Promoter Polymorphism in the Endotoxin Receptor (CD14) Is Associated With Increased Carotid Atherosclerosis Only in Smokers

The Carotid Atherosclerosis Progression Study (CAPS)

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Background and Purpose—The risk of atherosclerosis from endotoxemia is increased in smokers. Endotoxin is a potent mediator of inflammation, and smokers have elevated plasma levels of endotoxin. The endotoxin receptor CD14 can enhance the endotoxin-neutralization capacity of plasma. A functional polymorphism in the promoter region of the CD14 gene (CD14 \(-159\)C/T) was studied to determine its impact on common carotid artery (CCA) intima-media thickness (IMT) and any interactions with environmental inflammatory stimuli.

Methods—A community population (n=992; aged 50 to 65 years) underwent genotypic examination for the CD14 \(-159\) polymorphism by restriction fragment length polymorphism analysis.

Results—The CC genotype was associated with increased CCA IMT. The age- and sex-adjusted odds ratio for IMT above the 75th percentile was 1.63 (95% CI, 1.19 to 2.24; \(P=0.002\)) and 1.70 (95% CI, 1.18 to 2.44; \(P=0.004\)) after additional adjustment for conventional risk factors. This gene effect was found only in current smokers and ex-smokers. Multivariate analysis in this group (n=503) increased the odds ratio to 2.02 (95% CI, 1.23 to 3.34; \(P=0.006\)). No significant interactions were found in nonsmokers or with alcohol consumption.

Conclusions—The CD14 \(-159\) polymorphism is associated with increased CCA IMT in smokers from a general population. CD14 may modulate the inflammatory effects of smoking in atherogenesis. (Stroke. 2003;34:600-604.)

Key Words: antigens, CD14 ■ atherosclerosis ■ cigarette smoking ■ polymorphism

Carotid artery stenosis secondary to atherosclerosis accounts for approximately 15% of ischemic strokes. Conventional risk factors explain at most only half of the risk of atherosclerosis in the carotid artery. A recent family study has suggested that genetic factors account for a large portion of carotid atherosclerosis risk. Despite this, the responsible genes remain largely unknown. However, there is increasing evidence that gene-environment interactions may influence atherosclerosis risk.

Inflammation is now accepted as a major component of atherosclerosis. Certain conventional risk factors, including cigarette smoking and obesity, are associated with elevated levels of inflammatory markers. It has been suggested that these risk factors may exert part of their risk by promoting inflammation. In particular, 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. Circulating levels of endotoxin, in turn, have been shown to independently predict incident atherosclerosis measured by carotid ultrasound, but the ability of endotoxin to promote atherogenesis appears to be dependent on the degree of inflammatory response it provokes. The soluble form of the endotoxin receptor (sCD14) has been shown to enhance the endotoxin-neutralization capacity of plasma. A polymorphism in the promoter region of the CD14 gene has been associated with altered levels of this circulating endotoxin receptor.

We hypothesized that this functional polymorphism could predispose to atherosclerosis by modulating the host inflammatory response to endotoxin. In a large community population of middle-aged individuals, we studied the impact of the CD14 \(-159\) genotype on early atherosclerosis using common carotid artery (CCA) intima-media thickness (IMT), measured by ultrasound. We hypothesized that any effect of this polymorphism would be most pronounced in individuals who smoked and therefore had greater environmental exposure to bacterial endotoxin.

Subjects and Methods

Study Population

The study sample was drawn from participants in the Carotid Atherosclerosis Progression Study (CAPS), details of which have...
been published elsewhere. All members of a German primary healthcare service population aged 40 years or older (n=15 879) who lived 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 in the group aged 50 to 65 years (mean age, 57.9±4.4 years; 51.5% women) were included in this study. This number was chosen to allow us to detect a difference in IMT of 0.05 mm with a power of 0.9 and a significance of 0.01. Risk factors determined included the following: pack-years of smoking, current smoking status (defined as current smoker/ex-smoker or never-smoker), body mass index (BMI), mean systolic and diastolic blood pressure, and history of arterial hypertension, diabetes mellitus, or coronary heart disease. Socioeconomic status was measured with the use of a 4-point scale previously applied to German populations for coronary risk factor studies. Average alcohol intake was determined by a standardized questionnaire. Informed consent on the use of samples for analysis was obtained from all participants before entry, and the study was approved by the ethical review committee of the University of Dusseldorf Hospital.

