Angiotensin converting enzyme gene I/D polymorphism in essential hypertension and nephroangiosclerosis

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Angiotensin converting enzyme gene I/D polymorphism in essential hypertension and nephroangiosclerosis. An insertion/deletion (I/D) polymorphism of the angiotensin converting enzyme (ACE) gene significantly influences circulating ACE levels and plays a role in the development of target organ damage, that is, left ventricular hypertrophy in essential hypertension (EH), and microalbuminuria in diabetes mellitus. We have examined the role of the I/D polymorphism in essential hypertensive patients with renal involvement. The study was divided in two independent protocols. In protocol 1, we retrospectively analyzed the ACE genotypes in 37 essential hypertensive patients with a clinical and histopathological diagnosis of nephroangiosclerosis. In protocol 2, ACE genotypes as well as microalbuminuria and renal hemodynamic parameters were investigated in 75 patients with EH with normal renal function and a strong family history of hypertension. As control group, 75 healthy subjects with BP < 130/85 mm Hg and no family history of cardiovascular diseases were studied. The ACE variants were determined by PCR and the genotypes were classified as DD, DI, and II. In protocol 1, patients with nephroangiosclerosis displayed a significant difference in the genotype distribution (57% DD, 27% DI, 16% II) when compared to the control population (25% DD, 64% DI, 11% II; P < 0.001). There was no significant difference in genotype distribution between hypertensive patients with normal renal function (protocol 2; 33% DD, 59% DI, 8% II) and the control group. There were no differences in age, blood pressure, microalbuminuria, and duration of the disease among the three genotypes in the EH group from protocol 2. Taken together, these findings suggest that the DD genotype of ACE is associated with histopathologic-proven kidney involvement in patients with EH and that this polymorphism could be a potential genetic marker in hypertensives at risk of renal complications.

Arterial hypertension is the second most frequent cause of end-stage renal disease in patients entering a chronic dialysis program both in Europe and in the United States [1, 2]. Although pharmacological treatment of essential hypertension (EH) has decreased the incidence of cardiac and cerebral complications, renal involvement in this disease has not been reduced to the same proportion [1]. Therefore, besides high blood pressure (BP), other factors have been considered in the pathogenesis of the renal complications of hypertension.

Key words: genes, microalbuminuria, diabetes mellitus, target organ damage, blood pressure, genotype distribution, genetic marker.

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The renin-angiotensin system (RAS) plays a central role in salt and water homeostasis and in the maintenance of vascular tone. This system acts both as a circulating hormonal system, and as a local one as well, serving paracrine, autocrine and intracrine functions. In addition to the renal hemodynamic and tubular transport actions, the intrarenal RAS probably plays an important role as regulator of mesangial and endothelial cell function, acting as an inducer of cell growth [3]. There is a fair amount of evidence, supported both by experimental and clinical studies, that this system plays an important pathogenic role in the development of renal damage in EH, that is, nephroangiosclerosis, and that this effect is not exclusively related to hemodynamic factors [3, 4]. Specifically, transgenic animals expressing an additional renin gene show more rapid worsening of renal function than those without the additional gene, despite similar BP levels [5]. Moreover, recent gene transfer studies have shown that the addition of the human angiotensinogen and renin genes to the rat kidney induces glomerular sclerosis with no change in BP [6]. On the other hand, the use of angiotensin converting enzyme (ACE) inhibitors has been shown to have protective renal effects reducing the urinary protein excretion and slowing the progression of renal failure in several pathologies, independently of their BP lowering effect [3, 4]. About 15% of hypertensive patients show a decline in the renal function, in many cases despite a good BP control [7], pointing to an individual susceptibility in the development of renal damage in these patients.

