Association of an allelic variant of interleukin-6 with subclinical carotid atherosclerosis in an Australian community population


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Aims Atherosclerosis can be viewed as a low grade inflammatory process, and genetic polymorphisms within cytokines are candidate risk factors for the development of atherosclerosis. We examined the association of a common functional variant in the IL-6 gene with carotid intimal-medial wall thickness (IMT) and the presence of plaques in a randomly selected, cross-sectional Australian population.

Methods B-mode carotid ultrasound was performed on 1109 subjects aged 27–77 years, who were genotyped for the IL-6 polymorphism (-174G>C) and assessed for conventional cardiovascular risk factors.

Results The frequency of the IL-6 -174C allele was 0.41. Initial univariate analysis showed no association of the IL-6 -174G>C polymorphism with carotid IMT. Multivariate analysis however showed an association of the IL-6 -174C allele with increased IMT in subjects older than the median age of 53 years (P=0.005). Initial univariate analysis of the IL-6 -174G>C polymorphism and carotid plaque showed no association in the whole sample. In multivariate analysis the -174C allele was independently associated with an increased risk of carotid plaque in the whole sample (CC vs GG, OR=2.22, 95% CI=1.32 to 3.73, P=0.003).

Conclusions This study shows that the IL-6 -174G>C variant is independently associated with carotid plaque formation in the whole population and an increased carotid IMT in older subjects within a randomly selected, cross-sectional Australian population.

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KEYWORDS
Interleukin-6; Polymorphism; Intimal-medial thickness; Carotid ultrasound; Carotid atherosclerosis

Introduction

Atherosclerosis is a complex, multifactorial disease caused by the interaction of environmental factors and genetic components from many biological pathways; each component adding an increased risk of disease.
A variant in the IL-6 gene promoter and atherosclerosis

It has been hypothesized that low-grade inflammation is one process which may lead to the development and progression of atherosclerosis. Inflammatory cells have been shown to infiltrate atherosclerotic plaques at all stages of their development, from the fatty streak to advanced atheromatous lesions, plaque disruption and thrombosis.\(^1,2\) Circulating protein markers of inflammation such as interleukin-6 (IL-6) and C-reactive protein (CRP) have been found to be predictive for the risk of future coronary events in apparently healthy men and women.\(^3-5\)

IL-6 is a central mediator of the acute phase response and may therefore play a causal role in atherosclerotic disease. It is expressed in macrophages within human atheroma,\(^6\) has a stimulatory effect on smooth muscle cell proliferation\(^7\) and has the ability to accelerate atherosclerosis in murine models.\(^8,9\) Raised plasma concentrations have been found in patients with unstable angina,\(^10\) and in healthy subjects at risk of future cardiovascular events.\(^11\)

A functional genetic variant has been identified in the promoter of the interleukin-6 gene (-174G>C), which has been reported to regulate protein expression both in vitro\(^12,13\) and in vivo.\(^14,15\) The aim of this study was to investigate the involvement of this polymorphism in early subclinical atherosclerosis as measured by carotid artery intimal-medial wall thickness (IMT) and the presence of carotid plaque in the Perth Carotid Ultrasound Disease Assessment Study (CUDAS). This sample consists of randomly selected healthy subjects (n=1111), aged 27–77 years, with equal numbers of males and females and equal numbers in each age decile. All subjects underwent B-mode ultrasound and were genotyped for the IL-6 -174G>C variant.

Methods

Subjects

Subjects were original participants in the 1989 Australian National Heart Foundation Perth Risk Factor Prevalence Survey.\(^16\) This was a random electoral roll survey of 2000 people from the metropolitan area of Perth, Western Australia. Of these subjects 1111 (61% of those eligible) agreed to take part in the study described here. Subjects who had previous carotid artery surgery were excluded. Subjects who had cancer, chronic inflammatory disease or auto-immune disease were also not included in this study. This population is Caucasian with greater than 90% of participants recording Australia as their country of birth. Written informed consent was obtained from all study participants. The study protocol was approved by the Institutional Ethics Committee of the University of Western Australia.

