Interleukin-1 Receptor Antagonist Gene Polymorphism and Coronary Artery Disease

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Background—Cytokine gene variations are contributory factors in inflammatory pathology. Allele frequencies of interleukin (IL)-1 cluster genes [IL-1A(-889), IL-1B(-511), IL-1B(+3953), IL-1RN Intr 2 VNTR] and tissue necrosis factor (TNF)-α gene [TNFA(-308)] were measured in healthy blood donors (healthy control subjects), patients with angiographically normal coronary arteries (patient control subjects), single-vessel coronary disease (SVD), and those with multivessel coronary disease (MVD).

Methods and Results—Five hundred fifty-six patients attending for coronary angiography in Sheffield were studied: 130 patient control subjects, 98 SVD, and 328 MVD. Significant associations were tested in an independent population (London) of 350: 57 SVD, 191 MVD, and 102 control subjects. IL-1RN*2 frequency in Sheffield patient control subjects was the same as in 827 healthy control subjects. IL-1RN*2 was significantly overrepresented in Sheffield SVD patients (34% vs 23% in patient control subjects); IL-1RN*2 homozygotes in the SVD population (χ² carriage = 8.490, 1 df, P = 0.0036). This effect was present though not quite significant in the London population (P = 0.0603). A summary trend test of the IL-1RN SVD genotype data for Sheffield and London showed a significant association with *2 (P = 0.0024). No significant effect of genotype at IL-1RN was observed in the Sheffield or London MVD populations. Genotype distribution analysis comparing the SVD and MVD populations at IL-1RN showed a highly significant trend (P = 0.0007) with the use of pooled data. No significant associations were seen for the other polymorphisms.

Conclusions—IL-1RN*2 was significantly associated with SVD. A difference in genetic association between SVD and MVD was also apparent. (Circulation. 1999;99:861-866.)

Key Words: interleukins genes coronary artery disease

Although the pathogenesis of coronary artery disease (CAD) is multifactorial, family1 and twin2 studies suggest a heritable component. Studies indicate a greater genetic risk in monozygotic compared with dizygotic twins, and adoption studies have shown that much of the excess risk is genetic rather than environmental.3 Some of the known risk factors for CAD (disorders of lipid metabolism,4,5 vasoconstriction,6 and platelet function7) are genetically determined, but estimates of these known heritable risks explain less than half of the total inherited risk of CAD.8

The genetic basis of CAD may arise from a gene having a direct effect on the initiation of the disease process or a modifying effect on the development of the process after it is initiated. The importance of disease-modifying genes is exemplified in inflammatory and infectious illnesses in which polymorphic loci for cytokine genes have been shown to determine the clinical outcome of diseases such as alopecia,9 ulcerative colitis,10 diabetic nephropathy11 and malaria.12

It is clear that there is an important inflammatory component to atherosclerotic CAD,13 and peptide inflammatory mediators are likely to be involved.14 Arterial lesions contain inflammatory cells,15 and interleukin (IL)-1β15,16 and tumor necrosis factor (TNF)-α17 accumulate in atherosclerotic plaques. IL-1β has effects on the endothelium, including the induction of adhesion molecules,18 procoagulant activity19 and TGF-β expression,20 which may also contribute to the pathogenesis of CAD. Furthermore, there is now evidence that inflammation may determine the clinical presentation of CAD because levels of C-reactive protein (CRP) predict ischemic events21 and indicate a worse clinical outcome in unstable angina.22

In this study the impact of the IL-1 gene cluster and TNF-α polymorphisms known to influence the presentation and course of classic inflammatory and infectious diseases was examined in CAD. Because these genes could have a causative role in CAD or a modifying role, gene
Genotyping
DNA was extracted by standard methods from 20 mL EDTA anticoagulated blood. Genotyping was performed by a polymerase chain reaction (PCR)-based method. Briefly, genomic DNA was amplified with the use of Taq polymerase, dNTPs, and oligonucleotide primers synthesized on an ABI 373 DNA synthesizer. All the polymorphisms used in this study have been rigorously characterized and validated control subjects. Polymorphisms typed were TNFA (-308) a single base transition from G → A,21 IL-1A (-889) a C → T single base transition,24 IL-1B (-511) a C → T single base transition,25 IL-1B (+3953) a C → T transition in exon 5,26 and IL-1RN a variable number tandem repeat (86 bp) in intron 2 of the gene.27 The frequent allele was designated *1 and the rarer allele *2 for each gene variant. Table 3 shows the allele frequencies found in the 2 populations studied and their cohorts. Genotype frequencies in the patient and control subjects were not significantly different from those predicted under Hardy Weinberg equilibrium (HWE).