Biochemical Measurements
Fasting blood samples were taken for estimation of serum cholesterol, glycosylated hemoglobin A1c (HbA1c), and fibrinogen. Total serum cholesterol was determined enzymatically with the use of a commercial kit (Boehringer). LDL cholesterol was measured with the heparin-calcium precipitation method and HDL cholesterol with the phosphotungstic acid precipitation method. Glycosylated hemoglobin (glyc-Hb-%) was determined by the IMX method (Abbott). HbA1c concentration was calculated according to the formula HbA1c = (glyc-Hb-%)/1.76. Fibrinogen was determined from citrated plasma by the Clauss method (Multifibren, Behringwerke). C-reactive protein (CRP) concentration was measured by a highsensitivity in-house enzyme-linked immunosorbent assay (range, 0.6 to 40 mg/L). Ultrasound Imaging
The method used to determine CCA IMT has been described in detail before. In brief, we used a 7.5- to 10.0-MHz linear array transducer (P700SE, Phillips Medical System). IMT measurements were performed off-line with the use of automated imaging processing software that has been described previously. Blood/initial and medial/adventitial interfaces were automatically detected with the use of a gray value–based edge detection algorithm. The mean length of the arterial segment in which IMT was determined was 14.35 mm for the left CCA and 12.85 mm for the right CCA. Interobserver reliabilities were assessed in a sample of 15 subjects (54 arterial segments) in whom IMT was measured by 4 “blinded” observers. Linear regression analyses revealed high correlation coefficients (r=0.92 to 0.99), and, according to the method described by Bland and Altman, the ±2 SD of the difference between 2 observers varied between 0.03 and 0.06 mm. Intraobserver retest reliability was determined from repetitive examinations of 35 subjects (102 arterial segments) by 3 independent observers. Linear regression showed high coefficients (r=0.91 to 0.98), and the ±2 SD of the difference between the 2 examinations varied between 0.04 and 0.06 mm.

Laboratory Methods
DNA was available for 992 subjects. DNA was extracted from whole blood with the use of Nucleon kits (Tepnel Life Sciences). The polymorphism underwent genotypic analysis by polymerase chain reaction, resulting in a 497–base pair (bp) product, followed by restriction enzyme digestion with HaeIII (Bioline), producing a fragment of 199 bp, plus fragments of 298 bp for the T allele and 155 and 143 bp (appearing as a single band) for the C allele. Products were visualized on agarose gel stained with ethidium bromide. Genotypes were confirmed by sequencing. Polymerase chain reaction conditions and primers were selected according to the methods of Baldini et al.

Statistical Analysis
Data were analyzed with the use of SPSS (version 10). We analyzed the relationship between genotype and CCA IMT in 2 ways. First, we used CCA IMT as a continuous variable using linear regression. The mean of the right and left CCA IMT values was skewed, and therefore the reciprocal IMT was used to normalize the distribution. Geometric mean IMT values are given in the text for clarity. For the second analysis, we calculated odds ratios for IMT above the 75th percentile using multiple logistic regression. This second analysis was performed because previously published studies suggested that the relationship between IMT and risk of future vascular events is nonlinear and is strongest at higher values of IMT. In both analyses, a genotype-smoking interaction term was included to look for evidence of gene-environment interaction. The effects of genotype on CCA IMT were examined for additive (number of alleles), dominant (carriage of C allele versus TT homozygotes), and recessive (CC homozygotes versus carriage of the T allele) relationships. Age, BMI, mean systolic and diastolic blood pressures, and LDL/HDL cholesterol were normally distributed. We categorized pack-years of smoking by 10-year intervals. In this community population CRP was negatively skewed, with many subjects below the limits of measurement, and therefore CRP was categorized into quartiles.

Results
CCA IMT and Cardiovascular Risk Factors
Associations between cardiovascular risk factors and CCA IMT are shown in Table 1. On univariate analysis, age, sex, pack-years of smoking, BMI, history of arterial hypertension, history of diabetes mellitus, and CRP were all strongly associated with mean CCA IMT. Association with LDL and HDL cholesterol and alcohol intake >30 g/d was weaker but still significant. In multivariate analysis, age, sex, mean systolic blood pressure, low socioeconomic status, and CRP all remained significantly associated with mean CCA IMT.