A self administered questionnaire similar to that used by the 1989 Australian National Heart Foundation Risk Factor Prevalence Survey was used to record a history of hypertension, hyperlipidaemia, diabetes, angina pectoris, myocardial infarction (MI), stroke or a family history of premature-onset CHD by age 55 years in first degree relatives.\(^16\) Smoking lifetime exposure by pack-years was calculated. Anthropomorphic measurements and the lower of two resting blood pressures, measured with a mercury column manometer, were recorded by a trained research nurse.

Laboratory measurements

A fasting venous blood sample from each subject was obtained. Total cholesterol, HDL cholesterol and triglyceride levels were determined enzymatically with a Hitachi 747 autoanalyzer. LDL cholesterol was calculated using a method by Friedwald et al.\(^17\) Homocysteine was measured by HPLC and ferritin was measured on an ACS-180 (Bayer Diagnostics).

Genetic analysis

Genomic DNA was extracted from leukocytes using a salting out method. The G to C nucleotide substitution at -174 in the promoter region of the IL-6 gene created a restriction fragment length polymorphism (RFLP) and the genotype was determined by digestion of PCR fragments with Hpa II (Promega) and Sfi I (New England Biolabs) as previously described.\(^12\)

Carotid ultrasound

Bilateral carotid B-mode ultrasound was performed by two trained sonographers using a 7.5-MHz annular phased-array transducer on an Interspec (Apogee) CX 200 ultrasound machine as previously described.\(^18\) The IMT was defined as the distance between the characteristic echoes from the lumen-intima and media-advetitia interfaces on the far wall of the distal common carotid artery measured over a 1 cm segment length.\(^19\) A thorough search of the distal common carotid, carotid bulb, and internal and external carotid arteries was also made to determine the presence of focal plaque. Plaque was defined as a clearly identified area of focal increased thickness (≥1 mm) of the intima-media layer. Three end-diastolic images were analysed from the right and left distal common arteries at a site free of any discrete plaque and measurements averaged to give the mean IMT. Repeat measurements of randomly selected scans revealed no significant variation in the IMT measurements. Quality control measures included repeat scans on a subset of 30 subjects on two separate occasions 7–10 days apart. The intra-observer coefficient of variability was 2.9% for sonographer 1 and 4.8% for sonographer 2. The inter-observer coefficient of variability was 5.9%.

Statistical analysis

Outcome variables of the association analyses were log10 IMT levels and the presence of one or more carotid plaques. The principal explanatory variable was the genotyped polymorphism. The bi-allelic IL-6 polymorphism was coded into three classes and analysed categorically, with the most common homozygous genotype set as the baseline (GG=0, GC=1, CC=2). Sex, the presence of carotid plaques, physician-diagnosed diabetes, hypertension, and family history of myocardial infarction (MI) were analysed as binary variables. All other variables were analysed as continuous. Cigarette smoking status was analysed both as a binary variable (smoking ever) and as a continuous variable (pack years of smoking). IMT, body mass index (BMI), triglycerides, HDL, homocysteine and ferritin were not normally distributed and were therefore logarithmically (log10) transformed prior to analysis. Consistency of genotype frequencies with Hardy–Weinberg equilibrium was tested using a chi-squared goodness-of-fit test on a contingency table of observed versus predicted genotype frequencies.