Statistical Analysis
Differences in genotype distributions were assessed by first calculating separate heterozygote (ORhet) and homozygote (ORhom) odds ratios with their 95% confidence intervals. An ORhet indicates the increased risk to an individual carrying one putative disease allele compared with the risk of carrying none. If we define the number of individuals in the control population having genotypes AA, BB, and AB as a, b, and c, respectively, where B is the putative disease allele, and the number of individuals in the disease population having these genotypes as d, e, and f, respectively, then we can write ORhom = a.e/b.d. The homozygous OR is the analogous ratio for carrying 2 copies of the putative disease allele and can be expressed as ORhom = a/e/b.d. These ORs were used to determine whether a possible gene dosage effect existed. The appropriate χ² tests were then performed. In the case in which a trend was suggested, a χ² test for trend was performed,28 with the number of allele *2s used for weights, otherwise a standard χ² analysis for carriage. A P value of ≤0.05 was considered to indicate nominal statistical significance. For an overall type I error (false-positive rate) of 0.05, however, a corrected critical level of 0.0036 should be used to account for the multiple tests (14 independent analyses). Based on allele frequencies estimated from the control patients (Table 3) and the sample sizes available, the power to detect an increase in allele frequency of 0.1 was calculated for each analysis.

Differences between demographic details shown in Table 1 were assessed by Z test.

Methods
We studied 2 different white Caucasian UK populations: in Sheffield (Northern General Hospital) and London (St George’s Hospital). Patients attended for diagnostic coronary angiography or percutaneous transluminal coronary angioplasty (PTCA). Each participant gave written informed consent for the study, which was approved by the local Research Ethics Committee. Patient characteristics are detailed in Table 1.

Coronary angiograms were prospectively categorized in the following manner: A vessel was regarded as significantly diseased if it contained ≥1 stenosis involving >30% loss of lumen diameter estimated by eye from the angiographic appearance and if it supplied >10% of the myocardium (SVD). MVD was defined as ≥2 epicardial arteries having significant stenoses. Normal coronary arteries (patient control subjects) were defined as completely smooth coronary arteries or vessels containing irregularities causing <30% reduction in lumen diameter. Clinically, these control patients were subdivided into 2 groups: those with chest pain suggestive of ischemia and no other cardiac problem (ie, including patients with syndrome X) and those with valvular heart disease. The final groups were Sheffield, patient control subjects (130 patients), SVD (98 patients), MVD (328 patients); London, patient control subjects (102 patients), SVD (57 patients), and MVD (191 patients). Healthy blood donor control subjects (827) were also collected from the Regional Blood Transfusion Center, Sheffield by the Division of Molecular and Genetic Medicine, University of Sheffield.

Coexisting inflammatory diseases with a known association with polymorphisms of genes in the IL-1 cluster were at very low levels in the Sheffield population. Within the Sheffield population there were no cases of inflammatory bowel disease, Graves disease, or diabetic nephropathy, 1 case of multiple sclerosis, and 1 case of SLE and 4 cases of rheumatoid arthritis (2 in control subjects and 2 in the SVD group).

TABLE 1. Prevalence of Selected Risk Factors for Coronary Heart Disease Among Case Patients and Patient Control Subjects

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Sheffield</th>
<th></th>
<th></th>
<th>London</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case Patients</td>
<td>Control Subjects</td>
<td>Case Patients</td>
<td>Control Subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=98)</td>
<td>(n=130)</td>
<td>(n=57)</td>
<td>(n=102)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y*</td>
<td>58.9±0.9</td>
<td>60.8±1.13</td>
<td>61.1±1.1</td>
<td>64.4±0.64</td>
<td>57.4±0.95</td>
<td></td>
</tr>
<tr>
<td>Male sex, %</td>
<td>73</td>
<td>79</td>
<td>43</td>
<td>82</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Hypertension, %†</td>
<td>41</td>
<td>28</td>
<td>25</td>
<td>17</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Current or former smoker, %</td>
<td>71</td>
<td>64</td>
<td>55</td>
<td>63</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

*Mean±SEM of current age. †Hypertension defined as systolic blood pressure ≥160 mm Hg.

frequencies were tested in single-vessel disease (SVD), multivessel disease (MVD), and unobstructed coronary arteries (patient control subjects). A significant association between SVD and IL-1RN*2 homozygosity was determined with an odds ratio of 2.78.

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groups at both centers were also significantly raised above the control groups \((P<0.01)\).

