Interleukin-1 Receptor Antagonist Gene Polymorphisms in Carotid Atherosclerosis

Bradford B. Worrall, MD, MSc; Salman Azhar, MD; Paul A. Nyquist, MD; Robert H. Ackerman, MD; Theresa L. Hamm, RN; Thomas J. DeGraba, MD

Methods

Patients were recruited for 2 ongoing Institutional Review Board–approved studies designed to investigate the role of inflammation in carotid atherosclerotic plaque in a stroke-prone population. Controls were recruited from the community at large through print advertisement and from unaffected spouses. After informed consent was obtained, full medical history was obtained and a complete neurological examination was performed. Baseline information on stroke and transient ischemic attack, vascular risk factors, and related lifestyle conditions was obtained. Fasting blood glucose and lipid profile were performed on all subjects who had no known history of diabetes or hypercholesterolemia. All available cerebrovascular imaging was used to classify individuals. Controls underwent screening carotid duplex Doppler in an accredited laboratory. Patients with atrial fibrillation or highly suspected cardiac sources of emboli were excluded from these studies to avoid confusion between cardiac and carotid sources of ischemic events.

Blood Samples

The 314 blood samples collected between June 1997 and September 2001 were supplemented by 23 coded blood samples (with clinical data) from another hospital as part of an Institutional Review Board–approved collaboration. Nine samples were unusable because of degradation or undetectable DNA, leaving 328 for the analysis.

Results

Frequency of allele 2 was significantly greater in patients with atherosclerosis compared with nonatherosclerotic subjects. No difference was seen between symptomatic and asymptomatic atherosclerosis patients. Noncarriage of allele 2 was associated with reduced likelihood of atherosclerosis (odds ratio [OR], 0.44; 95% CI, 0.27 to 0.71). The homozygous carrier state for allele 2 was associated with greater likelihood of atherosclerosis (unadjusted OR, 7.30; 95% CI, 2.31 to 22.94; adjusted OR, 13.78; 95% CI, 1.94 to 97.9). A gene-dose effect was detected.

Conclusions

These data suggest that allele 2 of the IL-1ra gene represents a susceptibility factor in the development of carotid atherosclerosis. Further investigation appears warranted.

Key Words: atherosclerosis ■ carotid artery diseases ■ cytokines ■ interleukin-1 receptor antagonist ■ polymorphism

Despite a growing understanding of pathophysiological mechanisms involved in carotid atherosclerosis,1,2 variability exists among patients in plaque formation and instability unexplained solely by risk factor exposure. Differences in distributions of polymorphisms in genes coding for inflammatory factors may account for some of this variability in the pattern and behavior of carotid disease.

Interleukin-1 receptor antagonist (IL-1ra) is a counterinflammatory cytokine encoded by a polymorphic gene in the IL-1 gene family. Allele 2 of the variable number tandem repeat polymorphism of the IL-1ra gene (IL-1RN*2) has been associated with inflammatory diseases,3,4 including coronary atherosclerosis (CAD).5 Because of its biological plausibility for involvement in atherosclerosis, its association with inflammatory diseases, and its potential influence on IL-1ra and other cytokine production,6 we chose to investigate IL-1RN in carotid atherosclerosis. We hypothesized that (1) IL-1RN*2 occurs at a higher frequency in patients with atherosclerosis than in those without and (2) this allele is overrepresented among those with symptomatic compared with asymptomatic carotid atherosclerosis.

Methods

A total of 328 subjects identified as having carotid atherosclerosis or no atherosclerosis (controls) participated. Blood was obtained for DNA determination.

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All samples from cases and controls were handled in identical fashion.

Case-Control Criteria
Subjects were classified into atherosclerosis (>50% stenosis) or no atherosclerosis (<15% stenosis) groups on the basis of their vascular imaging. Individuals with 15% to 50% stenosis were excluded. The atherosclerosis group was further divided into symptomatic (stroke or transient ischemic attack referable to an ipsilateral plaque) or asymptomatic (never having ischemic symptoms) classifications on the basis of clinical history obtained from the patient and confirmed by medical records. A study neurologist adjudicated all cases to confirm group status. CT scans were obtained on all patients, and imaging studies were used to rule out silent infarcts in the asymptomatic population.