Univariate analysis used analysis of variance (ANOVA) to contrast mean IMT across the three levels of each genotype and \(\chi^2\) tests on contingency tables when comparing genotype to categorical variables.
Multivariate analysis used generalized linear models (GLMs) (linear and logistic regression) to model the effects of multiple covariates on the continuous and dichotomous outcomes. Both forwards and backwards stepwise modelling procedures were used to select a useful subset of independent predictors of an outcome of interest. Checks of goodness of fit included an investigation of the need for interaction or polynomial terms, analyses of Pearson residuals, and examination of the effect of observations with high regression leverage. Definitive final models were shown to provide a valid summary of the observed data. The stepwise regression models constructed included the following variables as possible explanatory covariates: age, gender, systolic blood pressure (systolic BP), diastolic blood pressure, body mass index (BMI), waist-hip ratio, total cholesterol, diastolic blood pressure, mmHg, HDL, LDL, smoking (pack-years), plasma homocysteine levels, alcohol consumption (g/day), exercise (sessions/week), physician-diagnosed diabetes, self-reported history of hypertension, myocardial infarction (MI) and family history of ischaemic heart disease or cardiovascular disease. Exploratory analyses for age-genotype and sex-genotype interactions were performed. Where an age-genotype interaction was detected on initial analysis, age was further analysed as a binary variable dichotomised at the median value (53 years in the whole population). In addition to the analyses of genotype as a categorical variable, evidence for a linear (genetically additive) effect on phenotype across the three genotypes of each polymorphism was sought by entering the genotypes as continuous covariates in the multivariate models. The GLMs were also used to estimate genotype specific means and 95% confidence intervals. Minitab for Windows v12.1 (Minitab Inc.) and SPSS version 10.1 for windows were used to manage and analyse data. Statistical significance was defined at the standard 5% level.

Results

DNA from 1109 of the total 1111 subjects was available for genotyping, and their characteristics have been presented previously. The mean age was 53.3 years (standard deviation [SD]=12.7 years). The sex ratio was balanced; 558 male (50.3%) and 553 female (49.7%) subjects were studied. Approximately 25.6% of the subjects had one or more detectable carotid plaques.

Genotype frequencies of the IL-6 -174G>C gene polymorphism in the whole sample were 15.4% for the CC genotype, 50.2% for GC, and 34.4% for GG. These genotype frequencies were consistent with Hardy–Weinberg equilibrium ($\chi^2=2.02, P=0.36$) and the frequency of the C allele was 40.5% [95% CI=37.5–43.5]. The genotype distributions and allele frequencies were not significantly different between males and females.

When the whole sample was analysed according to IL-6 -174G>C genotype (Table 1) a significant association with triglyceride levels was noted, with subjects carrying the CC genotype having 12% higher triglyceride levels than subjects with the GG or GC genotype combined (Table 1). All other variables were similar between IL-6 -174G>C genotypes.

Gene polymorphisms and carotid IMT

Univariate analysis showed that there was no association between the IL-6 -174G>C polymorphism and mean carotid IMT ($P=0.71$) in the sample as a whole, or in
females ($P=0.70$) or males ($P=0.19$) separately. Multivariate analysis, adjusting for known cardiovascular risk factors, also showed no association with mean IMT in the whole sample ($P=0.35$) (Table 2), but further analysis of the general linear model suggested an interaction between the IL-6 gene polymorphism and age ($P=0.006$). Exploratory analysis with the population stratified by median age (<53 years; ≥53 years) indicated that the association between the IL-6 -174C allele and increased IMT was only found in the older age group ($P=0.005$) (Table 2).

### Gene polymorphisms and carotid plaque

Univariate analysis of the whole sample showed that the IL-6 -174G>C polymorphism was not significantly associated with the presence of carotid plaques ($P=0.08$) (Table 1) and no significant associations were observed in either men ($P=0.21$) or women ($P=0.08$). However, multivariate modelling suggested that, after adjustment for age, gender, LDL, triglycerides, smoking (pack-years), hypertension, systolic BP, diabetes, and a previous history of myocardial infarction, there was a significant association of the IL-6 -174C allele with an increased likelihood of carotid plaque in the whole sample (GC vs GG, OR=1.39, 95% CI=0.96 to 2.03, $P=0.09$; CC vs GG, OR=2.22, 95% CI=1.32 to 3.73, $P=0.003$). Further exploratory analysis showed no significant sex-genotype interaction ($P=0.6$) however when the population was stratified by sex this association was significant in females, with a similar (non-significant) trend in males (Table 3). After adjustment for other risk factors, there was a significant linear (genetically additive) effect across the three IL-6 genotypes on risk of carotid plaque in females (OR=1.66, 95% CI=1.32 to 2.03, $P=0.01$) but not in males (OR=1.34, 95% CI=0.96 to 1.87, $P=0.08$).

