An Insertion/Deletion Polymorphism in the Angiotensin I-converting Enzyme Gene Accounting for Half the Variance of Serum Enzyme Levels

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Abstract

A polymorphism consisting of the presence or absence of a 250-bp DNA fragment was detected within the angiotensin I–converting enzyme gene (ACE) using the endothelial ACE cDNA probe. This polymorphism was used as a marker genotype in a study involving 80 healthy subjects, whose serum ACE levels were concomitantly measured. Allele frequencies were 0.6 for the shorter allele and 0.4 for the longer allele. A marked difference in serum ACE levels was observed between subjects in each of the three ACE genotype classes. Serum immunoreactive ACE concentrations were, respectively, 299.3±49, 392.6±66.8, and 494.1±88.3 μg/liter, for homozygotes with the longer allele (n = 14), and heterozygotes (n = 37) and homozygotes (n = 29) with the shorter allele. The insertion/deletion polymorphism accounted for 47% of the total phenotypic variance of serum ACE, showing that the ACE gene locus is the major locus that determines serum ACE concentration. Concomitant determination of the ACE genotype will improve discrimination between normal and abnormal serum ACE values by allowing comparison with a more appropriate reference interval. (*J. Clin. Invest.* 1990. 86:1343–1346.) Key words: gene polymorphism • genomic DNA hybridization

Introduction

Angiotensin I–converting enzyme (ACE)[1] (kininase II, EC 3.4.15.1) is a zinc metallopeptidase whose main known functions are to convert angiotensin I into the vasoactive and aldosterone-stimulating peptide angiotensin II, and to inactivate bradykinin (1). ACE is believed to have other physiological roles because of its wide enzymatic specificity and wide distribution (2). Thus, ACE is found as a membrane-bound enzyme in endothelial cells and different types of epithelial and neuroepithelial cells as well as in a circulating form in biological fluids, such as plasma and amniotic or seminal fluids. The mechanisms leading to the biosynthesis of the circulating form of ACE are still unclear, but all the data available indicate that its structures is very similar to that of the cellular form (3).

Plasma ACE measurement is widely used for the diagnosis and follow-up of sarcoidosis because an elevation of the enzyme is often observed in this disease (4). Although plasma ACE concentrations are remarkably stable when measured repeatedly in a normal subject, large interindividual differences make it difficult to interpret plasma ACE levels in a given patient, as the reference interval for normal values is large (5). The results of a study conducted in a large sample of healthy families showed an intrafamilial resemblance between ACE levels and also suggested that they are subject to the effect of a major gene (6).

Marker genotypes are useful tools to identify the alleles exerting a genetic effect on a quantitative trait (7). In an attempt to define the role of the ACE gene in the genetic control of circulating ACE we used the human endothelial ACE cDNA to detect DNA polymorphisms at the ACE gene locus. An insertion/deletion polymorphism was identified at this locus and used as a marker genotype in a population study designed to explore the relationship between this polymorphism and the serum ACE concentration.

Methods

Subjects

80 healthy Caucasians (38 males and 42 females) were selected for the study in a center for preventive medicine. Inclusion was based both on clinical characteristics and routine laboratory tests performed at the centre. Inclusion criteria were a body mass index < 28 kg/m^2, normal blood pressure on WHO criteria (systolic blood pressure < 160 mmHg and diastolic blood pressure < 90 mmHg measured in the sitting position after a 5-min rest), glycemia < 6 mmol/liter, plasma gamma glutamyl transpeptidase < 30 UI/liter, normal sedimentation rate, normal chest x-ray, and absence of acute or chronic disease and drug intake.

10 normal families comprising two parents and at least two children were selected according to the same criteria, to test the mendelian inheritance of the insertion/deletion polymorphism found at the ACE gene locus (see Results).

Materials and experimental protocols

Serum ACE measurement. Serum ACE concentration was measured in duplicate by direct radioimmunoassay (5).

DNA extraction. High-molecular weight DNA was isolated from peripheral blood leukocytes by standard techniques (8). DNA concentrations were measured by absorbance at 260 nm.

Human ACE cDNA probe. The complete human endothelial ACE cDNA (clones pG19-22 and pG21-11) was used in a first approach to

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1. *Abbreviations used in this paper:* ACE, angiotensin I–converting enzyme; BMI, body mass index; DBP, diastolic blood pressure; HMW, high molecular weight; RFLP, restriction fragment length polymorphism; RIA, radioimmunoassay; SBP, systolic blood pressure.