IL-1RN Polymerase Chain Reaction
Leukocyte DNA was extracted from whole blood with the QIAamp blood kit (Qiagen Inc) according to manufacturer’s instructions. The region containing the 86-bp variable number tandem repeat polymorphism was amplified by use of the flanking oligonucleotide primers 5'TCTAGCAACAAATAAT3' and 5'TTCTGGTGCTG-CAGGTA3' following standard protocols. Alleles were identified from electrophoretic migration compared with a 100-bp ladder.

Statistical Analysis
Nonparametric statistics, including Kruskal-Wallis \( \chi^2 \) tests, were used for our largely categorical data. These tests were chosen because of the occurrence of several rare alleles, leading to several expected small cell sizes. Because IL-1RN*2 has been associated with a variety of inflammatory diseases and because alleles 3 through 5 are all uncommon, allele frequency analyses compared IL-1RN*2 with non-IL-1RN*2. To explore a possible dose-response effect, we analyzed noncarriers, heterozygous carriers (1 copy), and homozygous carriers (2 copies) of IL-1RN*2 using \( \chi^2 \) tests for trends in binomial proportions. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated for specific allele carriage states. Differences were considered significant with a 2-tailed value of \( P=0.05 \). Statistical comparisons were made with S-PLUS 2000 Professional (Insightful Corp).

Multivariable logistic regression techniques were used, including 8 prespecified potential confounders (sex, race [white versus nonwhite], age, current smoking, presence or absence of hypertension, diabetes, hypercholesterolemia, and CAD) and IL-1RN*2 carrier status as independent variables to predict the presence of carotid atherosclerosis. Model discrimination was assessed by area under the ROC curve; an ROC curve area of 1.0 indicates perfect discrimination, an area of 0.5 indicates no discrimination, and areas between 0.5 and 1.0 indicate varying degrees of discrimination. Classification of overall predictive discrimination and was computed by the nonparametric method. An ROC curve area of 0.5 indicates no discrimination; an ROC curve area of 1.0 indicates perfect discrimination. Internal validation used bootstrap techniques with 100 samplings. To address potential ethnic stratification bias, we further prespecified an analysis restricting by race-ethnic group to control imaging populations.

Results
DNA samples from 206 subjects with atherosclerosis (97 symptomatic, 109 asymptomatic) and 122 individuals with no atherosclerosis were analyzed for IL-1ra polymorphisms. Vascular risk factors were more common among those with atherosclerosis. The atherosclerotic groups were very similar. The no atherosclerosis control group had more nonwhite controls and had more women than the other 2 groups (the Table).

Demographics and Risk Factor Information

<table>
<thead>
<tr>
<th>Symptomatic</th>
<th>Asymptomatic</th>
<th>No Atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=97</td>
<td>n=109</td>
<td>n=122</td>
</tr>
<tr>
<td>Mean age, y</td>
<td>73</td>
<td>75</td>
</tr>
<tr>
<td>Median age, y</td>
<td>72.3</td>
<td>73.8</td>
</tr>
<tr>
<td>White, %</td>
<td>93.8</td>
<td>99.0</td>
</tr>
<tr>
<td>Male, %</td>
<td>80.4</td>
<td>73.3</td>
</tr>
<tr>
<td>Hypertension*, %</td>
<td>87.6</td>
<td>89.0</td>
</tr>
<tr>
<td>Diabetes†, %</td>
<td>32.0</td>
<td>18.3</td>
</tr>
<tr>
<td>High cholesterol‡, %</td>
<td>64.9</td>
<td>77.1</td>
</tr>
<tr>
<td>Current smoker§, %</td>
<td>66.0</td>
<td>75.2</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td></td>
<td>50.5</td>
</tr>
<tr>
<td>History of stroke or TIA¶, %</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*Blood pressure ≥140/90 mm Hg for ≥1 year
†Taking an oral agent or being insulin dependent for ≥1 year.
‡LDL cholesterol ≥160 mg/dl untreated, fasting triglycerides >200 mg/dl, or on cholesterol-lowering medications for ≥1 year.
§More than 5 pack-years and smoking within last 12 months.
¶Any history of ischemic stroke or transient ischemic attack (TIA).

