Genetic Factors Associated With the Absence of Atherosclerosis in Octogenarians

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Background. Atherosclerosis (ATS) is a common age-related disease of large arteries. The prevalence of older subjects with vascular successful aging (VaSA), defined as the absence of clinical symptoms and instrumental signs of ATS, is low in Western countries. The possible contribution of genetics to the VaSA phenomenon is not known.

Methods. We investigated the distribution of four genetic polymorphisms (angiotensin converting enzyme [ACE], methylenetetrahydrofolate reductase [MTHFR], apolipoprotein E [apo E], and paraoxonase [PON] genes) in 30 subjects with VaSA, 30 subjects with moderate carotid atherosclerosis (ATS group), and 161 controls with a negative history for cardiovascular disease. Clinical examination; ultrasonographic examination of carotid, vertebral, abdominal aortic, iliac, and femoral arteries; and electrocardiogram were performed.

Results. The frequency of PON 192 B allele was lower in VaSA patients (13%) compared with ATS patients (37%) and controls (46%) (p = .06 and .006, respectively); B/B homozygotes were 27% in the ATS group, 12% in controls, and 0% in the VaSA group. The frequency of the MTHFR thermolabile + allele was higher in VaSA (0.51) compared with ATS (0.39) and controls (0.40) (VaSA vs C, p = .006). No differences in the distribution of ACE I/D and apo E alleles emerged between the three groups.

Conclusions. The low prevalence of the PON 192 B allele in the VaSA subjects suggests that this polymorphism might have an important role in VaSA, probably by hydrolyzing lipid peroxides and thus preventing low-density lipoprotein from undergoing the oxidative modification. This finding further supports the oxidative hypothesis of ATS.

Atherosclerosis (ATS), a progressive age-related disease of the large arteries characterized by the accumulation of lipids and fibrous elements, is the primary cause of coronary heart disease (CHD) and stroke, and represents the underlying cause of about 50% of all deaths in Western countries (1). The prevalence of ATS increases significantly with age, and only a few older individuals have no evidence of ATS when evaluated at one vascular site, for instance the carotid arteries (2). The percentage of older subjects without ATS in several vascular districts is not known, but it is conceivably much smaller. This highly selected group might represent a useful model to investigate the factors that protect against the development of ATS and allow the individual to achieve successful aging at the vascular level (3). In a previous study, we found that very old ATS-free subjects have higher plasma levels of vitamin E and lower levels of low-density lipoprotein (LDL) oxidation compared with age-matched controls with documented ATS (4). However, epidemiological studies have shown that not only environmental but also genetic factors contribute to the development of the atherosclerotic plaques. Indeed, it has been suggested that the fraction of disease explained by genetics within a population is high and often exceeds 50% (1).

ATS is considered a polygenic disease, and, in the past few years, a large number of genetic risk factors have been reported, including the polymorphisms in angiotensin converting enzyme (ACE) (5), methylenetetrahydrofolate reductase (MTHFR) (6), apolipoprotein E (apo E) (7), and paraoxonase (PON) genes (8). ACE converts angiotensin I into angiotensin II, a potent vasopressor peptide, and also inactivates bradykinin (9). The ACE gene has a common insertion/deletion (I/D) polymorphism in intron 16 (5), and the D allele has been associated with CHD and left ventricular hypertrophy in adult populations (9). MTHFR is a key enzyme in the metabolism of homocysteine, and elevated plasma levels of this amino acid have been associated with the development of ATS (10). A relatively common point mutation at nucleotide 677 of the MTHFR gene is responsible for a thermolabile variant of the enzyme that has been associated with increased homocysteine levels (10). Apo E is a polymorphic plasma protein that modulates the metabolism of triglyceride-rich atherogenic lipoproteins (11). A growing body of evidence indicates that apo E polymorphism plays a significant role in the susceptibility to developing ATS; indeed, individuals bearing the e4 allele have higher total cholesterol levels and an increased risk of developing CHD (7). PON is a high-density lipoprotein (HDL)-associated enzyme that might protect people from...
ATS by providing protection against the oxidative modification of LDL (12). A common mutation in the PON gene (192 Gln-Arg) affects the PON activity (13) and has been associated with cardiovascular disease (14).