An insertion/deletion (I/D) polymorphism of the ACE gene has been identified in humans [8, 10]. The ACE gene consists of 26 exons and spans 21 Kb on chromosome 17 [9], and the I/D polymorphism is characterized by the presence or absence of a 287 base pair fragment in intron 16 resulting in three genotypes (DD, DI, and II) [8]. The physiological importance of the I/D polymorphism relies on the fact that the DD genotype is associated with increased circulating [10] and tissue [11] ACE levels. This genotype has also been linked to EH [12], and left ventricular hypertrophy [13], although with varying results among populations [14, 15]. Relevant to the kidney, the DD genotype has more recently been related to the development of nephropathy in insulin and non-insulin dependent diabetes [16, 17], and to the antiproteinuric efficacy of ACE inhibition in patients with proteinuria [18]. Furthermore, it has recently been noticed that this genotype is associated with the progression of renal insufficiency in IgA nephropathy [19, 20]. However, there are no data about the
possible role of this genetic polymorphism in proved renal damage observed in EH. Therefore, we investigated whether the ACE gene I/D polymorphism is associated with the presence of renal damage in EH.

METHODS

Study subjects

The study was approved by the local Ethics Committee and all patients gave their written informed consent. The patients were recruited from the outpatient clinic of the Renal Unit at Hospital Clinic in Barcelona. In Protocol 1 we initially included in a retrospective analysis 47 patients with EH and histologic diagnosis of nephroangiosclerosis. The following inclusion criteria were applied: (a) diagnosis of EH prior to the development of renal insufficiency, (b) histopathologic diagnosis of nephroangiosclerosis [21], (c) age < 50 years, and (d) absence of diabetes mellitus. Five patients were excluded because by careful analysis the diagnosis was not unequivocal by clinical or pathological grounds and good quality DNA could not be obtained for genotyping in five additional patients. In Protocol 2, 75 treated essential hypertensive patients were included. The following selection criteria were applied: (a) diagnosis of moderate-severe EH with a systolic BP >160 mm Hg and diastolic BP >100 mm Hg, (b) age between 25 and 65 years, (c) family history of hypertension in at least one parent and one first degree relative, (d) absence of diabetes mellitus, (e) body mass index < 30 kg/m², and (f) alcohol intake < 50 g/day. Patients on oral contraceptives were excluded. As a control group 75 healthy subjects from the Hospital blood bank in our institution between 1975 and 1995 and were examined by the standard methods [22]. It has been demonstrated that in this case, polymerase chain reaction (PCR) of DNA from fixed, paraffin-embedded tissues provides a relatively simple and extremely sensitive method. The ACE I/D genotype was determined by PCR using published primers 5'-CTG GAG acct CCC ATC TTT TCT-3' and 5'-GAT GTG GCC ATC ACA TTG ATC AGA T-3' that flank the polymorphic region [8]. PCR was conducted in 25 µl volume containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 200 µM of each dNTP, 1 µM primers, 1U Taq polymerase and 100 ng of genomic DNA. After an initial denaturation at 96°C for five minutes, thermocycling consisted of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for one minute for 35 cycles followed by a final extension for five minutes. Amplification products were separated on 0.5% agarose/2.5% Nu Sieve (Amersham, Arlington Heights, IL, USA) and visualized by ultraviolet transillumination after ethidium bromide staining. This primer pair produced a ~490 bp product corresponding to the insertion and/or a ~190 bp fragment corresponding to the deletion. Subjects were classified as II, DD or heterozygote for insertion/deletion (DI). Mistyping of DI heterozygotes as DD due to preferential amplification of the D allele and inefficiency in amplification of the I allele has been described [23]. Therefore, all samples found to be DD were amplified with an insertion-specific primer pair that recognizes the inserted sequence: 5'-TGG GAC CAC AGC GCC CGC CAC TAC-3' and 5'-TCC CCA GCC GCC CTC CCA TGC CCA TAA-3' [19], with identical PCR conditions except for an annealing temperature of 64°C. The reaction yields a 335 bp product in the presence of an I allele.

