Inflammatory Gene Load Is Associated With Enhanced Inflammation and Early Carotid Atherosclerosis in Smokers

Paula Jerrard-Dunne, MRCPI; Matthias Sitzer, MD; Paul Risley, BSc; Alexandra Buehler, MD; Stefan von Kegler, MD; Hugh S. Markus, FRCP

Background and Purpose—Smoking acts as a pro-inflammatory stimulus. Inflammation may provide a key mechanism by which smoking causes atherosclerosis. If so, then the degree to which an individual mounts an inflammatory response is likely to influence atherosclerosis severity. This study examined the impact of inflammatory gene polymorphisms and gene–smoking interactions on common carotid artery intima-media thickness (IMT), a measure of early atherosclerosis.

Methods—In a community population (n=1000), mean IMT was determined using ultrasound. This population was genotyped for 6 polymorphisms in 4 inflammatory genes: IL-6-174, IL-6-572, and IL-6-597; IL-1-β-31; IL-1 receptor antagonist VNTR and CD14-159. Serum IL-6 levels were measured in the first 500 subjects. Genotypes/haplotypes associated with higher IL-6 levels were designated “inflammatory haplotypes.” A gene load score was calculated, in which 2 represented individuals homozygous for ≥2 inflammatory genotypes/haplotypes and 0 was homozygous for none.

Results—Increasing gene load of inflammatory genotypes was associated with a linear increase in serum IL-6 levels (P=0.018) and increased carotid artery IMT (P=0.003). There was a significant interaction between gene load and smoking status on carotid IMT (P for interaction=0.002). Specifically, in smokers, carriers of inflammatory haplotypes had significantly increased age- and sex-adjusted IMT (IL-6-174C/IL-6-572G/IL-6-597A, P=0.005; IL-1-β-31T/IL-1RN*2, P=0.04; CD14-159CC, P=0.028).

Conclusions—These findings support the hypothesis that inflammation and cytokine responses provide a key mechanism by which smoking causes atherogenesis. Secondly, they highlight the importance of gene–environment, and gene–gene–environment interactions in the pathogenesis of atherosclerosis. (Stroke. 2004;35:2438-)

Key Words: arteriosclerosis ■ cigarette smoking ■ genetics ■ gene expression ■ inflammation

Increasing evidence suggests that inflammation is crucial in the pathogenesis of atherosclerosis. In prospective studies, serum levels of inflammatory mediators such as IL-6 and C-reactive protein have been shown to be predictive of future vascular events.1,2

Although there is much interest in inflammatory markers as novel risk factors for atherosclerosis, it is important to recognize that certain conventional vascular risk factors appear to provoke a low-grade inflammatory response. Cigarette smoking is a potent mediator of inflammation,3 and this increased inflammation may be a key mechanism by which smoking mediates atherosclerosis.

Further evidence of a link between inflammation, smoking, and atherosclerosis comes from studies correlating inflammatory markers with atherosclerosis. C-reactive protein levels correlate strongly with subclinical atherosclerosis measured using ultrasound, but this association is weakened by adjustment for vascular risk factors such as smoking, suggesting that these conventional risk factors may mediate atherosclerosis in part through increased inflammation.4

If inflammation were on the intermediate causal pathway between these risk factors and atherosclerosis, then the degree to which an individual mounts an inflammatory response could influence the degree of atherosclerosis. Levels of inflammatory markers show marked interindividual variation, even after careful controlling for potential confounding factors. Twin and family studies suggest that a significant proportion of the interindividual variability in levels of inflammatory markers is determined by genetic factors.5 These findings suggest that certain individuals may have an “inflammatory phenotype” that predisposes them to atherosclerosis.

The gene loci encoding a number of inflammatory cytokines have been found to be polymorphic in regions that may affect their transcription. They are therefore potential candidate genes, polymorphisms of which might contribute to genetic susceptibility to atherosclerosis and large-vessel ischemic stroke. Candidate gene association studies examining these gene polymorphisms may offer a novel method to...
examine the hypothesis that inflammation is on the intermediate causal pathway between smoking and atherosclerosis.