No data are available concerning the genetic background associated with the absence of ATS in elderly subjects. In the present study, we compared the distribution of ACE, MTHFR, apo E, and PON gene polymorphisms in a sample of strictly selected older subjects without any clinical or instrumental evidence of ATS (vascular successful aging group—VaSA), in a group of older subjects with documented carotid ATS (ATS group), and in an older control population. We hypothesized that the “atherogenic” alleles of these candidate genes might be less prevalent in the VaSA group.

METHODS

Subjects

In the study period (1997–1998), 705 older community-dwelling subjects aged 75 years and older underwent duplex scanning; of these, 637 were not eligible to participate in the study because they met one or more of the exclusion criteria, and eight refused to participate. A total of 60 subjects, 30 with “vascular successful aging” (VaSA group) and 30 with signs of carotid atherosclerosis (ATS group), were enrolled. The subjects included in the VaSA group were selected by the following criteria: (i) negative history for cardiovascular disease, (ii) absence of clinical symptoms and ultrasonographic/Doppler signs (duplex examination) of carotid and extracoronary (abdominal aortic, iliac, and femoral) ATS, for example, lack of any focal protrusion (1.5 mm), and (iii) absence of clinical symptoms and rest electrocardiogram (ECG) signs of CHD (defined as codes 1-1-1 through 1-3-6 of the Minnesota code and any T wave and ST changes consistent with ischemia).

Subjects enrolled in the ATS group were selected by the presence of carotid ATS (i.e., a plaque inducing a 30% to 50% stenosis). These subjects may have a positive history for cardiovascular disease, provided that no acute episode occurred in the three months before inclusion in the study. Exclusion criteria for VaSA and ATS groups were dementia, heart failure, thyroid disease (clinical and laboratory screening), renal or hepatic insufficiency, inflammatory diseases, malabsorption, hypolipemic diet, treatment with lipid lowering drugs, and use of iron or antioxidant vitamin supplementation in the 6 months before enrollment.

All the subjects underwent a clinical examination, including an evaluation of cognitive (Mini-Mental State Examination [MMSE]) (15) and functional status (Barthel index and instrumental activities of daily living). A resting ECG was also recorded and evaluated according to the Minnesota code (16). The duplex examination was performed using an Aloka SSD 650 (Aloka Co, Tokyo, Japan); the degree of stenosis was evaluated using both image and Doppler data (2).

To analyze the frequency of the polymorphisms, the VaSA subjects were also compared with a sample of 161 “healthy” community-dwelling subjects who were older than 65 years (mean age: 79.4 ± 6.7 years, women: 59%). These subjects (control group) had a negative history for cardiovascular disease and were free from cognitive/functional impairment and major diseases. They did not undergo duplex scanning. The inclusion of an older control group was necessary to get the “normal” distribution of the four polymorphisms in the reference population (it might differ significantly from other populations reported in literature); furthermore, we could not simply use a sample of adult subjects, as aging itself might have contributed to select (positively or negatively) some alleles.

The study was approved by the local ethics committee, and all participants gave informed consent. Arterial hypertension was defined as a blood pressure >140/90 mm Hg or a documented history of hypertension or the current use of antihypertensive drugs. Diabetes mellitus was defined as the presence of fasting blood glucose levels >126 mg/dl in two or more determinations or a documented history of diabetes or the current use of antidiabetic drugs or insulin.

DNA Extraction and Polymorphisms Detection

Genomic DNA was extracted from blood leukocytes by the salting out method.

The insertion/deletion polymorphism in intron 16 of the ACE gene was evaluated by polymerase chain reaction (PCR) as described by Rigat and colleagues (5), with the addition of 5% dimethyl sulfoxide. The PCR products were separated by electrophoresis in a 2% TBE agarose gel and were visualized by ethidium bromide staining under ultraviolet (UV) light.

The MTHFR 677 polymorphism (Ala to Val substitution) was detected by the method of Frosted and colleagues (6). The PCR product (198 base pairs) was digested with the enzyme Hinf I, separated in a 2% TBE agarose gel, and visualized by ethidium bromide staining under UV light.

Apo E genotype was evaluated by the method of Hixon and Vernier (17). The PCR product (244 base pairs) was digested with the enzyme Hha I, separated in a NuSieve 5% TBE gel, and visualized by ethidium bromide staining under UV light.