Evaluation of the renal profile

In patients from protocol 1, renal biopsies were performed at our institution between 1975 and 1995 and were examined by the same pathologist by optic and immunofluorescence microscopy. Nephroangiosclerosis was diagnosed using widely accepted clinical and pathologic criteria [21]. Renal function in hypertensive patients from protocol 2 was evaluated by measuring plasma creatinine, glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and filtration fraction (FF). GFR and ERPF were measured by single shot clearance technique using intravenous injection of 125I-thalamate and 131I-hippurate respectively. Creatinine, glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and filtration fraction (FF). GFR and ERPF were measured by single shot clearance technique using intravenous injection of 125I-thalamate and 131I-hippurate respectively. Values were corrected for calculated body surface. The FF resulted from calculating the ratio of ERPF and GFR. To identify hypertensive patients at risk of cardiovascular and, possibly, renal morbidity, we measured urinary albumin excretion rate (UAE) in this group. UAE from two separate 24-hour urine collections was measured using a immunonephelometric assay (Boehringer Mannheim, Germany). Each urine sample was tested for urine infection by microscopic examination of the sediment and by culture. If infection was detected urine was sampled again once it resolved and examined. The subjects were told to continue their regular diet and treatment and to avoid heavy exercise on the days of urine collection. The values represent the average of the two different measurements. Microalbuminuria was defined as urinary albumin excretion rate between 20 and 200 μg/min.

Detection of angiotensin converting enzyme genotype

DNA was extracted from peripheral blood leukocytes as described previously [22]. In 18 of the 37 patients from protocol 1, where blood samples were not available, DNA was extracted directly from the paraffin-embedded renal tissues following standard methods [22]. It has been demonstrated that in this case, polymerase chain reaction (PCR) of DNA from fixed, paraffin-embedded tissues provides a relatively simple and extremely sensitive method. The ACE I/D genotype was determined by PCR using published primers 5'-CTG GAG acct CCC ATC TTT TCT-3' and 5'-GAT GTG GCC ATC ACA TTG ATC AGA T-3' that flank the polymorphic region [8]. PCR was conducted in 25 µl volume containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 200 µM of each dNTP, 1 µM primers, 1U Taq polymerase and 100 ng of genomic DNA. After an initial denaturation at 96°C for five minutes, thermocycling consisted of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for one minute for 35 cycles followed by a final extension for five minutes. Amplification products were separated on 0.5% agarose/2.5% Nu Sieve (Amersham, Arlington Heights, IL, USA) and visualized by ultraviolet transillumination after ethidium bromide staining. This primer pair produced a ~490 bp product corresponding to the insertion and/or a ~190 bp fragment corresponding to the deletion. Subjects were classified as II, DD or heterozygote for insertion/deletion (DI). Mistyping of DI heterozygotes as DD due to preferential amplification of the D allele and inefficiency in amplification of the I allele has been described [23]. Therefore, all samples found to be DD were amplified with an insertion-specific primer pair that recognizes the inserted sequence: 5'-TGG GAC CAC AGC GCC CGC CAC TAC-3' and 5'-TCC CCA GCC GCC CTC CCA TGC CCA TAA-3' [19], with identical PCR conditions except for an annealing temperature of 64°C. The reaction yields a 335 bp product in the presence of an I allele.

Statistical analysis

Quantitative variables are expressed as mean ± SEM. Genotype distribution and allele frequencies were compared between groups using the χ² test or the Fisher’s exact test. Differences between quantitative variables were analyzed by parametric test (analysis of variance). Only when the distribution was not normal (UAE rate) a non-parametric test (Kruskal-Wallis) was performed. A P value of less than 0.05 was considered significant.