Genotype Frequencies
Genotype frequencies were in agreement with the Hardy-Weinberg equilibrium. We conducted a global comparison of IL-1RN genotype distributions in the 3 groups. Because of low expected values, rare genotypes (occurring in ≤3% of all groups) were collapsed into a single category of “rare” to assess overall differences from the expected values. These rare genotypes accounted for 6% of symptomatic, 4% asymptomatic, and 3% of control individuals. The overall genotype distributions differed significantly (\( \chi^2=20.95 \) with 6 df; \( P=0.002 \)), justifying further investigation of specific intergroup differences.

Allele Frequencies
The frequency of allele 2 was significantly higher for the symptomatic atherosclerosis (33%; \( P=0.00002 \)) and the asymptomatic atherosclerosis (30%; \( P=0.0001 \)) groups compared with the no atherosclerosis group (15%; Figure 1). No
differences were observed in allele frequencies between the symptomatic and asymptomatic groups (P=0.96).

**Carrier Frequencies**

Because the atherosclerosis groups did not differ, they were collapsed for carrier frequency analyses. The frequencies of carrying at least 1 copy of IL-1RN*2 were 47% for the atherosclerosis group and 28% for the control group (P=0.0008). Homozygous IL-1RN*2 carrier frequencies were significantly different between the atherosclerosis group (15.5%) and the no atherosclerosis group (2.5%; P=0.0002; Figure 2). Heterozygous IL-1RN*2 carrier frequencies were 31% in the atherosclerosis group and 25% in the no atherosclerosis group, but this difference did not reach statistical significance (P=0.13; Figure 2). Overall, we found a significant gene-dose effect for IL-1RN*2 (χ² for trend, P=0.00015). Considering the different IL-1RN*2 carrier states, noncarrier of allele 2 was associated with reduced likelihood of atherosclerosis (OR, 0.44; 95% CI, 0.27 to 0.71). Furthermore, heterozygous (OR, 1.32; 95% CI, 0.80 to 2.18) and homozygous (OR, 7.30; 95% CI, 2.31 to 22.94) carrier states for IL-1RN*2 were both associated with greater likelihood of atherosclerosis.

Logistic regression controlling for the 8 prespecified variables demonstrated a continued association of IL-1RN*2 carrier status and carotid atherosclerosis with excellent discrimination (ROC=0.92). Internal validation demonstrated a bootstrap-corrected ROC=0.91. The effect of the polymorphism remained significant (OR, 13.78; 95% CI, 1.94 to 97.9) for homozygous carriers of IL-1RN*2. The gene-dose effect for IL-1RN*2 also persisted after adjustment (P=0.006). The analysis restricted to white cases and controls yielded nearly identical results for allele and carrier frequencies.

**Discussion**

IL-1RN*2 occurred more frequently among subjects with than in those without carotid atherosclerosis. The increased carriage of ≥1 copies of IL-1RN*2 in the atherosclerosis group suggests that allele 2 may represent a significant susceptibility gene in atherosclerotic development. Our data also support a gene-dose effect, with heterozygous carriers having intermediate risk compared with homozygous individuals. Ours is not the first study to link IL-1RN*2 to vascular disease. Cardiovascular researchers have demonstrated overrepresentation of IL-1RN*2 in individuals with CAD. Furthermore, other data support a link between IL-1RN and carotid atherosclerosis. A closely linked IL-1RN point mutation has been associated with greater intima-media thickness in a community-based black cohort. Newer data indicate that composite genotypes of the IL-1 gene family influence risk for cardiovascular and infectious disease, raising the possibility of epistatic genotype-genotype interactions modulating phenotype.