In this study, a candidate gene approach was used to determine whether potentially inflammatory gene variants are an independent risk factor for carotid atherosclerosis, and whether genetically determined differences in the response to the pro-inflammatory stimulus, smoking, are determinants of differences in interindividual atherosclerosis risk.

Subjects and Methods

Study Population

The study consisted of a sample drawn from participants in the Carotid Atherosclerosis Progression Study (CAPS), details of which have been published elsewhere. All members of a German primary health care service aged 40 years or older (n=15 879) living within a radius of 50 km from 5 study sites in Western Germany were invited to participate. Within a predefined time limit, 5460 agreed to participate. The first consecutive 1000 subjects of European ancestry in the age range of 50 to 65 years were included in this study. This number was chosen to allow detection of a difference in intima-media thickness (IMT) of 0.05 mm with a power of 0.9 and a significance of 0.01. Vascular risk factors were assessed using a standardized computer-assisted interview technique performed by a physician experienced in stroke medicine. Risk factors determined included pack-years of cigarette smoking and smoking category (never/ex/current smoker), history of arterial hypertension, history of diabetes mellitus, and body mass index. History of hypertension was defined as 3 measurements: >160 systolic, >95 diastolic, or receiving antihypertensive medication. Diabetes was defined as physician-diagnosed or currently receiving antidiabetic medication. Socioeconomic status was measured using a 4-point scale previously applied to German populations for coronary risk factor studies. The categories were 0 to 3, indicating lower, lower middle, upper middle, and upper classes. The mean value of 3 supine blood pressure measurements was taken as the actual arterial blood pressure. Average alcohol intake was determined using a standardized questionnaire. Fasting blood samples were taken for estimation of serum lipoprotein and high-density lipoprotein cholesterol, alcohol intake modeled as a continuous variable.

Statistical Methods

Data were analyzed using SPSS (Version 10.0). CCA-IMT values were averaged across the right and left sides. The reciprocal of the mean CCA-IMT values and log-transformed IL-6 levels were used to normalize their distributions before parametric analysis. Multiple linear regression analysis was used to determine relationships between genotypes and mean IMT and IL-6 levels. Linkage disequilibrium was tested using LINK DOS (http://www.med.unc.edu/genepop/linkdos.html). Haplotype frequencies were calculated using Estimating Haplotypes frequencies software (ftp://linkage.rocketei111.de/softwar117). And Haplotype Resolution using imperfect Phylogeny (http://www1.cs.columbia.edu/compbio/hap). Rare haplotype combinations (<1%) were excluded from analyses to minimize errors in haplotype prediction. Associations between genotypes/haplotypes and IL-6 levels were determined in ever-smokers (current/ex) and never-smokers. Additive and dominant models were tested to determine the effects of heterozygosity, and the assumption of an additive or a dominant effect on IMT was based on the relationship of the genotypes with IL-6 levels. Those genotypes/haplotypes that were associated with higher IL-6 levels were designated “inflammatory haplotypes.” Associations between these inflammatory haplotypes and mean IMT were then determined in ever-smokers and never-smokers. Age- and sex-adjusted and multivariate analyses adjusting for age, sex, and vascular risk factors were performed initially. Post hoc interaction terms were included in the overall model to confirm and produce a probability value for the smoking interactions that were observed in the separate analyses of the subgroups. To model the effects of increasing gene load of inflammatory genotypes on IMT, a gene load score was calculated based on the observed additive effects of gene–gene interactions on IL-6 levels and mean IMT. For tests of linear trend, gene load was modeled as a continuous variable.

