Angiotensin converting enzyme and endothelial nitric oxide synthase DNA polymorphisms and late onset Alzheimer's disease

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Abstract
Objectives—Several lines of evidence suggest that the endothelial constitutive nitric oxide synthase (ecNOS) and angiotensin converting enzyme (ACE) may have a role in Alzheimer's disease. ACE is widely expressed in the brain, and a DNA polymorphism at the ACE gene has been linked to the risk for late onset Alzheimer's disease. Nitric oxide (NO) production by microglial cells, astrocytes, and brain microvessels is enhanced in patients with Alzheimer's disease. There is a growing evidence that NO is involved in neuronal death in Alzheimer's disease, and the oxidative stress caused by NO in the brain could be a pathogenic mechanism in Alzheimer's disease. The objective was to determine if two DNA polymorphisms at the ecNOS and ACE genes that have been linked with different levels of enzyme expression, have some effect on the risk of developing late onset Alzheimer disease.
Methods—A total of 400 healthy controls younger than 65 years and 350 patients with Alzheimer's disease (average age 72 years) were genotyped for the ACE and ecNOS polymorphisms. To define a possible role for these polymorphisms in longevity 117 healthy controls older than 85 years were also analysed. Genomic DNA was obtained and amplified by polymerase chain reaction, and genotypes were defined following a previously described procedure. Gene and genotype frequencies between patients and controls were compared statistically.
Results—Gene and genotype frequencies for the ecNOS and ACE polymorphisms did not differ between both groups of healthy controls (<65 years and >85 years). EcNOS gene and genotype frequencies were similar between patients and controls. There was a slight but significantly increased frequency of the ACE-I allele among patients with Alzheimer's disease compared with controls (p=0.03; OR=1.28, 95%CI= 1.04;1.58).
Conclusions—The ACE-I allele was associated with a slightly increased risk of developing late onset Alzheimer's disease.
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Angiotensin converting enzyme (ACE) is a major component of the renin-angiotensin system. The enzyme catalyses the conversion of angiotensin I to angiotensin II (Agt II). Agt II exerts its biological functions through binding to two receptors, AT1R and AT2R. A polymorphism at intron 16 of the ACE gene (also known as the DCP1 gene), consisting in an insertion/deletion (I/D) of a 287 base pair sequence, is associated with ACE concentrations in blood, and the DD genotype has been linked to an increased risk for myocardial infarction and left ventricular hypertrophy.[1–4]

Several lines of evidence suggest that the renin-angiotensin system may have a role in Alzheimer's disease. Angiotensin converting enzyme is localised in the endothelium of cerebral blood vessels, epithelial cells of the choroid plexus, and the plasma membranes of astrocytes in the circumventricular organs.[5 6] In addition, ACE is expressed in neurons in the paraventricular and supraoptic hypothalamic nuclei and the dorsal vagal complex.[7 8] AgtII in the brain modulates pressor response, body fluid and electrolyte balance, neurohumoral function, and behaviour.[9-11] Apart from these actions through Agt II formation, ACE in the brain can also modulate central dopamine turnover, suggesting a possible involvement in Parkinson's disease, a movement disorder associated with a loss of dopamine synthesising neurons.[12] In accordance with a role for the renin-angiotensin system in Alzheimer's disease, the ACE-I allele has been associated with an increased risk of developing late onset Alzheimer's disease.[13]

Nitric oxide (NO) is synthesised from L-arginine by NO synthase (NOS).[14] Three isoforms of NOS have been described: inducible NOS, constitutive neuronal NOS, and endothelial constitutive NOS (ecNOS). There is a growing evidence that NO is involved in neuronal death in Alzheimer's disease. Brain microvessels in Alzheimer's disease produce high concentrations of NO.[15 16] Also, β amyloid protein (Aβ), the major component of the senile plaques that characterise Alzheimer's disease, induces NO production by microglial cells and astrocytes.[17–20] In addition, apolipoprotein E (APOE) stimulates NO production in monocyte derived macrophages,[21] a model of brain microglia, and it is well recognised that APOE plays a part in the development of Alzheimer's disease, with the APOE-ε4 allele being a major risk factor for late onset Alzheimer's disease. The oxidative stress

caused by NO production in the brain has been proposed as a pathogenic mechanism in Alzheimer's disease.[22]

In end stage Alzheimer's disease, ecNOS expression is strikingly increased in glial cells, which are responsible for maintaining the structural and functional integrity of the CNS.[16] The ecNOS is encoded by a gene located on chromosome 7q and comprises 26 exons.[23] Recently, a 27 bp repeat in intron 4 (the ecNOS4 polymorphism) has been associated with essential hypertension and coronary artery disease. Two alleles, of five and four repeats, have been described, and the five repeat allele would be at a higher frequency among patients with coronary artery disease compared with healthy controls.[24] In addition, the four repeat allele could be associated with essential hypertension.[25] The ecNOS4 polymorphism has been linked to different plasma NO concentrations, with 5/5 subjects having significantly higher concentrations than 4/5 and 4/4 subjects.[26]

To define a possible role for the ecNOS and ACE polymorphisms in late onset Alzheimer's disease we genotyped 350 patients that fullfilled the NINCDS-ADRDA criteria for clinically probable Alzheimer's disease, and 400 healthy controls younger than 65 years, from the same white population (Asturias, northern Spain). To define a possible role for both polymorphisms in longevity, we also analysed 117 healthy controls older than 85 years.