In the Sheffield population there was no significant difference in the genotypic distributions at the IL-1A(-889) and IL-1B (+3953) and TNFA(-308) loci between the patient controls, SVD and MVD groups. The frequency of allele 2 of the IL-1RN gene (*2) was, however, increased in the SVD patients: 0.34 versus 0.23 in patient control subjects (Figure, Table 3). Genotype distribution analysis also indicated a significant association between homozygosity for allele *2 and SVD \((\chi^2\text{ carriage}=8.490, 1\ \text{df}, \ P=0.0036)\) with a significant homozygote odds ratio \((P=0.0046)\) (Table 2). This trend was also seen in the separate London SVD population \((0.35\text{ vs }0.26)\) (Table 3) although at borderline significance \((\chi^2\text{ carriage}=3.53, 1\ \text{df}, \ P=0.0603)\) because of low statistical power.

As expected, when a summary test over the Sheffield and London data were performed, a trend was maintained \((P=0.0024)\) and the homozygote OR remained high at 2.78 \((95\%\ \text{CI }1.37\text{ to }5.65, \ P=0.0048)\) (Table 2). No association between genotype at IL-1RN and MVD was seen in either the Sheffield or London MVD populations studied. In consolidation, genotype analysis between SVD and MVD populations indicated a highly significant difference, as expected \((\chi^2\text{ trend}=11.456, 1\ \text{df}, \ P=0.0007, \text{ summary trend test, pooled data})\).

The possible confounding effect of hypertension and diabetes on the IL-1RN*2 association was assessed. Reanalysis of both cohorts with hypertensives and diabetics removed still shows overrepresentation of the IL-1RN*2 allele in the SVD patients, but with altered significance. In the Sheffield population, IL-1RN*2 frequency is 31% in SVD versus 24% in patient control subjects, but \(P=0.1\). However, in the London population, the allele IL-1RN*2 overrepresentation in SVD is now significant \((P=0.0439)\). The reduction of significance in the Sheffield population is probably a result of reduced power (14% for IL-1RN and 3% for IL-1B) after removal of hypertensives and diabetics.

In the Sheffield population, further analyses suggested a possible association between the IL-1B (-511) gene marker and both SVD and MVD: Allele 2 (*2) frequency was increased from 0.27 in the patient control subjects to 0.34 in SVD and 0.32 in MVD.

**Discussion**

There have been a number of reports of gene variants being associated with susceptibility to CAD. This study is the first to implicate cytokine gene variants in susceptibility to heart disease. These data suggest that carriage of *2 of IL-1RN is significantly associated with the presence of SVD.
One of the important findings is the different associations for SVD and MVD. This study deliberately, and prospectively, separated these 2 populations because the polymorphisms studied were in genes postulated to modify the process of CAD rather than being directly causative. This approach has previously been used to uncover the association of cytokine disease–modifying polymorphisms with conditions such as cerebral malaria. In a similar way, disease-modifying genes may be correlated with a phenotype of CAD rather than simply its presence or absence. The concept that the phenotype of CAD is modifiable is consistent with the observation that traditional risk factors such as smoking correlate poorly with the total burden of atheroma, arguing strongly that other factors influence the pattern of CAD development. The association of an IL-1 receptor antagonist gene variant with different patterns of CAD supports the hypothesis that polymorphisms in disease-modifying genes may determine the pattern of CAD.

Our results demonstrate that there is a difference in IL-1RN gene distribution between SVD and MVD in age- and sex-matched groups. Further, the angiographic control group is not statistically different from a blood donor control group, and this argues against the presence of bias within the patient control group caused by selection on the basis of chest pain. These results suggest a true genetic distinction between SVD and MVD. There are a number of possible explanations for this observation. First, SVD and MVD may have different causes and/or pathogenic mechanisms with the IL-1RN*2 protecting against progression to MVD or predisposing to SVD (ie, they can be different diseases). Second, the association may be with an aspect of CAD other than the angiographic findings, such as the mode or time of presentation, to which, unwittingly, our study was sensitive. Third, the association is only revealed when not overwhelmed by the presence of other pathogenic influences. This would be analogous to the way in which the genetic influences on coronary disease are lost with age even in monozygotic twins. Fourth, the SVD phenotype may be pathologically heterogeneous with a significant subset being influenced by the IL-1 system, whereas another subset may represent a early stage of a more widespread arterial disease that will, in time, affect all the coronary arteries (in the manner that some patients with SVD may be seen for PTCAs and remain adequately treated by this procedure for long periods of time, whereas others may progress to MVD relatively quickly). Fifth, the genetic association we found with SVD may be spurious and based on genetic admixture in the population or another confounding factor. We favor the explanation that at least in a subset-set of SVD patients, there is a significant association with the IL-1RN genotype, and this may indicate a pathogenic contribution of the IL-1 system. If the association is true the possibility that IL-1RN is a marker for a disease gene linkage disequilibrium cannot be ruled out but is not suggested by the haplotype relation between genes in this region.  

