Val64Ile Polymorphism in the C-C Chemokine Receptor 2 Is Associated With Reduced Coronary Artery Calcification


Objective—Studies in mice have shown that genetic disruption of monocyte chemotactic protein-1 or its receptor, the C-C chemokine receptor 2 (CCR2), inhibits atherosclerosis, but few data exist in humans to suggest that the monocyte chemotactic protein-1–CCR2 interaction is important in atherogenesis. A common polymorphism in the human CCR2 gene resulting in a substitution of isoleucine for valine (Val64Ile) has been associated with other disease phenotypes in humans.

Methods and Results—A cohort of first-degree relatives of persons with premature coronary artery disease was recruited and quantitatively phenotyped for the extent of CAC, a marker of coronary atherosclerosis, by using electron beam CT. The extent of CAC was significantly lower in subjects with the CCR2-Ile64 variant (Val/Ile and Ile/Ile genotypes) than in subjects carrying 2 Val64 alleles, even after adjustment for traditional risk factors.

Conclusions—This study provides genetic evidence linking CCR2 with coronary atherosclerosis in humans. (Arterioscler Thromb Vasc Biol. 2002;22:1924-1928.)

Key Words: atherosclerosis • chemokines • coronary artery calcification • polymorphism • coronary heart disease

Premature coronary artery disease (CAD) often clusters in families. Although some of the heritability of CAD can be explained by traditional risk factors with a genetic component, such as hypertension, diabetes, and hypercholesterolemia, many susceptibility and protective genes are yet to be identified. For example, a gene locus for myocardial infarction has recently been mapped to a region on chromosome 14 that does not contain any known cardiovascular risk factor genes.

Monocyte chemotactic protein (MCP)-1 and its receptor, CCR2, have been linked with atherogenesis. MCP-1 is synthesized by arterial smooth muscle cells and endothelial cells exposed to inflammatory stimuli, thus providing a chemotactic stimulus for the recruitment of monocytes into the arterial wall as one of the earliest cellular processes in atherogenesis. In the subsequent stages of lesion formation, macrophages themselves become important sources of MCP-1 synthesis. The functional effects of MCP-1 are mediated by CCR2. The important roles of MCP-1 and CCR2 in murine atherogenesis have been documented. Disruption of either of these genes in mice has been shown to suppress lesion formation in a gene dose–dependent manner. The mechanisms by which the MCP-1–CCR2 interaction promotes vascular lesion development are likely to go beyond simple monocyte recruitment to the vessel wall. Despite the large body of data linking the MCP-1–CCR2 interaction to atherosclerosis in mice, few data exist in humans directly linking MCP-1 or CCR2 to atherosclerosis.

One of the few common genetic variants in CCR2 that has been reported is a Gly to Ala substitution at base 190, resulting in a Val to Ile substitution at position 64. The allele frequency of this polymorphism has been described to be between 0.1 and 0.25, depending on the ethnic background. The Ile64 variant has been found to be associated with a lower risk of AIDS, pulmonary sarcoidosis, and of acute renal transplant rejection. These reports suggest that the Ile64 allele may be associated with reduced CCR2 function.

To search for gene variants associated with variation in coronary atherosclerosis, we recruited a cohort of healthy subjects with a family history of premature CAD, and we used electron beam CT (EBCT) to determine the extent of coronary artery calcification (CAC) as a phenotyping strategy. Calcification is a normal process in the development of coronary atherosclerotic plaque, and the degree of coronary calcification as assessed by EBCT is correlated with the extent of coronary atherosclerotic lesion. Therefore, quantification of CAC provides a noninvasive method for determining the extent of coronary atherosclerotic plaque burden in healthy subjects. In the present study, we determine that the CCR2-Ile64 polymorphism is associated with signif-
significantly lower CAC, suggesting that it may reduce the development of coronary atherosclerosis.