Methods

PATIENTS AND CONTROLS

DNA was obtained from 350 patients who fullfilled the NINCDS-ADRDA criteria for clinically probable Alzheimer's disease. A total of 400 healthy controls aged 65 years or younger were also analysed. These controls were blood bank donors, hospital staff, and eligible residents, and were recruited for the analysis of genetic factors involved in the risk of cardiovascular and Alzheimer diseases.[4] To define an association between the ecNOS and ACE polymorphisms and longevity, we also analysed 117 healthy controls aged 85 years or older. The presence of Alzheimer's disease in these healthy controls was excluded through a CAMCOG-CAMDEX test. Informed consent was obtained from people participating in this study.

ecNOS GENOTYPING

Genomic DNA was amplified by polymerase chain reaction(PCR) with primers 5'-CTA TGGTAGTGCCTTGGCTGGAGG and 5'-ACCGCCCAGGGAACTCCGCT. The PCR consisted of 30 pmol of each primer, 2 mM deoxynucleotide triphosphate, 2mM $MgCl_2$, 1×buffer, and 0.5 units Taq DNA polymerase, in a total volume of 30 µl. After an initial denaturation for 2 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 1 minute at 62°C, and 1 minute at 72°C, 10 µl was electrophoresed on a 3% agarose gel.

ACE GENOTYPING

For the analysis of the ACE-I/D polymorphism, genomic DNA was PCR amplified with primers 5'-CTGGAGACCACTCCCATCC TTTCT-3' and 5'-GATGTGGCCATCACA TTCGTCAGAT-3' (anealing at 58°C), as described for the ecNOS genotyping. Allele sizes were 490 bp (I) and 190 bp (D). By using these primers flanking the insertion allele, mistyping of the DD genotype may occur. Therefore, we confirmed each DD genotype by using an insertion specific primer, following a previously described procedure.[27]

STATISTICAL ANALYSIS

Differences between allele and genotype frequencies were assessed using a χ^2 test (with Yates' correction), and p≤0.05 was considered as significant. Odds ratios (ORs) and their 95% confidence intervals (95% CIs) were also calculated, using the computer program BMDP New Systems (BMDP Statistical Software, Cork, Ireland).

Results

We genotyped 350 patients with late onset Alzheimer's disease (average age 72 (SD 9) years; 195 women, 155 men), and 400 healthy controls younger than 65 years (range 21 to 65), for the ecNOS and ACE polymorphisms.

ACE genotype frequencies were in Hardy-Weinberg equilibrium in patients and controls. Patients showed a significantly increased frequency of the ACE-I allele compared with controls (0.43 and 0.38, p=0.03, table). Thus the ACE-I allele would confer a slightly increased risk of developing late onset Alzheimer's disease in our population (OR=1.28; 95%CI 1.04–1.58). Carriers of the I allele (II+ID) were at a slightly but non-significantly increased frequency in patients compared with controls (p=0.058; OR=1.35; 95%CI 0.99–1.82). Both groups of healthy controls, older than 85 and younger than 65 years, had similar genotype and gene frequencies, suggesting that this polymorphism is not associated with longevity in our population (table).

In addition to the four and five repeat ecNOS alleles, we identified two new alleles, of three and six repeats (figure). EcNOS genotype frequencies were in Hardy-Weinberg equilibrium in patients and controls, and are summarised in table. Gene and genotype frequencies did not differ between patients and controls. No difference was found between controls younger than 65 years or older than 85, indicating that the ecNOS polymorphism is not involved in longevity in our population.

Patients were also genotyped for the APOE (ε2, ε3, ε4) polymorphism, and a total of 166 patients (48%) were APOE-ε4 carriers. ACE and ecNOS genotypes were at similar frequencies between carriers and non-carriers of APOE-ε4 (data not shown).

Discussion

There is a growing evidence that the renin-angiotensin system plays a part in the pathogenesis of Alzheimer's disease. ACE, a major component of the renin-angiotensin system, is widely expressed in the brain, and has been implicated in cognitive processes.[28] Several lines of evidence suggest that ACE may play a

Frequencies of the ACE and ecNOS polymorphisms in patients with Alzheimer's disease, healthy controls younger than 65 years, and healthy controls older than 85 years.