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the detection of restriction fragment length polymorphism (RFLP) on the human ACE gene (9). For subsequent detection of the insertion/deletion polymorphism, a 584-bp *Ban* I restriction fragment corresponding to nucleotide positions 2123 to 2707 of the published cDNA sequence (9) was routinely used. Inserts of plasmids pG19-22 and pG21-11, and the *Ban* I fragment of plasmid pG19-22, were isolated from low-gelling temperature agarose gels (SeaPlaque, FMC Bioproducts, Rockland, ME) and labeled at high specific activity by the random-primer labeling method (10) using a commercial kit (Amersham International, Amersham, UK).

RFLP detection. Individual high-molecular weight DNAs were digested by restriction enzymes (New England Biolabs, Beverly, MA) under the conditions advised by the supplier, and were submitted to electrophoresis on 0.7% agarose gels. DNAs were transferred by capillary blotting with alkali on nylon membranes (Hybond N+, Amersham International) (11) and hybridized to labeled probes, according to previously described protocols (8). Hybridization and filter washing were done under high stringency conditions (9).

Statistical methods. One-way analyses of variance were used to compare group means for the different parameters studied. To account for the association between means and variances of Ir-ACE levels in the three genotype groups, log-transformed values were used for the statistical test. Allele frequencies were estimated by the gene counting method and Hardy-Weinberg's equilibrium was checked by the chi-square test. Correction for biases of the estimated contribution of the locus to the phenotypic variance of ACE was computed according to Boerwinkle et al. (12). Pearson correlation coefficients were also computed when necessary.

Results

Clinical parameters. The values for age, body mass index (BMI), systolic and diastolic blood pressure (SBP and DBP) for men and women are listed in Table I. BMI, which was slightly greater for men than for women, was the only parameter that differed significantly between sexes ($P < 0.01$).

Serum ACE concentrations. Serum ACE concentrations did not differ significantly between men and women and were not correlated to age, SBP, DBP, or BMI (Table I). Consequently, these variables were not adjusted for subsequent analysis (Table II). The mean value and dispersion of serum ACE levels in this population were similar to those observed in previous studies of healthy subjects (5, 6, 12a).

Identification of an insertion/deletion polymorphism at the ACE gene locus. To detect DNA polymorphisms, gel-blot hybridization experiments were made on genomic DNA in 15 individuals, using the complete endothelial ACE cDNA as a probe. A two-allele RFLP at the ACE locus was detected with the following restriction enzymes: *Xba* I, *Hind* III, *Bgl* II, *Kpn* I, and *BamH* I. Analysis of the fragment sizes obtained with these enzymes showed that RFLPs resulted from a ~ 250-bp insertion/deletion polymorphism. Hybridization of the filters with different parts of the cDNA showed that this polymorphism could be detected by a 584-bp *Ban* I restriction fragment, located between positions 2123 and 2707 of the cDNA sequence (9). For the rest of the study this fragment was therefore used as a probe, and the polymorphism was detected with the restriction enzyme *Hind* III. The insertion/larger allele is designated *I*, and the deletion/shorter allele, *D*.

Genetic characterization of the insertion/deletion polymorphism. Mendelian inheritance of the genetic polymorphism at the ACE gene locus was established in 10 normal nuclear families. Allele segregation in one nuclear family, analyzed by DNA gel-blot hybridization, is shown in Fig. 1. In this family, the father was homozygote DD, the mother was homozygote II, and both children were heterozygotes ID for the polymorphism.

ACE genotypes were determined for all subjects (Table II). Allele frequencies were 0.406 for the I allele and 0.594 for the D allele. The observed genotype distribution (II = 0.18, ID

Table I. Clinical Data and Serum ACE Concentrations of 80 Healthy Subjects

	Men $n = 38$	Women $n = 42$
Variable		
Age	43.3±7.0	45.8±7.3
BMI (Kg/m^2)	23.6±2.5	21.9±2.4*
SBP (*mmHg*)	124.7±8.5	122.9±11.3
DBP (*mmHg*)	78.2±7.4	75.9±6.7
Ir-ACE (*μg/liter*)	408.5±98.2	417.1±103.5

Ir-ACE, serum ACE concentrations measured by direct RIA of ACE. Results are means ± SD.
* Comparison between men and women: $P < 0.01$.

