDDM cases than in IDDM control subjects: 549 ± 148 μg/L ($n = 47$) vs. 461 ± 130 μg/L ($n = 51$) ($P = 0.0022$) and higher than in a reference population (7) in both instances ($P < 0.001$). Plasma ACE immunoreactivities were not different between the cases with incipient or established nephropathy: 540 ± 122 vs. 566 ± 188 μg/L. Plasma ACE immunoreactivities were related to genotype in IDDM cases ($P = 0.047$) and in IDDM control subjects ($P = 0.0146$). To analyze whether plasma ACE immunoreactivity was higher in IDDM cases than in IDDM control subjects independently of ACE genotypes, the odds ratio (Maentel-Haenzel estimate) of diabetic nephropathy associated with plasma ACE ≥ 461 μg/L (the mean value of IDDM control group) was calculated after adjustment for ACE genotype: 2.02 (95% CI, 0.824–4.949, $P = 0.124$).

The ACE genotype distribution or plasma levels were not related to diabetic retinopathy. Among 32 subjects without retinopathy, 12 were DD homozygotes, 17 were ID, and 3 were II homozygotes, whereas in 52 subjects

TABLE 1
Clinical and biological characteristics of IDDM subjects in study 2

	Cases with nephropathy	Control subjects without nephropathy	Significance
Sex (M/F)	37/25	30/32	NS
Age (years)	39 ± 14	43 ± 18	NS
Diabetes duration (years)	20 ± 11	22 ± 12	NS
Retinopathy			
Zero	16	16	NS
Background	11	11	
Preproliferative	7	11	
Proliferative	28	24	
BMI (kg/m^2)	23.2 ± 2.8	23.3 ± 3.4	NS
HbA_{1c} (%)	8.4 ± 1.7	8.7 ± 1.6	NS
Mean arterial pressure (mmHg)	98 ± 12	90 ± 8	$P < 0.0001$
GFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$)	103 ± 45	105 ± 22	NS

Data are means ± SD. IDDM cases with incipient ($n = 37$) or established ($n = 25$) diabetic nephropathy and IDDM control subjects with normal UAE, blood pressure, and GRF are indicated. GFR reduced in subjects with established versus those with incipient nephropathy: 62 ± 35 vs. 129 ± 29 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$ ($P < 0.0001$).

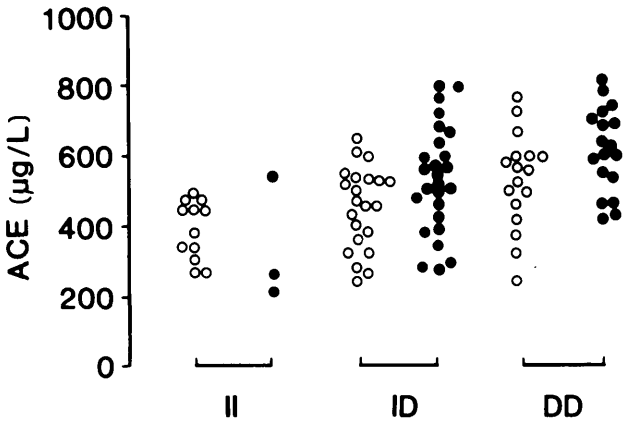

FIG. 2. Individual plasma ACE levels according to ACE genotype in study 2. Ninety-eight IDDM subjects with (●) and without (○) diabetic nephropathy. This was presented the same as in Fig. 1. See RESULTS for analysis.

with proliferative retinopathy these frequencies were 16, 28, and 8, respectively (NS). Plasma ACE levels were 509 ± 114 µg/L in subjects without retinopathy vs. 496 ± 168 µg/L in those with proliferative retinopathy (NS). In IDDM cases with incipient diabetic nephropathy and their matched IDDM control subjects, diabetic retinopathy severity was related to diabetes duration ($r' = 0.85$, $P = 0.0001$) and to plasma prorenin ($r' = 0.38$, $P < 0.009$), but not to plasma ACE ($r' = 0.05$, $P = 0.68$). Plasma prorenin in IDDM cases with incipient diabetic nephropathy was not different from their matched IDDM control subjects: 354 (65–1,985) vs. 287 (112–601) ng/L ($P = 0.677$).

DISCUSSION

We observed an imbalance of ACE-insertion polymorphism in IDDM subjects with nephropathy compared with those without nephropathy. The II genotype is a genetic basis for low ACE activity in the general population, and we propose that it can protect against the onset of diabetic nephropathy in IDDM.