Risk factors included in the multivariate IMT analyses were age, sex, body mass index, mean systolic and diastolic blood pressures, history of arterial hypertension, history of diabetes mellitus, serum cholesterol and glycosylated hemoglobin A1c, pack-years of smoking (analyzed by 10-year categories), smoking category, low-density lipoprotein and high-density lipoprotein cholesterol, alcohol intake (grams per day), and socioeconomic status.

Results

Baseline demographic characteristics of the population are given in Table I (available at http://www.strokeaha.org). All genotypes for the polymorphisms studied were in Hardy–Weinberg equilibrium. The IL-6-174C, IL-6-597A, and IL-6-572G alleles were in strong allelic association (P<0.001). There was also significant linkage disequilibrium between the IL-1β-31T and the IL-1RN*1 alleles (P<0.001). Allele and haplotype frequencies are shown in Tables 1, 2, and 3. Figure 1a shows the relationships between IL-6 levels and the different haplotypes. The CC genotype of the CD14-159 polymorphism was associated with higher IL-6 levels, but the effect was confined to smokers (P=0.034). The IL-1 haplotype IL-1RN*2/IL-1β-31T was also associated with higher IL-6 levels in smokers (P=0.011). Trends toward higher IL-6 levels were seen for IL-6-174 CC homozygotes (P=0.051), IL-6-572G GG (P=0.087) homozygotes, and IL-6-597 AA homozygotes (P=0.17), with no heterozygote effects. The
**TABLE 1. IL-6 Allele and Haplotype Frequencies**

<table>
<thead>
<tr>
<th>Allele frequencies</th>
<th>IL-6-174</th>
<th>IL-6-572</th>
<th>IL-6-597</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.43</td>
<td>0.08</td>
<td>0.43</td>
</tr>
<tr>
<td>G</td>
<td>0.57</td>
<td>0.92</td>
<td>0.57</td>
</tr>
<tr>
<td>Haplotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGG</td>
<td>0.442</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGA</td>
<td>0.371</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCG</td>
<td>0.066</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGA</td>
<td>0.060</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGG</td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCG</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCA</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCA</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated with Estimating Haplotype frequencies (EH) software (ftp://linkage.rockefeller.edu/software/eh).

**TABLE 2. IL-1 Allele and Haplotype Frequencies**

<table>
<thead>
<tr>
<th>Allele frequencies</th>
<th>IL-1β-31</th>
<th>IL-1RN</th>
<th>Haplotype Frequency*</th>
</tr>
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<tbody>
<tr>
<td>C</td>
<td>0.33</td>
<td>2.010</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.77</td>
<td>1.089</td>
<td></td>
</tr>
<tr>
<td>Haplotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T*1</td>
<td></td>
<td>0.622</td>
<td></td>
</tr>
<tr>
<td>C*1</td>
<td></td>
<td>0.276</td>
<td></td>
</tr>
<tr>
<td>C*2</td>
<td></td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>T*2</td>
<td></td>
<td>0.049</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated with Estimating Haplotype frequencies (EH) software.

IL-6 haplotype IL-6-174C/IL-6-572G/IL-6-597A, (CGA) was associated with higher IL-6 levels (P=0.035) overall, with no differential effect in smokers. (Figure 1a)

Figure 1b and Table II (available at http://www.strokeaha.org) shows the relationship between these gene variants and mean IMT in smokers and nonsmokers. The CD14 CC genotype was associated with significantly higher mean CCA-IMT in smokers (age- and sex-adjusted P=0.036; multivariate P=0.035). There was a trend toward higher CCA-IMT in smokers with the IL-1RN*2/IL-1β-31T haplotype (age- and sex-adjusted P=0.049; multivariate P=0.119). The IL-6 CGA haplotype was associated with significantly higher mean CCA-IMT overall (age- and sex-adjusted P=0.018), and the effect was again strongest in smokers (age- and sex-adjusted P=0.004; multivariate P=0.010) (Figure 1b). The magnitude of the effects of each of these gene variants on IMT was similar, with a difference in mean IMT in the region of 0.04±0.01 mm in smokers.