### Discussion

This study used a large cross-sectional community-based sample of adults (n=1109) from Western Australia to study the association of a polymorphism in the IL-6 gene with common carotid IMT and the presence of carotid plaque. We have shown for the first time, that the -174G>C polymorphism is independently associated with an increased intimal-medial thickening in older subjects and with an increased risk of having more than one carotid plaque in the whole sample.

Studies have shown that mean IMT of the common carotid artery is a good indicator of generalized atherosclerosis. Carotid IMT has also been demonstrated to predict an increased risk of future myocardial infarction and stroke in adults. Our findings suggest that the IL-6 -174G>C polymorphism is therefore associated with the development of atherosclerosis as measured by carotid IMT. Subgroup analysis showed that this association was restricted to older subjects (i.e. greater than the median of 53 years). This perhaps is not surprising, as

#### Table 2

<table>
<thead>
<tr>
<th>Genotype (n)</th>
<th>Adjusted means (95% CI)*</th>
<th>$P$ value (F statistic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole sample</td>
<td>GG (380) 0.69 (0.68, 0.70)</td>
<td>$P=0.35$ (F$_{2,110}=1.06$)</td>
</tr>
<tr>
<td></td>
<td>GC (556) 0.70 (0.69, 0.71)</td>
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<tr>
<td></td>
<td>CC (171) 0.70 (0.69, 0.72)</td>
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<tr>
<td>&lt;53 years</td>
<td>GG (192) 0.63 (0.62, 0.64)</td>
<td>$P=0.18$ (F$_{2,554}=1.72$)</td>
</tr>
<tr>
<td></td>
<td>GC (279) 0.63 (0.62, 0.63)</td>
<td></td>
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<tr>
<td></td>
<td>CC (83) 0.61 (0.61, 0.63)</td>
<td></td>
</tr>
<tr>
<td>&gt;53 years</td>
<td>GG (188) 0.76 (0.75, 0.78)</td>
<td>$P=0.005$ (F$_{2,553}=5.37$)</td>
</tr>
<tr>
<td></td>
<td>GC (277) 0.78 (0.76, 0.79)</td>
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<tr>
<td></td>
<td>CC (88) 0.81 (0.79, 0.83)</td>
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*Genotype-specific means from GLM model; adjusted for age, gender, LDL, HDL, triglycerides, systolic blood pressure, waist hip ratio and cigarette smoking (pack years)

#### Table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>OR (95% CI) and $P$ value for the whole population</th>
<th>OR (95% CI) and $P$ value for males</th>
<th>OR (95% CI) and $P$ value for females</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>GC</td>
<td>OR=1.39 (95% CI=0.96 to 2.03) $P=0.09$</td>
<td>OR=1.52 (95% CI=0.92 to 2.53) $P=0.10$</td>
<td>OR=1.24 (95% CI=0.70 to 2.20) $P=0.47$</td>
</tr>
<tr>
<td>CC</td>
<td>OR=2.22 (95% CI=1.32 to 3.73) $P=0.003$</td>
<td>OR=1.84 (95% CI=0.90 to 3.77) $P=0.09$</td>
<td>OR=3.10 (95% CI=1.39 to 6.90) $P=0.006$</td>
</tr>
</tbody>
</table>

*Genotype-specific associations relative to baseline genotype in study population from logistic regression model; adjusted for age, gender, LDL, triglycerides, systolic BP, smoking (pack-years), and previous history of hypertension, diabetes or myocardial infarction.
thickening of the carotid intimal-medial wall is strongly associated with age; older subjects having thicker IMT.\textsuperscript{22} A genetic risk of increased IMT may therefore only be detectable as subjects age and their risk of developing atherosclerosis increases.\textsuperscript{22}

Two other published studies are contradictory to our findings in that they have shown the GG genotype, and not the CC genotype to be associated with thicker carotid IMT. The most recent of these studies only 87 subjects of mixed race, with a mean age of 70 years,\textsuperscript{26} which is considerably older than the CUDAS sample. The frequency of the C allele in this study was lower (20\%) than in the present study (40\%). Others have also shown a difference in the C allele frequency between races.\textsuperscript{27}

It is therefore possible that a small sample size and population stratification in this study caused a different association to be seen. An earlier study on a group of 109 Finnish men showed an association between the IL-6 genotype and carotid IMT only in univariant analysis ($P=0.04$ for trend). When adjusted for other cardiovascular risk factors (such as age, BMI, LDL, systolic BP and smoking) no association was observed.\textsuperscript{28} Our study is the largest to date and warrants confirmation in other large populations.

