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ASSOCIATION OF A POLYMORPHISM OF THE ANGIOTENSIN I-CONVERTING ENZYME GENE WITH ESSENTIAL HYPERTENSION

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SUMMARY: **Angiotensin I-converting enzyme (ACE) is responsible for production of angiotensin II and breakdown of kinins, leading to increased blood pressure (BP). Furthermore, ACE inhibitors are effective anti-hypertensive agents. A 287 bp insertion/deletion polymorphism in intron 16 of the ACE gene (*ACE*) was examined by PCR in a cross-sectional study of 80 hypertensive (HT) and 93 normotensive (NT) subjects whose parents had a similar BP status at age ≥50. The frequency of the insertion allele was 0.56 in HTs and 0.41 in NTs, and the difference between observed alleles in all subjects in each group was significant (χ^2 = 7.6, P<0.01). The data thus provide evidence in favour of an association of HT with a polymorphism at the *ACE* locus (17q23), so implicating this locus, and possibly a genetic variant of *ACE* itself, in human essential hypertension.**

The relationship of the renin-angiotensin system with essential hypertension (HT) has been studied extensively over many years, but firm conclusions about a causal role have not been forthcoming [1]. The biologically active effector hormone of the system, angiotensin II, is produced in the bloodstream and regional vascular beds by a dual enzyme cascade involving renin, secreted by the kidney, and angiotensin I-converting enzyme (*ACE*), a membrane-bound dipeptidyl caboxypeptidase ectoenzyme located in the endothelial lining of blood vessels throughout the body [2]. The early cloning of the human renin gene (*REN*) [3] permitted initial molecular genetic examination of the involvement of *REN* in essential HT, where genetic factors are regarded as predominating over environmental influences in onset of the disease [4,5]. Studies to date have, however, failed to show association or linkage of restriction fragment length polymorphisms (RFLPs) of *REN* with essential HT in caucasian populations [6-10].

The second enzyme, ACE, is an effective target for antihypertensive medication in HT patients. ACE inhibitors also prevent the development of HT

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in the spontaneously HT rat (SHR), in which increase in blood pressure (BP) with age is accompanied by a selective rise in vascular ACE activity that appears to play a pathogenic role in the HT [11]. Recently, studies examining a large collection of polymorphisms in crosses of the stroke-prone SHR (SHRSP) with normotensive WKY rats have mapped a major effect on BP to a locus that is closely linked to the rat ACE gene [12,13]. This part of the rat genome has synteny to human chromosome 17, so that a human homolog of the gene responsible might be expected to lie in the same region, where the human *ACE* locus is at 17q23 [14]. In a study of 80 normotensive (NT) caucasian subjects a *Hin*dIII insertion/deletion RFLP of *ACE* was found to account for 47% of the total phenotypic variance of serum ACE activity [15]. The latter is a probable index of vascular wall ACE concentration [2], which in turn may be largely determined by the level of gene expression, so that the polymorphism has been suggested as being in linkage disequilibrium with *ACE* regulatory elements [15].

The present report describes results of a cross-sectional study in which we used a polymerase chain reaction (PCR) technique [16] to detect the 287 bp insertion/deletion polymorphism located in intron 16 of *ACE* [16,17] in groups of HT and NT subjects. In order to reduce the environmental component of HT and to help overcome problems of penetrance we tested two genetically predisposed populations, viz. the HT offspring of HT parents and, as control, the NT offspring of NT parents.

METHODS

Study design: Adult caucasian subjects were used for the study. Those in the HT group had been diagnosed according to conventional criteria, which included a diastolic pressure of >90 mmHg and a systolic pressure of >140 mmHg on 3 occasions spanning 2 months. Since the study was directed at molecular analysis of genomic DNA structure rather than physiological parameters, the only criteria for selection of patients was that they had been correctly diagnosed as having essential HT and that they were the offspring of two HT parents. NT subjects were selected, with the aid of the Sydney Red Cross Blood Bank, on the basis of both parents being NT at age ≥50. Although obtaining sufficient numbers of subjects in each category was made considerably more difficult by the rigid entry criteria we applied, such a course was regarded as necessary to improve the statistical power of the analyses. The age of subjects in each group did not differ significantly: for the HT patients it was 50 ± 14 S.D. (range 22-82 years) and for NT subjects it was 46 ± 9 (range 30-71 years). Blood samples of ~20 ml were drawn from the antecubetal fossa of each person, placed in heparinized tubes, and white cells were separated and stored at –70°C prior to DNA extraction.