**Methods**

**Subjects**

First-degree relatives of subjects with premature CAD (defined as documented CAD before the age of 60 years in men and before the age of 70 years in women)\(^{20,21}\) were recruited and phenotyped. A subset of this cohort has been previously described.\(^{22}\) Study subjects were recruited through a preventive cardiology clinic, through newspaper and radio advertisements, and by word of mouth. Men aged 30 to 65 years and women aged 35 to 70 years were included in the present study. Exclusion criteria for entry into the study included the following: (1) clinical diagnosis of diabetes mellitus; (2) total cholesterol >300 mg/dL; (3) cigarette smoking (>1 pack per day); (4) poorly controlled hypertension defined as systolic blood pressure >150 and/or diastolic blood pressure >95 mm Hg; (5) body mass index (BMI) >37; (6) severe renal insufficiency defined as serum creatinine >3.0 mg/dL; and (7) chronic illness that could affect cardiovascular risk or atherosclerosis, such as active cancer, autoimmune disease, and dementia. Only unrelated subjects were used for this analysis. Subjects of all races and ethnic backgrounds were recruited, but the present analysis was limited to non-Hispanic white subjects. The University of Pennsylvania Institutional Review Board approved the study protocol.

Subjects were asked to come, after a 12-hour overnight fast, to the General Clinical Research Center of the University of Pennsylvania Medical Center. A questionnaire regarding medical and family history was completed by the study subjects and reviewed by the research nurse. Information on current medications was recorded. Height and weight were measured with a wall stadiometer and a beam balance. Systolic and diastolic blood pressures were measured in both arms with a random-zero sphygmomanometer. For determination of CAC, individuals were placed in a supine position on the scanning bench of the EBCT scanner (Imatron). Forty contiguous 3-mm-thick computed tomograms (transverse 2D images) were obtained from the root of the aorta to the apex of the heart. ECG gating was used so that all images were obtained at the same time during the cardiac cycle. Image acquisition time was 100 ms. Scans were obtained with a 30-cm\(^2\) field of view and transferred into 512×512 reconstruction matrix in which 1 pixel=0.343 mm\(^2\). A minimal size of 0.67 mm\(^2\) was used to define calcification. Scan results were reviewed for technical quality and accuracy of the computer-assisted identification of CAC. A global CAC score was determined according to the method of Agatston et al\(^{23}\) with the use of software expressly designed for this purpose (Imatron).

**Analytical Methods**

Plasma total cholesterol, HDL cholesterol (HDL-C), and triglyceride levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems Inc) with the use of Sigma reagents (Sigma Chemical Co), and LDL cholesterol (LDL-C) was calculated by the Friedewald formula: LDL-C=total cholesterol−HDL-C−(triglycerides/5). When triglyceride levels were >400 mg/dL, LDL-C was not calculated.

**Genotyping**

Genomic DNA was extracted from whole blood by using a nonenzymatic high-salt procedure.\(^{24}\) Allele-specific amplification primers for the CCR2-Val64Ile polymorphism were used in conjunction with a common forward primer. Two polymerase chain reaction reactions were conducted for each DNA sample (1 for each allele-specific oligonucleotide) under the following conditions: 94°C for 40 seconds and 60°C for 30 seconds for 35 cycles in 20 μL reactions containing 10 pmol of each primer, 0.2 mmol/L of each dNTP, 1 U TaqExpress (GenPak Ltd), 50 mmol/L Tris HCl (pH 9.1), 16 mmol/L ammonium sulfate, 3.5 mmol/L MgCl\(_2\), and 150 μg/mL BSA, plus 25 to 100 ng DNA. For each genotype, the 2 allele-specific amplification reactions were run independently on 3% agarose gels, and each lane was scored for the presence and absence of each allele. The allele-specific primers used were CCR2B-64IRC (5′-TTGGCAGTTTATAGATCGGAC-3′) and CCR2B-64IRT (5′-TTG CAG TTT ATT AAG ATG CGG AT-3′). The common forward oligo was CCR2B-64IF (5′-TAC CAA CGA GAG CGG TGA AGA GT-3′).