RESULTS

Protocol 1

The group of patients from protocol 1 included 35 men and two women, aged 42 ± 1 year with systolic blood pressure (SBP) of 169 ± 5 and diastolic blood pressure (DBP) of 105 ± 2 mm Hg at the time of diagnosis, serum creatinine of 0.327 ± 0.40 µmol/liter (3.69 ± 0.5 mg/dl) and with 1.97 ± 0.5 g/day proteinuria. The mean duration of known hypertension prior to the biopsy was 5 ± 1 year, with a mean treated SBP of 147 ± 2.5 and DBP 91 ± 2 mm Hg. The distribution of the ACE genotype in the nephroangiosclerosis group was 21 (57%) patients with DD genotype, 10
(27%) with DI and 6 (16%) patients with II genotype (Table 1). This distribution was clearly different to the one in the control group, where the ACE genotype distribution was: 19 DD (25%), 48 DI (64%) and 8 II (11%; \( P = 0.001 \)). The hypertensive patients with histological evidence of nephroangiosclerosis were also characterized by an excess in the frequency of the D allele (70%), while in the control group was only present in 57% (\( P < 0.05 \); Table 1).

**Protocol 2**

The 75 hypertensive patients from protocol 2 included 30 male and 45 female, aged 55 ± 1 year, with SBP of 182 ± 25 and DBP of 109 ± 1.1 mm Hg at the time of diagnosis. The mean creatinine level was 76 ± 2 μmol/liter (0.9 ± 0.02 mg/dl) and the mean duration of hypertension was 10 ± 1 year with a mean treated SBP of 142 ± 1.5 and DBP of 88.6 ± 1 mm Hg. Microalbuminuria was detected in 14 patients (19%) and the mean urinary albumin excretion rate was 19.6 ± 12.5 μg/min. The renal hemodynamic study disclosed a GFR of 98 ± 14 ml/min/1.73 m² and a ERPF of 438 ± 13 ml/min/1.73 m², resulting in a FF of 0.22, not significantly different from the normal values of the laboratory.

The genotype distribution in hypertensive patients, 25 DD (33%), 44 DI (59%) and 6 II (8%), was not significantly different from that of the control group. The allele frequencies were also not different between the two groups, with 94 (63%) allele D and 56 (37%) allele I in the hypertensive patients and 86 (57%) allele D and 64 (43%) allele I in the control group. By using more stringent inclusion criteria, that is, early onset of the disease (age ≤ 40 years) or higher BP levels (BP ≥ 180/110), the genotype distribution was neither significantly different between hypertensive and control subjects (data not shown). There was no difference in the genotype distribution between males and females (data not shown).

No differences in age, BP level, known duration of hypertension, microalbuminuria and UAE rate were found between any of the different ACE genotypes (Table 2). Similarly, plasma renin activity and the renal hemodynamic parameters measured in hypertensive patients were similar among the three genotypes (Table 2).

**DISCUSSION**

The kidney is a main target organ in EH. In early stages of this disease, functional changes due to an increase of renal vascular resistance, with reduction in RPF, normal or subnormal GFR accompanied by raised FF, are observed in many patients [24]. Later in the course of the disease, GFR decreases probably due to loss of functioning nephrons, representing the onset of irreversible morphologic damage due to nephroangiosclerosis [24]. There are several mechanisms, both hemodynamic and non-hemodynamic, involved in the development of these alterations, and there is agreement about the important role of the RAS in this process [3]. On the other hand, there is an individual susceptibility to develop end-stage renal disease due to hypertension which, in some patients, occurs even after a good control of their hypertension has been achieved [7]. Differences in the activation of the renal tissue RAS, not detectable by clinical tests, could explain the differences in the incidence of renal insufficiency among hypertensive patients.