PCR method for detection of ACE polymorphism: DNA was isolated from peripheral blood leukocytes as described previously [18] and 80 ng was used in a PCR under rigorous conditions [19] to avoid the possibility of results arising from PCR-product carry-over. The sense oligonucleotide primer was 5' CTG GAG ACC ACT CCC ATC CTT TCT 3' and the antisense primer was 5' GAT GTG GCC ATC ACA TTC GTC AGA T 3' [16]. Reactions were performed in a final total volume of 50 µl containing 10 pmol of primer, 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 0.1 mg/ml gelatin, 0.5 mM of each dNTP, and 1 unit of *Taq*

polymerase (Perkin-Elmer/Cetus, Norwalk, CT). Amplification was carried out in a Perkin-Elmer/Cetus DNA Thermal Cycler for 30 cycles with steps of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. PCR products were electrophoresed in agarose gels and DNA was visualized by ethidium bromide staining.

Statistical analysis: Data were compiled according to genotype, and allele frequencies were calculated. Statistical analysis required calculation of observed number of alleles from genotype data in each group. The difference between groups was then tested by χ^2 analysis with 1 degree of freedom.

RESULTS

The polymorphism detected by the PCR was evident as a 490 bp PCR product in the presence of the insertion and as a 190 bp fragment in the absence of the insertion. Thus each DNA sample yielded one of three possible patterns after electrophoresis: for each homozygote, either a 490 bp band or a 190 bp band was seen, and for heterozygotes both a 490 bp band and a 190 bp band appeared (Fig. 1). In addition, as seen by others [16], a smaller quantity of a third fragment was present in PCR products from heterozygotes and has been suggested to correspond to a hybrid.

Genotype and derived allele frequencies are shown in Table 1. Most notable in the case of genotype data was a 50% reduction in 'D D' homozygote frequency in the HT group compared with NTs and a 45% increase in 'I I'. In examining data for NT and HT groups the correct data for analysis was the observed

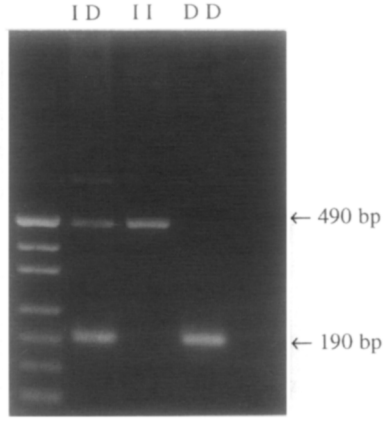

Fig. 1. Polymorphism of angiotensin I-converting enzyme gene demonstrated using PCR. Shown are examples of the 3 patterns of PCR products seen after staining gels with ethidium bromide. These represent the 3 genotypes for the *ACE* insertion/deletion polymorphism examined, viz. 'I I', 'D D', and 'I D' for the 490 bp insertion ('I') homozygote, the 190 bp deletion ('D') homozygote, and the 490-bp/190-bp insertion/deletion ('I'/'D') heterozygote, respectively. The first lane shows size markers (pUC19 cut with *HpaII*) and the last lane shows results for negative control in which PCR was carried out in the absence of genomic DNA.

Table 1. Genotype and allele frequencies for the insertion/deletion polymorphism of *ACE* in essential hypertensive (HT) and normotensive (NT) groups

		Frequencies						
		Genotypes			Alleles		Total alleles on all chromosomes	
	n	I I	I D	D D	I	D	I	D
HT	80	26	38	16	0.56	0.44	90	70
NT	93	21	35	37	0.41	0.59	77	109
							$\chi^2 = 7.6$ ($P<0.01$)	

numbers of alleles, i.e., the sum of the number of alleles on each chromosome of all of the subjects in each group, calculated as described previously [20]. Statistical analysis of this data (Table 1) showed that values for the HT group differed significantly from those for the NT group ($\chi^2 = 7.6$; $P<0.01$).