**Gene–Gene Interactions**

When the combined effects of the inflammatory genotypes were explored, the data were found to fit an additive model for IL-6 levels and mean IMT, with no evidence of supra-additive interaction effects. To examine the effect of overall gene load, a sum score for the 3 inflammatory gene haplotypes was calculated. The range of this gene load score was 0 to 2, in which 2 represented individuals homozygous for ≥2 inflammatory genotypes/haplotypes and 0 represented individuals homozygous for none. Figure 2 illustrates the effect of gene load on IL-6 levels and on mean IMT. Increasing gene load was associated with higher IL-6 levels (age- and sex-adjusted P=0.025; multivariate P=0.018). The gene load score of inflammatory haplotypes was also associated with increased IMT (age- and sex-adjusted P=0.001; multivariate P=0.003).

**Gene–Environment Interactions**

To determine whether smoking influenced the effects of gene load on IMT, interaction terms for smoking were included in the regression model. The interaction terms was significant for gene load score and smoking status (P=0.008), and pack-years of smoking (P=0.001). The association between increased IMT and gene load score was stronger in smokers (age- and sex-adjusted P<0.0005; multivariate P<0.0005) and absent in nonsmokers (age- and sex-adjusted P=0.554; multivariate P=0.709). Age- and sex-adjusted mean IMT values (95% CI) according to increasing gene load score were 0.77 (0.75 to 0.78), 0.76 (0.74 to 0.78), and 0.80 (0.75 to 0.85) mm for nonsmokers and 0.76 (0.75 to 0.78), 0.80 (0.78 to 0.82), and 0.84 (0.80 to 0.88) mm for smokers. This association in smokers was consistently seen in both sexes and when measures from either the right or the left common carotid arteries were analyzed individually. Similar to the effects seen on IMT, the observed association between gene load score and IL-6 levels was also only significant in smokers (age- and sex-adjusted P=0.030; multivariate P=0.007) versus nonsmokers (age- and sex-adjusted P=0.341).

To examine the association with more advanced atherosclerosis, odds ratios for IMT above the 75th percentile compared with the lower 3 quartiles were determined using binary logistic regression. Table 4 shows the odds ratios for an IMT in the highest quartile according to the number of gene load score in smokers and nonsmokers. In smokers, there was a graded increase in odds ratio with increasing number of at-risk genotypes (P=0.001), but no association was found in nonsmokers.

**Discussion**

This study suggests that genetic variants in genes encoding components of the inflammatory response are a risk factor for early carotid atherosclerosis in individuals who smoke. The effects of the 3 inflammatory gene haplotypes were additive, with combinations of at-risk genotypes and alleles leading to a significantly increased risk of elevated IMT. Significant gene–smoking interactions were identified. Specifically, the studied polymorphisms were associated with increased IMT in smokers but showed no association in individuals who had never smoked.

Additive gene load was also associated with higher IL-6 levels, and again this effect was only significant in smokers. These parallel findings support the hypothesis that the effects of these genes on IMT may be mediated by enhanced inflammation in the presence of a pro-inflammatory stimulus, in this case smoking.

The systemic inflammatory response is heightened in smokers and relates to pack-years of smoking.13 Bacterial endotoxin, a potent mediator of inflammation, has been identified as an active component of cigarette smoke, and...
smokers have elevated plasma levels of endotoxin. The increased risk of respiratory tract infection seen in smokers may also act as a pro-inflammatory stimulus in this group.

The factors influencing interindividual variance of IMT are complex, but with a strong genetic component. The phenotypic variance of IMT can be considered to consist of the combined effects of environment, genes, and gene–environment interactions. The effects of conventional vascular risk factors on carotid IMT have been well-described, but these explain <50% of the overall variance. A number of studies have also examined the role of candidate genes in IMT variability. To date, very few studies have looked specifically for the effects of gene–environment interactions.