We also show that the -174G>C polymorphism is independently associated with the presence of plaque in the whole sample. Exploratory subgroup analysis showed this association to be statistically significant in females, with a similar but non-significant trend in males. However, numbers in this subgroup analysis were small, and with larger numbers the effect may be significant in both sexes. To our knowledge this is the first time that the -174G>C polymorphism has been associated with plaque and hence requires confirmation in other populations.

The -174G>C polymorphism in the promoter of the IL-6 gene has been shown to be functional in vitro.\textsuperscript{12,13} Fishman et al.\textsuperscript{12} showed that the -174G allele at basal levels and after stimulation had the highest IL-6 levels in HeLa cells. However another study\textsuperscript{13} suggested that this may be cell type specific and in endothelial and macrophage cell lines it is the -174C allele that produces higher reporter gene expression. This study also suggested that the -174G>C polymorphism is not the only functional polymorphism in the IL-6 promoter and haplotype analysis suggests a complex interaction between several polymorphisms.\textsuperscript{13} Recent in vivo studies have reported that in healthy subjects there is little or no difference in plasma IL-6 levels by genotype,\textsuperscript{29} but after coronary artery bypass surgery (CABG),\textsuperscript{14} in abdominal aortic aneurysm patients,\textsuperscript{15} and in babies after the stress of a vaginal delivery,\textsuperscript{30} subjects with the -174CC genotype consistently have higher IL-6 protein levels. This suggests that it is the -174C allele and not the G allele that is more responsive to inflammatory stimuli in vivo. This in vivo data supports our findings that the -174C allele is a genetic risk factor in subclinical atherosclerosis, a low-grade inflammatory disease.

However, there are inconsistencies in the studies published, with other groups finding that the G allele is associated with increased IL-6 protein levels and hospitalization periods after CABG,\textsuperscript{31} and an increased risk of peripheral artery occlusive disease.\textsuperscript{32}

Several studies have reported higher cardiovascular risk in middle aged subjects carrying the –174C allele,\textsuperscript{15,33,34} and in a study in the US,\textsuperscript{35} a similar association was observed in elderly men and women. By contrast a recent large study showed the IL-6 –174G>C polymorphism was not associated with the risk of coronary artery disease or myocardial infarction.\textsuperscript{36} It is likely that these inconsistencies arise because the IL-6 –174G>C is not a strong determinant of cardiovascular risk, and that its effect is modulated by age, other genetic and environmental factors.

One weakness of this study is that plasma IL-6 protein measurements were unavailable. Previous studies have suggested that the effects of genotype on IL-6 protein levels are likely to be too small to detect in healthy subjects who have very little atherosclerotic burden and no prior inflammatory stimulation.\textsuperscript{29,30} It is also uncertain that plasma levels would accurately reflect local tissue production of IL-6 protein. The different findings between the univariate and multivariate analysis in this study suggest that the effect of the IL-6 –174G>C polymorphism is small and only after adjusting for confounders in the multivariate model is an association observed. Whilst our study is relatively large, we realise that subgroup analysis (by age and sex) and analysis of subjects with plaque (only 25\% of the population) reduce numbers dramatically and subgroup findings should be considered as exploratory. Therefore our findings need further confirmation in other large studies. Haplotype analysis of the IL-6 promoter may have provided a clearer picture of how IL-6 expression is controlled, but other reported promoter variants are either in complete allelic association in Caucasians (-597G>A)\textsuperscript{14} or are rare (-572G>C)\textsuperscript{14} and in order to have enough power to detect a statistically significant effect of these haplotypes a much larger sample than that described here would be required.

In conclusion, we have shown that the IL-6 -174C allele is associated with an increase in common carotid IMT in older subjects and the presence of plaques in this large cross-sectional community-based sample of men and women. This finding supports a role for inflammation in the early development of carotid atherosclerosis.

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