The reanalysis of the data with the hypertensives and diabetics removed suggests that an association in the 2 populations studied still exists for the IL-1RN*2 allele and SVD, though statistical significance is lost in the Sheffield cohort. We believe that this is the result of reduced statistical power. We cannot exclude, however, that there is an association for this allele with hypertension but this study population cannot answer this. The need for a prospective study is, however, indicated.

The definition of CAD on the basis of coronary angiography has advantages and disadvantages. The one advantage of an angiographic database is the assurance that the control group is free from significant coronary atheroma. Coronary angiography, in addition, allows an assessment of the burden of disease and has been used in several other studies as a measure of the severity of coronary artery disease. A disadvantage, however, is the classification of the angiograms. In this study, patient control subjects were defined to include completely smooth coronary arteries as well as irregular arteries with narrowing <30% of lumen area. A narrowing of >30% was arbitrarily used to indicate CAD, although there is no implication that this would be flow limiting. If flow-limiting definitions were used, arteries with lesions between 30% and 50% would be included as patient control subjects, which is clearly inappropriate, or be removed from the analysis and so introduce selection bias. Further, the reliability of our angiographically based patient control allele frequency was the same as that in healthy blood donor control subjects.

Within the population studied, the allele frequencies were not significantly different from those expected under HWE, although IL-1B (-511) in the Sheffield population was unusually biased. The possibility of genotyping error has been examined and excluded. There was no obvious ethnic diversity or evidence of consanguinity within the populations studied. This effect could be due to small sample size but also may reflect selection pressure in the Sheffield population.

### Table 3. IL-1RN and IL-1B(-511) Population Allele Frequencies

<table>
<thead>
<tr>
<th>Population Cohort</th>
<th>Sheffield</th>
<th>London</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1RN</td>
<td>Control</td>
<td>SVD</td>
</tr>
<tr>
<td>Allele *2</td>
<td>0.2269</td>
<td>0.3418</td>
</tr>
<tr>
<td>Allele *1</td>
<td>0.7731</td>
<td>0.6582</td>
</tr>
<tr>
<td>IL-1B(-511)</td>
<td>Allele *2</td>
<td>0.2654</td>
</tr>
<tr>
<td>Allele *1</td>
<td>0.7346</td>
<td>0.6582</td>
</tr>
</tbody>
</table>
Several studies support a role for IL-1 in the pathogenesis of CAD. IL-1 regulates mitogenesis of arterial wall cells, leukocyte adherence, and LDL metabolism. Increased synthesis of IL-1 has been demonstrated in human arterial plaques, and serum concentrations are raised in patients with minimal CAD and angina. Our data support an emerging hypothesis that IL-1 cytokines are important in atherogenesis.

The biological control of IL-1 is complex. IL-1 production can be inhibited by anti-inflammatory cytokines as well as prostaglandins and glucocorticoids. The actions of IL-1 are also regulated by a nonsignaling receptor IL-1R II, which can be membrane bound or soluble, and also by the polypeptide antagonist IL-1ra, which binds without agonist activity to signaling receptor IL-1R I.

IL-1ra is induced in a number of cell types by cytokines and bacterial products and levels of IL-1 and IL-1ra in vivo vary in parallel, suggesting coordinate regulation. IL-1ra is an acute phase protein. It can be detected in the endothelium of diseased coronary arteries (data not shown) and it also inhibits the fatty streak formation in the apolipoprotein E knockout mouse. This strongly implicates IL-1ra in the control of inflammation in the vessel wall. The data presented here add weight to the potential importance of IL-1 biology in coronary atherosclerosis.

The functional effect of the IL-1RN polymorphism is not yet fully understood but may depend on the cell type stimulated. Phenotypic data for the disease-associated allele in some instances gives rise to production of more IL-1ra and in others less.

Polymorphisms within genes at the IL-1 locus have been associated with a number of inflammatory and infectious diseases, including periodontitis. The recent suggestion of a link between periodontitis and CAD might be by an association at the IL-1 locus. This raises the possibility that the data presented here showing an association between IL-1RN2 and SVD may be indicating either multiple and complex relations between the IL-1 locus and CAD or that there is another gene, as yet unidentified, within the IL-1 locus, which is in linkage disequilibrium with both IL-1RN and IL-1B. As mentioned previously, we cannot eliminate this possibility.

In conclusion, the results reported here do suggest an association between IL-1RN2 and CAD. The association with a particular pattern of CAD, SVD, raises important questions regarding the impact of genetic influences on CAD as well as the role of the IL-1 family of cytokines in coronary atherosclerosis.

**Acknowledgments**

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