In any complex polygenic disease, the overall effect of any individual gene is likely to be small and may be masked by the influence of gene–environment interactions. This study demonstrates that in isolation, the individual effect of any one polymorphism on IMT was small and unlikely to be of major relevance. However, when the additive effects of these genes and gene–environment interactions were considered, differences of greater potential relevance were found. This method of modeling gene–gene and gene–environment interactions may be a useful aid to a better understanding of the complexities of the genetic determinants of carotid atherosclerosis. However, the score used to calculate gene load was based on a post hoc analysis of those genotypes/haplotypes that showed an association individually with IL-6 levels and with elevated IMT. As such, these data would require confirmation in an independent sample. This would require larger sample sizes to allow for low frequencies of haplotypes, subgroup analyses, and adjustment for multiple statistical comparisons, which are all acknowledged limitations of the present study.

Although the term “inflammatory” has been used for convenience to describe those genotypes/haplotypes that were associated with higher IL-6 levels, this is an oversimplification of the complex inflammatory and anti-inflammatory effects of the genes studied. It is notable that an association was found between the CC genotype of the CD14-159 polymorphism and increased inflammation and IMT, whereas it is the TT genotype that has been associated with higher levels of soluble CD14. This would be consistent with recent data suggesting that soluble CD14 has anti-inflammatory properties.

Acute or chronic inflammatory processes were not specific exclusion criteria for entry into the study and could have

Figure 1. A, Gene effects on IL-6 levels according to smoking (error bars show 95% CI of geometric mean, age- and sex-adjusted). B, Gene effects on mean CCA IMT according to smoking (error bars show 95% CI of harmonic mean, age- and sex-adjusted).
influenced the results. In the presence of acute or chronic inflammatory states, IL-6 levels typically increase many-fold above the upper limit of detection of the high-sensitivity assay used. The range of detected IL-6 levels in the study population was 0.156 to 8.821 pg/mL, suggesting that it is unlikely that the data were influenced by individuals with active acute inflammation.

It has been proposed that lower degrees of IMT thickening appear to reflect a nonatherosclerotic adaptive response to changes in shear and tensile stress rather than atherosclerosis per se. A very elevated IMT may be a better marker of clinical risk than mean IMT values. Therefore, in addition to comparing mean IMT values, odds ratios for a very elevated IMT (above the 75th percentile) were determined and the results were consistent.

In conclusion, the findings of this study support the hypothesis that inflammation and cytokine responses provide a key mechanism by which smoking causes atherogenesis. Secondly, they emphasize the importance of gene–environment and gene–gene–environment interactions in the pathogenesis of atherosclerosis.

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References
10. Tarlow JK, Blakemore AL, Lennard A, Solarri R, Hughes HN, Steinkasserer A, Duff GW. Polymorphism in human IL-1 receptor an-

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**Table 3. CD14 Allele and Genotype Frequencies**

<table>
<thead>
<tr>
<th>CD14 Genotype</th>
<th>CD14-159 Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele frequencies</strong></td>
<td>0.50</td>
</tr>
<tr>
<td><strong>F</strong> 0.50</td>
<td>0.244</td>
</tr>
<tr>
<td><strong>TT</strong></td>
<td>0.508</td>
</tr>
<tr>
<td><strong>CC</strong></td>
<td>0.248</td>
</tr>
</tbody>
</table>

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**Table 4. Gene–Smoking Interactions**

<table>
<thead>
<tr>
<th>Gene Load Score</th>
<th>% of Population</th>
<th>OR for IMT &gt;75th Percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers, n=512</td>
<td>Nonsmokers, n=488</td>
</tr>
<tr>
<td>1</td>
<td>34</td>
<td>144</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

Trend P=0.001 Trend P=0.073

The odds ratios (OR) for an IMT in the highest quartile (cutoff 0.83 mm) according to gene load score (adjusted for age and sex).


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