Sample bias may be present in such a case-control study of subjects regularly attending a diabetes clinic, but this is unlikely to explain our results. First, ACE genotype distribution may have been different in IDDM subjects compared with the general population, but this did not appear so from study 1. Second, genotype association with a confounding variable such as diabetic retinopathy is unlikely because this complication was not found to be associated with the ACE genotype or phenotype. Conversely, diabetic retinopathy severity was proportional to diabetes duration, a classical finding (15), and to plasma prorenin, as reported previously (16). These findings suggest that the ACE genotype and/or phenotype are truly linked to diabetic nephropathy. Larger studies are nevertheless required to confirm the lack of association between ACE genotype and IDDM or between ACE genotype and diabetic complications other than nephropathy.

Another problem is the misclassification of IDDM subjects according to their risk of diabetic nephropathy, when the assessment is based on functional and not on anatomical changes, which are sometimes dissociated in subjects with diabetic nephropathy (17). Clinical proteinuria may be attributable to kidney disease other than diabetic nephropathy. However, concomitant diabetic retinopathy, as in this study, renders the diagnosis of diabetic nephropathy >95% certain (1,2). Regarding incipient diabetic nephropathy, microalbuminuria indicates ongoing established diabetic nephropathy with >80% positive predictive value (11). However, this assumption was recently challenged in subjects with IDDM of long duration (18). This is why we matched IDDM subjects with incipient diabetic nephropathy with control subjects of the same age, sex, duration of diabetes, and diabetic retinopathy severity. On the other hand, some subjects with IDDM of long duration can display anatomical signs of diabetic nephropathy and reduced GFR, but normal UAE (19); however, our normoalbuminuric control subjects with severe diabetic retinopathy all had normal GFR. Some of our control subjects may nonetheless have been misclassified as such, when in reality they were at risk of developing diabetic nephropathy. This is because, with improved patient care, there has been a calendar decline in the incidence of this complication (20). If this were the case, we may have underestimated the link between the ACE gene polymorphism and diabetic nephropathy.

These data were obtained in white French subjects. Other studies are required to be established if these data can be applied to other ethnic or national groups.

This observation is consistent with a role for ACE in regulating renal circulation through vasoactive peptide metabolism: ACE levels may be critical for angiotensin II production in the kidney (21), although it may also have other unknown functions caused by its low enzymatic specificity (4). Experimentally, renal hemodynamic changes induced by angiotensin II or diabetes display features that both lead to intraglomerular hypertension, which is a basis for future glomerulosclerosis (3,22). Alterations in renin and kallikrein were related to renal hemodynamic abnormalities in diabetic subjects (16,23). Although a pathogenetic role for these enzymic alterations is not proven, they provide support for a role of ACE in modulating renal hemodynamic alterations in diabetic subjects. Plasma and cellular ACE levels depend on I/D polymorphism (7,24), which is probably a marker in linkage disequilibrium with a causal variant. Identification of this variant may improve knowledge of the relationship between ACE and diabetic nephropathy. The ACE gene is a candidate for vascular events that result from local circulatory disturbances. Recently, the ACE genotype was related to myocardial infarction, suggesting a deleterious effect of this enzyme in the coronary circulation (8,25). Interestingly, myocardial infarction is the main cause of premature death in subjects with diabetic nephropathy (1,2). Albuminuria can reflect widespread vascular damage in IDDM (26), and this may be modulated by the ACE genotype. ACE inhibitors reduce microalbuminuria and prevent or reduce evolution of diabetic nephropathy (27,28) by their renal as well as systemic effects (29). Further studies are required to test

if the ACE gene polymorphism could be a basis for abnormal renal hemodynamics in IDDM.

The ACE gene polymorphism may not account for all the genetic susceptibility to diabetic nephropathy. Genetic predisposition to diabetic nephropathy was reported to be associated with familial hypertension and an intermediate phenotype, i.e., high sodium-lithium countertransport in erythrocytes (30).

Plasma ACE levels were mildly elevated in these IDDM subjects compared with control populations, independently of diabetic retinopathy or nephropathy. This diabetes-related ACE elevation confirms previous findings (9,10) and was not attributable to glycemic control because it was independent of HbA_{1c} levels. Also, the correlation between ACE activities and immunoreactivities suggested that elevated enzyme activity may not be secondary to altered activity of the enzyme attributable to glycosylation. Plasma ACE half-life is not known, especially in diabetes. This enzyme is probably trapped by the reticuloendothelial system (4), which is altered in diabetes-reduced ACE catabolism may be a basis for its elevation in diabetes. On the other hand, endothelial function is altered in diabetes, which may increase ACE gene expression or secretion. If plasma ACE levels remain high in subjects with diabetic nephropathy after adjustment for genotype (see RESULTS), this could indicate either that subjects with the highest ACE levels are at increased risk of nephropathy or, conversely, that diabetic nephropathy induces a further increase in plasma ACE levels. A longitudinal study of kidney function according to ACE genotype and plasma levels is required in IDDM to clarify this point, confirm our hypothesis, and establish these variables as risk factors for diabetic nephropathy.

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