The I/D polymorphism of the ACE gene has been associated in some studies [12, 13] but not in others [14, 15] with various forms of cardiovascular disease. More recently, although this polymorphism has not yet been associated with primary forms of renal disease, it has been closely linked to the progression of chronic renal diseases of different etiologies [19, 20]. However, the I/D polymorphism has not yet been investigated in proven hypertensive renal disease. The present study shows a significant association between the ACE DD genotype and the presence of biopsy-proven nephroangiosclerosis in EH that, to our knowledge, has not been reported before. The histological vascular renal changes observed in hypertension, particularly segmental hyalinosis within afferent arterioles and interlobular arteries, has also been described as a phenomenon of aging, as a feature of diabetic nephropathy and as a vascular complication of cyclosporine therapy [21]. Nevertheless, it has been shown that the vascular changes on the renal segmentary arteries usually do not appear before the age of 50 years, which was the cut-off age in our study. In addition, none of the patients we included had diabetes mellitus nor had they received cyclosporine. Although the clinical

**Table 1.** ACE genotype distribution and allele frequency in the hypertensive patients with nephroangiosclerosis from protocol 1 and in the control group

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Nephroangiosclerosis</th>
<th>Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>21 (57%)</td>
<td>19 (25%)</td>
<td>37</td>
</tr>
<tr>
<td>DI</td>
<td>10 (27%)</td>
<td>48 (64%)</td>
<td>58</td>
</tr>
<tr>
<td>II</td>
<td>6 (16%)</td>
<td>8 (11%)</td>
<td>14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency</th>
<th>D</th>
<th>I</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephroangiosclerosis</td>
<td>52 (70%)</td>
<td>22 (30%)</td>
<td>74</td>
</tr>
<tr>
<td>Controls</td>
<td>86 (57%)</td>
<td>64 (43%)</td>
<td>150</td>
</tr>
</tbody>
</table>

\( \chi^2 \) tests were performed for nephroangiosclerosis vs. controls for analyses of genotype distributions and allele frequencies.

\( ^a P < 0.001, ^b P < 0.05 \)

**Table 2.** Clinical and renal features of hypertensive patients from protocol 2 grouped by genotype

<table>
<thead>
<tr>
<th>N</th>
<th>Age years</th>
<th>Sex M/F</th>
<th>SBP mm Hg</th>
<th>DBP mm Hg</th>
<th>PRA ng·ml⁻¹·hr⁻¹</th>
<th>Duration of HT years</th>
<th>Microalbuminuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>25 (33%)</td>
<td>15/10</td>
<td>178 ± 3</td>
<td>111 ± 2.5</td>
<td>0.56 ± 0.15</td>
<td>9 ± 1</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>DI</td>
<td>44 (59%)</td>
<td>27/17</td>
<td>188 ± 4</td>
<td>109 ± 2</td>
<td>0.43 ± 0.08</td>
<td>11 ± 1</td>
<td>6 (43%)</td>
</tr>
<tr>
<td>II</td>
<td>6 (8%)</td>
<td></td>
<td>186 ± 11</td>
<td>107 ± 5</td>
<td>0.25 ± 0.15</td>
<td>8 ± 2</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>GFR ml/min/1.73 m²</th>
<th>ERPF ml/min/1.73 m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>104 ± 5</td>
<td>434 ± 12</td>
</tr>
<tr>
<td>DI</td>
<td>92 ± 7</td>
<td>420 ± 14</td>
</tr>
<tr>
<td>II</td>
<td>100 ± 2</td>
<td>522 ± 2</td>
</tr>
</tbody>
</table>

Abbreviations are: NS, not significant; M, male; F, female; SBP, systolic blood pressure; DBP, diastolic blood pressure; PRA, plasma renin activity; HT, hypertension; UAE, urinary albumin excretion; GFR, glomerular filtration rate; ERPF, effective renal plasma flow. One-way ANOVA was performed to compare group means for different parameters.
and pathological diagnosis of nephroangiosclerosis was fairly unequivocal in our patients, it has to be considered that the presence of proteinuria with a normal sediment in a patient with essential hypertension is not a routine indication of renal biopsy. Therefore, in spite of the striking association found between the D allele with nephroangiosclerosis, our retrospective sample of patients with nephroangiosclerosis may not be representative of the general hypertensive population with milder clinical presentations of kidney organ damage. Although there is a clear predominance of males in our nephroangiosclerosis group, a difference in the distribution of genotypes among sex groups has not been observed in this or any other study.