CD14 Polymorphism and Cardiovascular Risk Factors
The genotype distribution was 24.8% for CC, 50.8% for CT, and 24.4% for TT. This was in Hardy-Weinberg equilibrium (P=0.999). Analysis of the relationships between genotype and the aforementioned cardiovascular risk factors revealed no significant associations.

CCA IMT and Genotype
When IMT was analyzed as a continuous variable, the C allele of the CD14 polymorphism was associated with increased CCA IMT. This finding was significant when we used an additive model (allele frequency) (P=0.049) and for CC homozygotes versus CT/TT combined (P=0.022) (Table 2).

Analysis of IMT by highest quartile showed that the CC genotype was significantly more common in the top quartile; 33.3% of the CC genotype was found in this quartile compared with 23.7% and 23.1% of the CT and TT genotypes, respectively (P=0.009 for CC compared with TT and P=0.011 for linear trend). The odds ratio for IMT above the 75th percentile was 1.63 (95% CI, 1.19 to 2.24; P=0.002) for CCA compared with both the other genotypes combined. After multivariate analysis controlling for conventional risk factors, the odds ratio for the CC genotype was not weakened at 1.70 (95% CI, 1.18 to 2.44; P=0.004).
Environment Interactions

There was a significant interaction between CD14 genotype and smoking (P for interaction=0.008). Specifically, the relationship between genotype and IMT was stronger in smokers and absent in those who had never smoked (Table 3 and Figure). Additionally, there was evidence of a graded relationship between the number of pack-years smoked and the effect of CD14 genotype on IMT. Increasing pack-years of smoking resulted in a linear increase in IMT with carriage of the C allele (P<0.001, adjusted for cardiovascular risk factors). The increase in IMT was highest in the TT homozygotes. In contrast, there was no linear relationship in the CC homozygotes (P=0.704, adjusted for cardiovascular risk factors). The relationship with smoking was not affected by potential confounding of factors, including exercise, total energy expenditure per day, or use of antihypertensive treatment.

No significant associations were shown between increased IMT, CD14 genotype, and BMI or level of alcohol consumption.

Discussion

This study found that the C allele of the functional CD14 (endotoxin receptor) −159C/T polymorphism was associated with increased CCA IMT in a general community population but that this association was limited to smokers. The C allele has previously been associated with lower levels of circulating sCD14,15 and individuals with lower levels of sCD14 have reduced capacity to neutralize endotoxin.12–14 Interestingly, this association was limited to smokers, who are known to have increased endotoxin exposure.9,10 These findings support a potential role for this polymorphism in modulating endotoxin-mediated atherosclerosis and demonstrate a potentially important gene-environment interaction between smoking and CD14 genotype in the pathogenesis of early atherosclerosis.

Our study is one of the first to demonstrate gene-environment interactions in the pathogenesis of carotid atherosclerosis. There are a number of pathophysiological explanations for the interaction with smoking. Previous work has shown that TT homozygotes for the CD14 −159 polymorphism have elevated levels of sCD1415 and that carriage of the T allele results in upregulation of transcription of CD14.21 Increases in sCD14 can decrease monocyte response to endotoxin by clearance via lipoproteins. This leads to a decreased inflammatory response.22 A strong correlation between sCD14 and the endotoxin-neutralizing capacity of

### Table 2: Association Between the CD14 Polymorphism and Mean CCA-IMT Analyzed According to Smoking Status

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Mean CCA-IMT (mm)</th>
<th>Additive No. of C Alleles</th>
<th>Dominant CC/CT vs TT</th>
<th>Recessive CC vs CT/TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0.768</td>
<td>0.749</td>
<td>0.748</td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>0.752</td>
<td>0.738</td>
<td>0.756</td>
<td></td>
</tr>
<tr>
<td>Ever smokers</td>
<td>0.789</td>
<td>0.758</td>
<td>0.738</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>P_m</th>
<th>P_u</th>
<th>P_m</th>
<th>P_u</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>0.049</td>
<td>0.255</td>
<td>0.363</td>
<td>0.955</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>0.747</td>
<td>0.284</td>
<td>0.212</td>
<td>0.087</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>0.002</td>
<td>0.011</td>
<td>0.023</td>
<td>0.072</td>
</tr>
</tbody>
</table>

*P_m indicates univariate P value; P_u, multivariate P value.