PON 192 polymorphism was detected as described by Sanghera and colleagues (18). The PCR product (100 bp) was digested with the enzyme Alw I, separated in a 3% NuSieve gel, and visualized by ethidium bromide staining under UV light. The + (B) and − (A) alleles corresponded to Arg and Gln at position 192, respectively (Figure 1).

Statistical Analysis

Means were compared by the unpaired t test or the Mann-Whitney test, as appropriate. Prevalences were compared by the chi-square test or Fisher’s exact test. Systat for Windows, version 5.0 (Systat, Inc, Evanston, IL), and SPSS for Windows, version 7.0 (SPSS, Inc, Chicago, IL) statistical packages were used.

RESULTS

The principal characteristics of the VaSA and ATS subjects are reported in Table 1. No differences emerged in age, MMSE score, Barthel index score, or total, LDL, and HDL cholesterol values. Female gender was more frequent in the VaSA group (82%) compared with ATS (50%) (p = .01), while hypertension was more frequent in the ATS group (70%) compared with the VaSA group (50%), but the difference...
was not significant. The prevalence of diabetes was 13.3% in the ATS group, while no VaSA subject was diabetic. The ATS group had more smokers (53.5%) compared with the VaSA group (20.5%) \((p < .01)\). Triglycerides were lower in the VaSA group compared with the ATS group \((p < .01)\).

Table 2 reports the frequencies of the PON 192, MTHFR 677, ACE I/D, and apo E gene polymorphisms in the VaSA and ATS groups and in controls.

The frequency of PON + (B) allele was lower in the VaSA group (13%) compared with the ATS group (37%) and controls (46%) \((p = .05\) and .006, respectively). The homozygotes for the B allele were 27% in the ATS group and 12% in controls, while no VaSA subject was BB homozygous.

The frequency of the MTHFR thermolabile + allele was higher in the VaSA group (0.51) compared with the ATS group (0.39) and controls (0.40) \((p = .006)\); the prevalence of + + homozygotes was also higher in the VaSA group (35%) compared with both the ATS group (10%) and controls (13%) \((p = .01)\).

No differences in the distribution of ACE gene I/D alleles emerged among the three groups; the frequency of the DD homozygotes was slightly higher in the ATS group (53.8%) compared with the VaSA group (42.8%) and controls (47.2%), but the difference was not significant.

The apo E alleles distribution was not different between the three groups. The frequency of the e4 allele was 0.11 in the VaSA group, 0.09 in the ATS group, and 0.085 in the control group. The frequency of the e2 allele was about double in the VaSA group (0.075) compared with the ATS group (0.03) and controls (0.035).

**DISCUSSION**

In this study, we tested the hypothesis that some genetic polymorphisms might be associated with “vascular successful aging,” defined as the absence of clinical symptoms and instrumental signs of ATS in very old, community-dwelling subjects. The most interesting finding is the low prevalence of the PON B allele in VaSA subjects.

Human PON is an ester hydrolase, associated in serum with HDL particles, which catalyzes the hydrolysis of certain nonphysiological substrates, such as paraoxon and phenylacetate, and is able to protect LDL from oxidation by hydrolyzing lipid peroxides in the lipoproteins (18). Human paraoxonase exists in two major allelic forms (A and B) that differ in the amino acid at position 192 (18). Originally, the B allele (Arg 192) was associated with a higher PON activity for hydrolyzing phenylacetate and paraoxon (13). However, it has been demonstrated that the PON active site required for LDL protection is not exactly equal to that involved in the paraoxonase activity and that PON Arg 192 is less effective in protecting LDL from oxidative modifications, thus providing a potential biological link between PON 192 polymorphisms and ATS (18). Moreover, oxidized LDL lipids can reduce the ability of PON to protect LDL against oxidation (20), and PON Arg192 seems to be more sensitive to lipid peroxides (19). The activity of PON is preserved by antioxidant flavonoids in vitro (20), and it is restored by vitamin E supplementation in smokers with a reduced pretreatment activity (21).