	ACE					ecNOS				
	Genotypes			Alleles#		Genotypes			Alleles	
	DD	ID	II	D	I	55	45	44	5	4
Patients (%)	119 (34)	161 (46)	70 (20)	0.57	0.43	273 (78)	70 (20)	7 (2)	0.85	0.15
Mean age (SD)	72 (9)	72 (8)	72 (7)			71 (10)	72 (9)	71 (8)		
Controls (%)	164 (41)	176 (44)	60 (15)	0.62	0.38	296 (74)	97 (24)	7 (2)	0.86	0.14
Controls >85 (%)	43 (37)	58 (49)	16 (14)	0.62	0.38	86 (73)	29 (25)	2 (2)	0.86	0.14

In addition to the four and five repeat ecNOS alleles, there were also two rare alleles of three and six repeats Two patients were ecNOS-56 and three controls were ecNOS-35). The ACE-I allele was at a significantly higher frequency in patients (Yates' χ^2, p=0.03, OR=1.28 (95% CI 1.04-1.58)
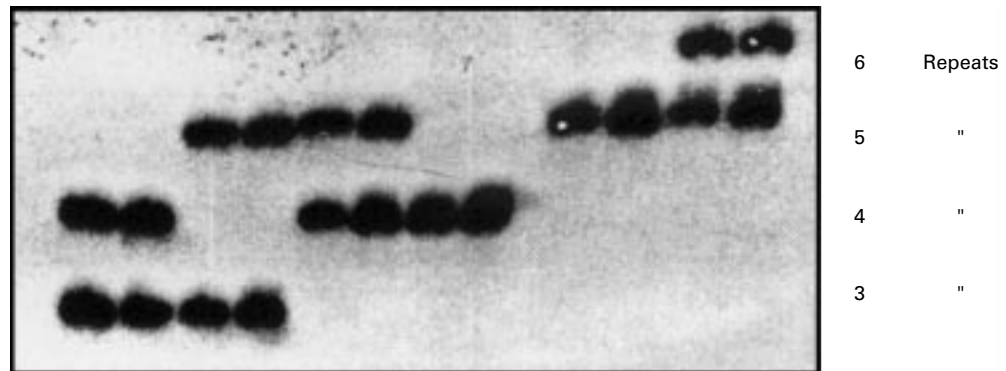
Genotypes for the ecNOS polymorphism. In addition to the four and five repeat alleles, previously described, we found two new rare alleles of three and six repeats.

part in Alzheimer's disease by modulating inflammation.[29] In accordance with this, an increased frequency of the I allele among patients with Alzheimer's disease has been described by Kehoe *et al.*[13] These authors analysed three different populations (Cardiff, London, and Belfast), and found an increased frequency of the I allele: ORs ranged from 1.82 to 2.71. We also found an association between the I allele and Alzheimer's disease in our population. However, the difference between patients and controls was less significant in our population, with an OR for the presence of the I allele of 1.28. The fact that a similar result has been found in four different populations suggests that the ACE-I allele is truly involved in the development of Alzheimer's disease.

The ACE-I/D polymorphism has been associated with longevity.[30] To define a possible role of this polymorphism in longevity as a consequence of a decreased risk of developing Alzheimer's disease, we genotyped 117 healthy controls older than 85 years. Gene frequencies were almost identical between controls younger than 65 and older than 85 years, indicating that the ACE-I/D polymorphism is not related with longevity in our population.

The oxidative stress caused by NO in the brain has been proposed as a pathogenic mechanism in Alzheimer's disease. EcNOS, one of the enzymes involved in NO synthesis, is expressed at increased concentrations in glial cells from patients with Alzheimer's disease.[15 16] In addition, NO production by microglial cells is enhanced by β amyloid.[17–20] The ecNOS polymorphism analysed in this study has been linked to different plasma NO concentrations.[26] Thus subjects homozygous for the five repeat allele would produce more ecNOS, having higher NO concentrations. Taken together, these data suggest that the oxidative stress that characterises Alzheimer's disease could be genetically regulated, and the ecNOS polymorphism could modulate the susceptibility to develop Alzheimer's disease. However, our data indicate that this polymorphism is not linked to an increased risk of developing Alzheimer's disease.

A recent large genome scan showed that Alzheimer's disease is a genetically complex disorder involving many genes.[31] One gene that is clearly implicated is that encoding APOE.[32] Other genes, such as those encoding α-2 macroglobulin and lipoprotein receptor related protein, have also been linked to Alzheimer's disease.[33 34] Our work suggests that the ACE gene is also involved in late onset Alzheimer's disease, and warrants further studies in other populations. DNA polymorphisms at other renin-angiotensin system components (angiotensinogen and angiotensin receptors) have been described. Studies directed to define a possible role for these polymorphisms in the risk of developing Alzheimer's disease would be of special interest.

Finally, the treatment of several diseases involves drugs that target the renin-angiotensin system components, such as ACE inhibitors and AT1R antagonists. If the genetic studies finally confirm the involvement of the renin-angiotensin system in Alzheimer's disease, the pharmacological targeting of the components of this system could be a new promising approach for the treatment of this common progressive neurodegenerative disorder.

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