Table II. Clinical Parameters and Serum ACE Values in Groups with Different ACE Genotypes

Genotype	II $n = 14$	ID $n = 37$	DD $n = 29$
Age	44.4±7.4	44.3±7.5	45.0±7.0
BMI (Kg/m^2)	22.9±3.1	23.2±2.6	22.0±2.3
SBP (*mmHg*)	124.3±11.1	124.6±9.5	122.4±10.5
DBP (*mmHg*)	77.1±8.2	76.9±6.7	77.1±7.3
Ir-ACE* (*μg/liter*)	299.3±49.0	392.6±66.8	494.1±88.3
Ir-ACE Ln (*μg/liter*)	5.69±0.15‡	5.96±0.17‡	6.19±0.19‡

I corresponds to the larger allele and D to the shorter allele. Ir-ACE, serum concentrations obtained by direct RIA of ACE. Ir-ACE Ln, logarithm of serum ACE concentrations. Results are means ± SD.
* For Ir-ACE comparisons, the test was only carried out on log-transformed values.
‡ Comparison between genotype groups; $P < 0.001$.

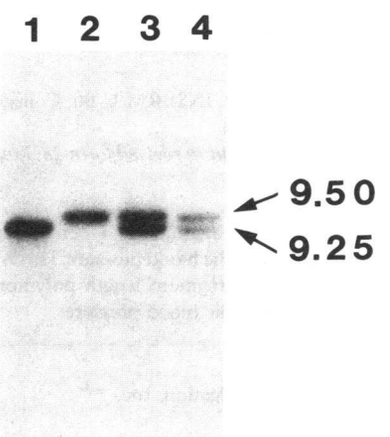

Figure 1. DNA gel-blot hybridization experiments with the *Ban* I–*Ban* I restriction fragment of the ACE cDNA. Genomic DNA digestion of members of a nuclear family by the restriction enzyme *Hind* III. DNA from the father, mother, and two children are shown, respectively, in lanes *1, 2, 3,* and *4*. DNA fragment sizes, in kilobases, are indicated by numbers on the right.

= 0.46, and DD = 0.36) was in agreement with the Hardy-Weinberg proportion.

Relationship between the ACE genotype and serum ACE level. Mean serum ACE levels were then compared among the three groups defined by the ACE gene's polymorphism. The results of this comparison, given in Table II and Fig. 2, show a significant relationship between the polymorphism and the level of ACE in the serum ACE level, with an additive effect of the alleles. The polymorphisms accounted for 47% of the variance in serum ACE levels.

Discussion

The stability of the circulating ACE level in a given individual allows study of the factors influencing its long-term regulation. A large-scale study of plasma ACE levels conducted in normal nuclear families has shown important interindividual variation and demonstrated the presence of a familial aggregation of ACE values due to a genetic control (6). To test the hypothesis that an allelic variant of the ACE gene itself was responsible for this control, we designed a study to explore the relationship between circulating ACE levels and ACE gene polymorphisms.

We detected RFLPs at the ACE gene locus using an ACE cDNA probe that spanned complete endothelial ACE mRNA sequence. With this probe, we previously showed that a single gene codes for ACE in man (9). Here, with most of the restriction enzymes tested, we detected a two-allele RFLP corresponding to an insertion/deletion polymorphism, with an allelic frequency of 0.6 for the shorter allele and 0.4 for the larger allele. Analysis of polymorphic fragment segregation in ten nuclear families demonstrated the mendelian inheritance of the two identified ACE gene alleles (Fig. 1).

The polymorphic insertion (250 bp long) was located inside the ACE gene as it hybridized with internal fragments of the cDNA. Failure to detect the insertion/deletion polymorphism with some of the restriction enzymes using the cDNA as probe indicates that the polymorphic insertion is not present in the cloned cDNA and is probably flanked by intronic sequences. Gene polymorphisms caused by the presence or absence of sequence insertions have been described in several mammalian genes, for instance in the 5′ flanking region of the human insulin-receptor gene and the 5′ flanking region of the rat prolactin gene (13, 14). In both cases, sequence analysis revealed that DNA insertions consisted of repetitive elements.

All subjects included in the study were tested for ACE gene polymorphisms by DNA gel-blot hybridization using the restriction enzyme *Hind* III. When the level of serum ACE was compared in subjects of the three genotype classes (II, DD, and ID), a marked difference was found between the three groups. Subjects with the deletion polymorphism had higher ACE levels than those with the insertion polymorphism. Intermediate levels were observed in heterozygotes (Table II and Fig. 2). The genetic effect accounted for 47% of the total variance of serum ACE. As the presence of each allele had an additive effect on serum ACE and two alleles had twice the effect of one, each allele was codominant.