To our knowledge, this is the first observation of a significant association between PON polymorphism and the absence of ATS in older individuals.
Table 1. Principal Characteristics of the VaSA and ATS Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>VaSA</th>
<th>ATS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>80.9 ± 5.4</td>
<td>81.8 ± 5.6</td>
</tr>
<tr>
<td>Female gender (%)</td>
<td>82%**</td>
<td>50</td>
</tr>
<tr>
<td>Barthel index score</td>
<td>26.4 ± 2</td>
<td>26.6 ± 2.3</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>93.1 ± 10.6</td>
<td>90.7 ± 12.3</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>20.5**</td>
<td>53.5</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.0</td>
<td>6.4 ± 1.31</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.42 ± 0.45**</td>
<td>1.81 ± 0.61</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.92 ± 1.0</td>
<td>4.33 ± 0.9</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.72 ± 0.4</td>
<td>1.45 ± 0.4</td>
</tr>
</tbody>
</table>

Notes: VaSA = vascular successful aging; ATS = atherosclerosis; MMSE = Mini-Mental State Examination; LDL = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol. **p < .01.

Table 2. Distribution of PON 192, MTHFR 677, ACE I/D, and Apo E Polymorphisms in VaSA, ATS, and in Controls

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>VaSA</th>
<th>ATS</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON 192</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (+) allele</td>
<td>0.13*†</td>
<td>0.37</td>
<td>0.46</td>
</tr>
<tr>
<td>A (−) allele</td>
<td>0.87†</td>
<td>0.63</td>
<td>0.54</td>
</tr>
<tr>
<td>B/B and B/A</td>
<td>25%</td>
<td>47%</td>
<td>79%</td>
</tr>
<tr>
<td>B/B homozygous</td>
<td>0%</td>
<td>27%</td>
<td>12%</td>
</tr>
<tr>
<td>MTHFR 677</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ allele</td>
<td>0.51‡</td>
<td>0.39</td>
<td>0.40</td>
</tr>
<tr>
<td>− allele</td>
<td>0.49</td>
<td>0.61</td>
<td>0.60</td>
</tr>
<tr>
<td>+/− and +/-</td>
<td>68.2%</td>
<td>68.2%</td>
<td>68.3%</td>
</tr>
<tr>
<td>+/+ homozygous</td>
<td>35%**</td>
<td>10%</td>
<td>13%</td>
</tr>
<tr>
<td>ACE I/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D allele</td>
<td>0.64</td>
<td>0.69</td>
<td>0.68</td>
</tr>
<tr>
<td>I allele</td>
<td>0.36</td>
<td>0.31</td>
<td>0.32</td>
</tr>
<tr>
<td>D/D and D/I</td>
<td>85.7%</td>
<td>84.6%</td>
<td>88%</td>
</tr>
<tr>
<td>DD homozygous</td>
<td>42.8%</td>
<td>53.8%</td>
<td>47.2%</td>
</tr>
<tr>
<td>Apo E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e2 allele</td>
<td>0.075</td>
<td>0.03</td>
<td>0.035</td>
</tr>
<tr>
<td>e3 allele</td>
<td>0.815</td>
<td>0.88</td>
<td>0.88</td>
</tr>
<tr>
<td>e4 allele</td>
<td>0.11</td>
<td>0.09</td>
<td>0.085</td>
</tr>
<tr>
<td>3/4 and 4/4</td>
<td>23%</td>
<td>17%</td>
<td>15.3%</td>
</tr>
</tbody>
</table>

Notes: PON = paraoxonase; MTHFR = methylenetetrahydrofolate reductase; ACE I/D = angiotensin converting enzyme insertion/deletion; apo E = apolipoprotein E; VaSA = vascular successful aging; ATS = atherosclerosis. *p = .006 vs C. †p = .05 vs ATS. ‡p = .01 vs C.

Taken together, our data do not contradict the concept that ACE D and apo E e4 alleles increase the risk of developing ATS in adult populations; conversely, the lack of a reduction in their frequency in the VaSA subjects suggests that these two alleles might have only a minor role in the VaSA phenomenon.

Finally, two important limitations of the present study should be acknowledged. First, as a consequence of strict inclusion criteria, the number of VaSA subjects was very small, despite the fact that we screened a large number of individuals. Therefore, our results need to be replicated in a larger sample of older people. Second, because we did not measure PON activity in our subjects, we don’t know whether it was higher in the VaSA subjects or not. However, it is known that the PON 192 Arg allele is associated with a lower ability to inhibit LDL oxidation (20).

In conclusion, in a sample of older subjects, the PON 192 and apo E e4 alleles increase the risk of developing ATS in adult populations; conversely, the lack of a reduction in their frequency in the VaSA subjects suggests that these two alleles might have only a minor role in the VaSA phenomenon.

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M615

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REFERENCES


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