Significance values are shown for additive (allele frequency), dominant (carriage of C allele vs TT), and recessive (CC vs T allele carriage) models and also after adjustment for all cardiovascular risk factors (see text).
plasma has also been demonstrated.\textsuperscript{23} Data from the Bruneck atherosclerosis study showed that subjects with high endotoxin levels were at risk of increased IMT only if they also had increased inflammatory markers.\textsuperscript{11} Individuals with the \textit{CC} genotype express lower levels of CD14\textsuperscript{21} and therefore may also be less able to clear endotoxin and other CD14 ligands, resulting in chronic low-grade inflammation. Conversely, carriage of the T allele upregulates CD14 expression and may enable a more robust response to acute challenge but better clearance of ligand and reduced chronic inflammation. The effect of smoking on IMT may arise from the bioactive endotoxin found in cigarette smoke.\textsuperscript{9} The increased risk of lower respiratory tract infection seen in smokers\textsuperscript{24} may also increase the antigenic load in this group. Risk of atherosclerosis from endotoxemia has been found to be increased in current and past smokers compared with nonsmokers.\textsuperscript{10} A genetic predisposition that reduces clearance of endotoxin would therefore be expected to enhance the inflammatory consequences of smoking. The \textit{CC} genotype may be such a genotype. The measurement of endotoxin alone may not be sufficient as an indication of potential CD14 stimulation. The CD14 receptor is known to respond to a range of factors and has been proposed as an “ante-antibody” for the innate immune system.\textsuperscript{25} If this is the case, then activation of CD14 may arise from many different stimuli. In this cohort no significant association was found between CRP and CD14 genotype. We do not believe that this lack of association invalidates our findings because the gene-environment interactions observed may be operating via alternative inflammatory pathways or more localized inflammation within atherosclerotic plaque.

The association we found was stronger when relationships were studied with an increased IMT, indicated by a value in the highest quartile, compared with when IMT values across the range were correlated with genotype. This is consistent with previous studies suggesting that risk factors and future risk correlate better with an increased IMT above a certain value rather than with IMT treated as a continuous variable. For example, the Atherosclerosis Risk in Communities (ARIC) Study found that the relationship between IMT and risk of coronary heart disease was nonlinear and that it was strongest at the higher values of IMT.\textsuperscript{26}

In this community population, the number of subjects with carotid plaque was relatively low, at only 6.3%. Therefore, there were too few cases to allow meaningful statistical analysis of the association between genotype and plaque. Previous similar studies have reported higher incidence of plaque.\textsuperscript{27,28} The low frequency we found may reflect a true lower prevalence in our population, but direct comparison is difficult because of differences in both study groups and in definition of plaque. We defined plaque as an IMT >1.7 mm, while other studies have used definitions ranging from 1.0 to 1.7 mm.\textsuperscript{27,29,30} This study therefore addresses the mechanisms involved in the initiation of carotid atherogenesis, not the later progression of established plaques. The pathogenesis of atherosclerosis is complex and multifactorial. Although the relationship between the CD14 gene and carotid IMT remains circumstantial at this time, the findings of this study support the hypothesis that gene-environment interactions are potentially important determinants of atherosclerosis. The role of “conventional risks” is well documented, but the input from the subject’s genes has only recently begun to be explored.

Measurement of increased CCA IMT has previously been used as a measure of subclinical atherosclerosis in a general population.\textsuperscript{29} CCA IMT measured by ultrasound correlates well with histological measurements of the artery wall.\textsuperscript{30} Prospective studies have shown that increased IMT independently predicts future cardiovascular event risk, including stroke.\textsuperscript{31,32} Measurement of CCA IMT, as opposed to other sites, appears to provide the best reproducibility.\textsuperscript{33}

These findings support a role for the CD14 endotoxin receptor in atherosclerosis. Genetic variation in the endotoxin

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Age and Sex Adjusted</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(P)</td>
<td>OR</td>
</tr>
<tr>
<td>All</td>
<td>0.007</td>
<td>1.58</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>0.014</td>
<td>1.79</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>0.188</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Significance values are shown after age and gender adjustment and after adjustment for all cardiovascular risk factors (see text).

Relationship between CCA IMT and CD14 genotype by smoking status. A graded response was seen in current smokers and ex-smokers but was absent in individuals who had never smoked.

\(\text{CC} \text{ vs } \text{TT} \ p=0.036\)
receptor may act to mediate the risk of smoking on vascular disease.

Acknowledgments

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References

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