Cambien et al. recently proposed a model for the genetic control of plasma ACE levels, based on the results of a family study (6). In this study, the genetic analysis of familial phenotypes suggested that these levels are affected by a major gene, which was estimated to account for 29% of the total phenotypic variance of plasma ACE in adults. The present results confirm this major gene's effect as a clear difference in serum ACE levels was observed between the three genotype classes and the ACE gene was found to be the major gene responsible for this effect. In adults, the insertion/deletion polymorphism accounted for about half the total phenotypic variance, a proportion somewhat larger than the 29% estimated from the family study referred above. As only 47% of the serum ACE variance found here was due to the allele effect, other genetic or environmental factors may be involved in the interindividual variations in circulating ACE. However, in a study of several candidate parameters (12a), no clear hormonal or environmental influence was detected.

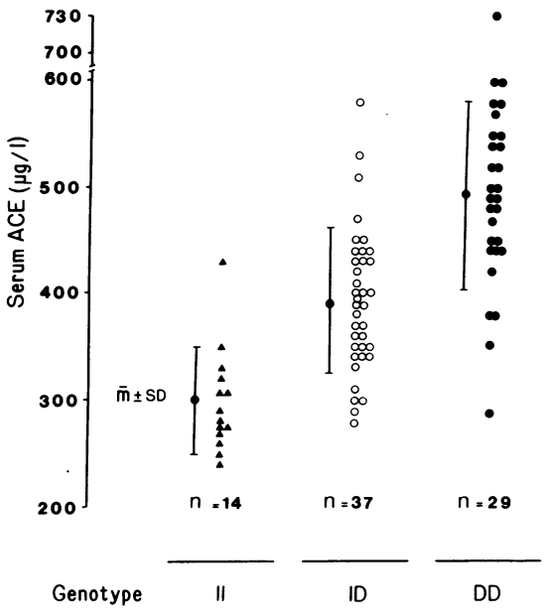

Figure 2. Serum immunoreactive ACE concentrations (µg/liter) for individual with the II, ID, and DD genotypes, respectively, shown in left, middle, and right panels. Solid vertical bars indicate mean concentration and standard deviation for each group.

Gene alleles have been shown to exert quantitative effects on plasma concentrations of other proteins. Thus, a *Pvu* II polymorphism of the lipoprotein lipase gene was found to be associated with variations in the concentrations of serum triglycerides, but no significant relationship was found with other polymorphisms of the same gene (15). Genetic variation at the fibrinogen locus accounted for part of the variation in plasma fibrinogen phenotypes (16). However, in both these cases, the genetic effect appeared to be weaker than the serum ACE-genotype relationship, probably due in part to the greater stability of serum ACE concentrations which are less influenced by environmental factors than fibrinogen and triglyceride levels.

Circulating ACE probably originates from the vascular endothelial cells (1). ACE is a membrane-bound ectoenzyme of the cell surface, to which it is anchored by a carboxyterminal hydrophobic domain (9). The biochemical mechanism of circulating ACE secretion is not clear, but several hypotheses can be made including proteolytic cleavage of the hydrophobic anchor and passive leakage of the membrane-bound enzyme. Whatever the mechanism involved, it is likely that the observed genetic control of serum ACE level is exerted at the

transcriptional level. In that case, the insertion/deletion itself may not play a direct part in controlling ACE transcription but is more likely to be in linkage disequilibrium with regulatory elements of the ACE gene.

The present results of DNA gel-blot hybridization indicate that the insertion/deletion polymorphism is localized inside an intron. However, we cannot exclude the possibility that as a result of different ACE pre-mRNA splicing, depending on the presence or absence of the insertion, part of this sequence might be present in the mature RNA, thus modifying its stability or giving rise to the presence of an additional peptide inside the protein, and consequently altering its secretion or stability. Such polymorphic insertion was recently described in the signal peptide of the apolipoprotein B gene (17). Lastly, the insertion itself might modify the splicing process of the ACE precursor mRNA by interfering with the lariat formation step (18).

The observation of a genetic polymorphism that explains much of the interindividual variability in plasma ACE levels has clinical implications, particularly for the diagnosis of granulomatous diseases, such as sarcoidosis in which circulating ACE levels are high because of increased ACE secretion by monocyte-derived cells (19). By determining the genotype of patients, it may be possible to reduce the size of the reference interval to which a given measurement of the plasma ACE level should be compared (Fig. 2). This would allow more accurate conclusions to be drawn about the significance of plasma ACE levels in individual patients (20). As ACE is implicated in vasoactive peptides metabolism, the ACE gene is a candidate gene for essential hypertension. Consequently, the ACE gene polymorphism described here constitutes a potential tool for genetic studies of hypertension.

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