**Statistical Methods**

The model used to calculate 10-year absolute risk was developed by Wilson et al.\(^{25}\) To test whether the distribution of genotypes was in accordance with Hardy-Weinberg equilibrium and whether the frequencies were different in groups of subjects with higher or lower CAC scores, a χ\(^2\) analysis was used. Linear regressions were performed to compare the distributions of risk factors in the 2 CCR2 genotypes. To examine the role of the CCR2 genotype as a predictor of CAC, logistic regression models were fitted by using cut points of 1, 5, 10, and 20, which have been previously used for analysis of cohorts with CAC as a phenotype.\(^{26,27}\) These models included the CCR2 genotype (Ile/Ile=0, Val/Val=1), sex (male=1, female=0), age, BMI, plasma levels of triglycerides (logarithmically transformed), smoking status, and LDL-C and HDL-C levels. ANCOVA was used to compare the mean CAC score for the 2 CCR2 genotypes. The ANCOVA statistic was chosen because of its ability to incorporate additional factors as well as covariates into the statistical model. Age, sex, BMI, lipid-lowering medication, smoking status, triglycerides (logarithmic scale), LDL-C, and HDL-C were used as covariates in the ANCOVA, as was an interaction term for CCR2 genotype and sex. The outcome

<table>
<thead>
<tr>
<th>TABLE 1. Descriptive Statistics</th>
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<tr>
<td><strong>Men (n=367)</strong></td>
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<tr>
<td>---------------------------</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td>Age, y</td>
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<tr>
<td>Systolic BP, mm Hg</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
</tr>
<tr>
<td>BMI, kg/m(^2)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
</tr>
<tr>
<td>CAC</td>
</tr>
<tr>
<td>10-year risk, %</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
</tr>
<tr>
<td>Lipid-lowering medication, n(%)</td>
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</table>
variable used was the natural logarithm of the CAC score + 1. Assumptions of homogeneous variance were not violated in the current data set. Statistical analyses were performed using the S-Plus package 2000, release 3 (Insightful Corp.).

Results
Cardiovascular risk factors, CAC scores, and CCR2 genotype were determined in a total of 662 white subjects (295 women and 367 men). The demographics of the study group are described in Table 1. The distribution of CAC in the cohort is described in Table 2. Eighty-two percent of the men and 54% of the women had CAC scores >0. The maximum CAC score was 1327 in women and 3538 in men. Twenty subjects included in this analysis had documented CAD.

In this cohort, the CCR2 allele was in Hardy-Weinberg equilibrium, with genotype frequencies of 81% Val/Val, 18.1% Val/Ile, and 0.9% Ile/Ile. No significant associations were found between CCR2 genotype and individual cardiovascular risk factors. The average 10-year absolute CAD risk was 1327 in women and 3538 in men. Twenty subjects included in this analysis had documented CAD.

The frequency of carriers of the CCR2-Ile64 allele was significantly higher in those subjects with CAC ≤20 (22.3%) than those subjects with CAC >20 (15%, P<0.05). When the probability of having a CAC score >1 was evaluated by logistic regression while controlling for other cardiovascular risk factors, a significant association with the CCR2-Val64Ile genotype was found, with an odds ratio of 1.65 for Val64 relative to Ile64 (P=0.041, Table 3). When the same analysis was performed with the use of CAC >20 as a cut point, the association remained significant, with an odds ratio of 2.7 (P=0.007, Table 3). Analyses using cut points of 5 and 10 produced similar significant results (data not shown). Logistic regression analysis after exclusion of the 20 subjects with a clinical history of CAD produced similar significant results. There was no evidence of a genotype-by-sex interaction as assessed by logistic regression. Therefore, regardless of the CAC cut points used and independent of sex and other cardiovascular risk factors, persons with lower CAC scores were significantly more likely to be carriers of the Ile64 variant than persons with higher CAC scores.

Among subjects with CAC scores >0, we found by ANCOVA that the presence of at least 1 CCR2-Ile64 allele (Ile/X genotype) was associated with a significantly lower mean CAC score in both men and women (Figure), even after other CAD risk factors were included in the statistical model (P=0.0055). These results indicate that the association of CCR2-Ile64 with lower CAC cannot be explained by differences in traditional risk factors between the 2 genotype groups. By ANCOVA, no genotype-by-sex interaction was observed (P<0.84), indicating that the sex of the subject does not influence the association between CCR2 genotype and CAC. The total model explained 40.5% of the variance in CAC, and the CCR2 genotype accounted for 1.4% of the variance in CAC in this cohort.