DISCUSSION

Analysis by PCR of a 287 bp insertion/deletion polymorphism in intron 16 of the 21 kb, 26 exon human angiotensin I-converting enzyme gene [16,17] has demonstrated a statistically significant association of this polymorphism with essential HT. It is unlikely that this is a spurious association arising by random chance or is due to imperfect matching of HT and NT groups, since the frequency of the minor ('I') allele in our NT group, 0.41, was similar to a value of 0.43 reported by Rigat *et al.* for a group of 199 unrelated individuals in France [16]. This group presumably contained a minor proportion of HT individuals. Since 20% of the adult population have HT and, on the basis of our data, have an 'I' allele frequency of 0.56, the slightly higher 'I' allele frequency found by Rigat *et al.* is consistent with our findings. These workers found, moreover, co-dominant segregation of the *ACE* polymorphism in 50 nuclear families [16]. The present data suggest that possession of the insertion ('I') allele might be a marker for predisposition to HT, although, since not all 'I I' genotypes were hypertensive, the 'I' allele alone would appear to be insufficient, i.e., there is likely to be a co-requirement of predisposing alleles of other genes elsewhere in the genome. Alternatively, other loci could act to oppose the development of HT in certain 'I I' individuals.

Since the insertion/deletion in *ACE* is in an intron, and there is no evidence to date for differential splicing that might incorporate this sequence into a primary transcript, the polymorphism itself would be unlikely to influence the dipeptidyl carboxypeptidase activity of the encoded enzyme. It also remains to be shown whether this DNA might influence the splicing of primary transcripts that leads to mature mRNA. Another possibility is that the insertion might

contain an enhancer that influences transcriptional activity. In this regard it is of interest that there is also a smaller ACE, so far found only in testis, which arises from use of a promoter in intron 12 [17]. Since the insertion is in intron 16 [16], which spans ~1.9 kb of DNA [17], such an enhancer could affect either the proximal promoter located 2.2–4.1 kb upstream or that positioned 9.9–11.8 kb 5'. Alternatively, the polymorphism may be a linkage marker of change(s) elsewhere that have a critical influence on ACE active site conformation, substrate recognition, or catalysis, or of differences in other, yet-to-be identified DNA elements that control *ACE* transcription in HT patients.

The other possibility is that the genetic variant responsible for HT is another gene located not far from the ACE gene. The *ACE* locus, at 17q23, is near that of the growth hormone gene (*GH*), at 17q22-q24 [21], raising the possibility that the *ACE* polymorphism is in linkage disequilibrium with a *GH* variant. Growth hormone (GH) enhances overall somatic growth, so that a genetic variation in GH production could increase the rate and extent of growth of cardiac and vascular smooth muscle cells or regional vascularization at an early age, so as to increase responsiveness to environmental stimuli later in life. Indeed, increased heart weight and thickened vessel walls have been seen in very young SHRs, leading to a conclusion that cardiovascular hypertrophy may precede HT [22,23]. In SHRSP x WKY crosses, linkage analyses of microsatellite markers in a F_2 population placed the '*RatACE*' locus outside the interval defined by markers for the '*RatGH*' locus at a recombination distance of 2% and demonstrated significant linkage of the *RatACE* locus with BP [12]. *RatACE* fell within a region of 10:1 odds from the gene influencing BP in analysis of BP after NaCl-loading and within a region of 100:1 odds for basal BP phenotype [12]. Furthermore, a polymorphism detected in *RatACE* by PCR was used to demonstrate close linkage of this gene to a dominant locus causing HT [13].

Although a noticeable difference in plasma angiotensin II has not been seen in essential HT, differences in the operation of local renin-angiotensin and kallikrein-kinin systems in regional vascular beds in HT, as well as in other tissues such as the adrenal and CNS [24,25], may not be reflected in circulating angiotensin concentrations. Furthermore, ACE acts on a variety of peptide substrates [26], so its action that leads to HT could involve some other peptide.

The present finding for *ACE* extends our previous work which showed a significant association of a RFLP of the insulin receptor gene with HT [20]. It is expected, however, that more than two genes contribute to the disease [4,5], so that further work is needed to identify all of the loci that together cause HT.

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