In a separate protocol, we investigated the functional renal profile of a group of hypertensives. It is a widely accepted notion that microalbuminuria in essential hypertensive patients may be secondary to the renal functional abnormalities that accompany hypertension. ACE inhibition has been demonstrated to be effective in reducing the urinary excretion of albumin in several diseases [18], but it has been difficult to prove a relationship between microalbuminuria and specific changes in the renal hemodynamics observed in some patients with EH [25]. Although microalbuminuria is not completely proven to be a risk factor for the future development of proteinuria and worsening of renal function in nondiabetic patients with EH, there is evidence indicating that it is a marker of increased cardiovascular risk in these patients [26]. Interestingly, an association between the D allele of the ACE gene with microalbuminuria [27, 28] as well as retinopathy and left ventricular hypertrophy has recently been reported, with results suggesting that the deletion polymorphism might be an independent risk factor for the development of organ damage in hypertensive patients [27].

The lack of association between ACE I/D polymorphism and BP observed in our study is in agreement with most reported data [14] and supports the notion that the mechanisms of kidney damage by the RAS may be mediated by mechanisms probably additive to the pressor, hemodynamic effect. In protocol 2 we were not able to demonstrate any relation between renal functional parameters and the ACE genotype, probably because these parameters were within the normal range in our hypertensive patients. Similarly, the distribution of genotypes was not different between hypertensives with or without microalbuminuria, probably due to the low number of patients with this abnormality. We can only speculate about the possible link between the DD genotype of the ACE gene with the presence of renal damage in hypertension. The ACE I/D polymorphism has been associated to both plasma and tissue ACE levels, the DD genotype being the one related to the highest levels and the II to the lowest [10]. It has been assumed that the tissue ACE levels determine the local production of angiotensin II. Since all the components of the RAS have been detected in the kidney [3], subjects with the DD genotype might have higher local activity of ACE and higher levels of angiotensin II that could lead to increased intraglomerular pressure, stimulate different proto-oncogenes and growth factors inducing cell hypertrophy and proliferation, and increases in cell matrix protein. It is important to note that a molecular variant of the ACE gene determines plasma ACE activity in congenic strains of rats, demonstrating that a quantitative trait locus confers concordant effects in humans and rats [29]. However, data from a family study suggest that the I/D polymorphism may be in fact a genetic marker strongly associated with an unknown functional variant (ACE S/s) [30] located within or near the ACE gene and which explains a large portion of the interindividual variance of plasma ACE levels. Therefore, the molecular mechanism by which variants in the ACE gene may influence target organ damage in hypertension is still unknown.

In conclusion, the present study demonstrates the presence of an association between the I/D polymorphism of the ACE gene with biopsy-proven renal involvement occurring in hypertensive patients. This suggests that genetic factors may be important in determining the renal damage observed in patients with essential hypertension and that the DD genotype of the ACE gene could be a useful genetic marker with important clinical, therapeutic and prognostic implications in recognizing hypertensive subjects that are at greater risk of renal damage. However, larger prospective studies will be necessary to assess the role of the DD genotype in the renal outcome in essential hypertensive patients with and without renal involvement.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin converting enzyme; BP, blood pressure; DBP, diastolic blood pressure; EH, essential hypertension; ERPF, effective renal plasma flow; FF, filtration fraction; GFR, glomerular filtration rate; I/D, insertion/deletion; PCR, polymerase chain reaction; RAS, renin-angiotensin system; SBP, systolic blood pressure; UAE, urinary albumin excretion rate.

REFERENCES

23. SHANMUGAM V, SELL KW, SAHA BK: Mistyping ACE heterozygotes. PCR Meth Appl 3:120–121, 1993

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