We found no evidence of genetic stratification in the cohort or of any correlation between CAC or CAC amount and 5 random loci (see Appendix). This indicates that the genetic association observed between the CCR2-Val64Ile alleles and CAC cannot be explained by population substructure.

Discussion
In the present study, we used CAC, as measured by EBCT, to noninvasively estimate the extent of coronary atherosclerosis in order to address the hypothesis that genetic variation in CCR2 is associated with variation in coronary atherosclerosis. The results indicate that the Val64Ile variant in CCR2 is

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**TABLE 2. CAC Distribution**

<table>
<thead>
<tr>
<th></th>
<th>Men (n=367)</th>
<th>Women (n=295)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAC=0</td>
<td>65 (17.7%)</td>
<td>135 (45.9%)</td>
</tr>
<tr>
<td>CAC 1–10</td>
<td>123 (33.5%)</td>
<td>87 (29.5%)</td>
</tr>
<tr>
<td>CAC 11–20</td>
<td>23 (6.3%)</td>
<td>9 (3.0%)</td>
</tr>
<tr>
<td>CAC 20–80</td>
<td>55 (15.0%)</td>
<td>30 (10.2%)</td>
</tr>
<tr>
<td>CAC &gt;80</td>
<td>101 (27.5%)</td>
<td>34 (11.5%)</td>
</tr>
</tbody>
</table>

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**TABLE 3. Logistic Regression Results Where the Outcome Compared Is Either CAC>1 or CAC>20**

<table>
<thead>
<tr>
<th></th>
<th>CAC &gt;1</th>
<th>CAC &gt;20</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>β</td>
<td>SE</td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-12.77</td>
<td>1.66</td>
</tr>
<tr>
<td>Age, y</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>Male sex*</td>
<td>1.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Smoker†</td>
<td>0.45</td>
<td>0.33</td>
</tr>
<tr>
<td>Hypertension‡</td>
<td>0.04</td>
<td>0.25</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>0.14</td>
<td>0.03</td>
</tr>
<tr>
<td>Triglycerides, log(mg/dL)</td>
<td>0.41</td>
<td>0.23</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>-0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>CCR2§</td>
<td>0.50</td>
<td>0.25</td>
</tr>
</tbody>
</table>

β is the parameter estimate, SE is standard error, and OR is odds ratio.

*0= female, 1= male; †0= non smoker 1= smoker; ‡0= no hypertension 1= hypertension; §Ile/X=0; Val/Val=1.
significantly associated with CAC. The rarer Ile64 variant was found to be associated with reduced CAC independent of traditional cardiovascular risk factors. This same variant was found to be associated with a delay in developing AIDS in subjects infected with HIV-1,12 was less frequent in patients with pulmonary sarcoidosis than in healthy control subjects, 14 and was associated with a significant decrease in the risk of acute renal transplant rejection.15 The finding in the present study is consistent with these previous reports and with the concept that the Ile64 variant may have reduced function compared with wild-type CCR2 and therefore have a protective effect regarding the development of atherosclerosis.

CAC is a normal process in the development of coronary atherosclerotic plaque, 16,17 and the amount of CAC as assessed by EBCT is correlated with the extent of atherosclerotic lesion.18,19 Therefore, CAC provides a readout of the extent of coronary atherosclerotic plaque burden. EBCT is a noninvasive procedure and is therefore suitable for use in phenotyping asymptomatic subjects in an attempt to determine genes associated with premature coronary atherosclerosis. Of note, a recent report using CAC as a phenotyping strategy for linkage analysis reported loci on chromosomes 6 and 10 that were linked to CAC in a population of subjects recruited for hypertension.28

The association of the CCR2-Val64Ile variant with CAC could not be explained by an association with any of the known major risk factors for CAD or by population stratification. Although the analysis was performed on subjects with a CAC score of ≥1 (54% of the women, 85% of the men), inclusion of all subjects did not affect this result (not shown). Furthermore, when the frequency of the Ile64 allele was compared in groups with a lower and a higher CAC score with the use of different CAC cut points, the frequency of the 64Ile variant in the lower CAC group was always higher. Therefore, the apparent protective effect of the CCR2-64Ile allele on CAC was not dependent on the analysis of the data using a particular CAC cut point and was not confounded by association with other traditional risk factors.