These findings could have important ramifications in our growing understanding of the pathophysiology of atherosclerosis and implications in individual patients. The “response to injury” model in atherosclerosis highlights the role of inflammatory cytokines such as tumor necrosis factor-α and IL-1β in the response of endothelial cells to risk factors. These inflammatory cytokines play an integral role in increased migration of inflammatory cells and the proinflammatory, procoagulant nature of developing atherosclerotic plaques. Counterinflammatory cytokines such as IL-1ra are believed to be effective in the local control of inflammatory processes. It can therefore be hypothesized that a polymorphism that affects the efficiency of a key counterinflammatory cytokine could increase the likelihood of the development of atherosclerotic disease and thus increase the risk of heart attack and stroke.

The IL-1RN alleles may affect the inflammatory environment in the vascular endothelium. Compared with homozygotes for IL-1RN*1, homozygotes for IL-1RN*2 have a 2- to 3-fold-lower production of the endothelium-derived isoform of IL-1ra; heterozygotes produce an intermediate level. In contrast, leukocyte stimulation experiments have demonstrated hypersecretion of IL-1ra and IL-1β among those carrying IL-1RN*2 compared with IL-1RN*1 homozygotes. IL-1RN alleles may influence the efficiency of IL-1 receptor blockade. Knockout mouse studies demonstrate that hemizygous IL-1RN mice cannot fully control vascular inflammation despite the presence of a normal gene product.

This polymorphism may directly or indirectly interact with other known vascular risk factors to influence the development and progression of atherosclerosis. Our atherosclerosis groups had expectedly higher rates of vascular risk factors. However, our logistic regression analysis indicated an independent association of IL-1RN genotype and carotid atherosclerosis. Although the model requires external validation, our bootstrapping techniques suggested excellent internal validation. The potential for effect modifications between IL-1RN genotype and vascular risk factors warrants further exploration. The lack of association between IL-1RN genotype and symptomaticity suggests that other factors predominate in determining the symptomatic phenotype.

The candidate gene approach represents a primary avenue of investigation into genetic contributions to complex dis-
cases albeit with notable limitations. The association reported here needs to be studied in a range of populations to confirm broad applicability. Similar methodological limitations confront the case-control design, especially with regard to selection biases. Ethnic stratification may occur within the white population, but recent studies suggest that ethnic stratification bias is smaller than previously suspected and is generally quantifiable. Nonetheless, Italian investigators were unable to replicate the association of IL-1RN*2 with coronary disease found in an American cohort, raising the possibility of differences in background allele frequencies, variation in the relative importance of this pathway in atherogenesis, or methodological differences. In our study, despite slight imbalances in racial makeup among experimental groups, the restricted analysis suggested that this imbalance did not introduce bias.

In summary, IL-1RN*2 occurs at a higher frequency in those with than in those without carotid atherosclerosis. This association, taken in the context of the role of IL-1ra in regulating the inflammatory cascade and the association of IL-1RN*2 with inflammatory diseases, suggests that carriage of IL-1RN*2 should be investigated as a risk factor for the development of carotid atherosclerosis in larger prospective, racially diverse cohorts. Other polymorphic cytokine genes also merit investigation with specific attention given to composite genotypes and epistatic interactions.

Acknowledgments

Dr Worrall was supported in part by the American Academy of Neurology Clinical Research Training Fellowship Grant. We wish to thank the participants in these protocols, Diann Richko for her assistance in data management, and Drs Zsolt May and Javier Romero for their assistance in collecting the clinical data and samples for the Massachusetts General Hospital participants. Dr Worrall wishes to thank William A. Knaus, MD, who served as master’s thesis advisor and Karen C. Johnston, MD, and Viktor E. Bovbjerg, PhD, who served as thesis readers on this project.

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*Stroke*. 2003;34:790-793; originally published online February 20, 2003; doi: 10.1161/01.STR.0000057815.79289.EC
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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