The allele frequency of Ile64 was 0.1 in the present study of white subjects, similar to what has been described previously for other white populations (0.07 to 0.1).12,13 Higher frequencies have been reported for American blacks of African origin and Hispanics (0.15) and for Asians (0.25).12,14 Additional studies will be required to determine whether the association with CAC reported in the present study is found in these populations as well.

There are some limitations to the present study. As in any observational study, the present study is potentially subject to ascertainment bias. Quantification of CAC by EBCT is sensitive, but at very low CAC scores, it could result in artifact being counted as CAC. Importantly, the association of the CCR2 genotype with CAC does not prove a causal relationship. Although CAC is strongly correlated with coronary atherosclerosis burden, the CCR2 genotype could somehow affect the propensity of the atherosclerotic plaque to develop calcification rather than the development of plaque itself. The genetic analysis included the genotyping of only 1 gene, but clearly, many genes may have an impact on coronary atherosclerosis, and the association of CCR2 genotype with coronary atherosclerosis will need to be studied in the context of other CCR2 variants and other genes.

In summary, our results indicate that the common Val64Ile variant in CCR2 is significantly associated with reduced CAC as a marker of the extent of coronary atherosclerosis. This provides evidence that CCR2 is important for atherogenesis in humans and is consistent with the concept that the CCR2-Ile64 variant may have a reduced function compared with wild-type CCR2 and therefore may have a protective effect regarding the development of atherosclerosis.

Appendix
Genetic stratification within the cohort could result in a false-positive genetic association. That could happen if, for example, most subjects with CAC ≥1 had a different ethnic origin from subjects with a CAC of 0. In such a situation, a gene would artifactualy appear to be associated with the clinical end point under study (eg, see Kittles et al29). We addressed the genetic stratification issue by genotyping 5 microsatellites in a random subset of 200 samples from the cohort. Five microsatellite markers (D8S47 [chromosome 8], RH93303
[chromosome 8], D12S329 [chromosome 12], D2S173 [chromosome 2], and D18S42 [chromosome 18] were chosen at random with the sole criteria being (1) no close fall near genes with a known role in cardiovascular disease and (2) to be in a different chromosomal region from the CCR2 gene. The fluorescently labeled polymerase chain reaction products were electrophoretically separated with an ABI 377 sequence (PE Biosystems). Alleles were identified by using TRUEALLELE (Goldstein et al. 20). There was no significant difference in terms of risk factors or CAC between the 200 samples included in the genetic stratification analysis and those left out. If the association of CCR2 genetic variation with CAC is due to genetic stratification, allele sharing at other genetic markers in the genome should be higher in subjects with CAC ≥1 than if pairs of subjects with CAC ≥1 and CAC 0 are compared. A shared allele distance computed reflects the number of nonshared alleles per locus. 30 If 2 individuals have the same genotype at all 5 loci, the distance is 0; if they have no alleles in common, the distance is 10. We compared 55 subjects with CAC 0 and 145 subjects with CAC ≥1. The mean ± SD genetic distance within the CAC 0 was 5.32 ± 1.64; within the CAC ≥1 group, it was 5.39 ± 1.67; and between the CAC 0 and CAC ≥1 groups, it was 5.46 ± 1.68. Among CAC ≥1 subjects, we took 60 individuals with CAC ≥20 (high CAC) and 60 individuals with CAC <10 (low CAC). In the high CAC group, the mean ± SD genetic distance was 5.47 ± 1.73; in the low CAC group, the distance was 5.54 ± 1.64; and between the low and high CAC groups, the distance was 5.51 ± 1.67. These results indicate that in this cohort, there is no genetic stratification that is correlated with CAC status; therefore, the genetic association observed between the CCR2-Val64Ile alleles and CAC cannot be explained by population substructure.

Acknowledgments

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References

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