Interleukin-6 Gene $-174G>C$ and $-572G>C$ Promoter Polymorphisms Are Strong Predictors of Plasma Interleukin-6 Levels After Coronary Artery Bypass Surgery


Abstract—Interleukin-6 (IL-6) synthesized in response to diverse stimuli may play an important role in bridging the inflammatory and atherosclerotic processes. The acute-phase response after coronary artery bypass graft surgery (CABG) is associated with the induction and release of cytokines, such as IL-6. We have examined the effect of common polymorphisms in the IL-6 gene promoter ($-174G>C$, $-572G>C$, and $-597G>A$) on IL-6 levels after elective CABG. DNA extracted from the peripheral blood of 127 patients was amplified by polymerase chain reaction. IL-6 genotypes were resolved by gel electrophoresis after restriction enzyme digestion. Serum IL-6 was measured before surgery and in serial samples at 6, 24, 48, and 72 hours after CABG. Genotype distribution was as expected for a population in Hardy-Weinberg equilibrium for all polymorphisms. Rare allele frequencies ($\pm 95\%$ CIs) were similar to those reported previously: $-597A$ 0.36 (0.30 to 0.42), $-572C$ 0.07 (0.04 to 0.10), and $-174C$ 0.37 (0.31 to 0.43). The $-174G>C$ and $-597G>A$ genotypes were in strong allelic association ($A=0.97$, $P<0.001$). Baseline IL-6 levels did not significantly differ between patients with different genotypes for any polymorphism. However, 6 hours after CABG, peak IL-6 levels were significantly higher ($P=0.03$) in carriers of the $-572C$ allele than in those of the $-572GG$ genotype (355±67 versus 216±13 pg/mL, respectively) and in those with genotype $-174CC$ compared with $-174G$ allele carriers (287±31 versus 227±15 pg/mL, respectively; $P=0.04$). These effects remained statistically significant after adjusting for possible confounders, including age, sex, smoking, duration of cardiopulmonary bypass, aortic cross-clamp time, and total duration of surgery. These data demonstrate that IL-6 promoter polymorphisms influence peak IL-6 production after CABG, suggesting that these polymorphisms, which are functional in vitro, are also functional in vivo, suggesting a genetic influence on IL-6 levels after acute severe injury. (Arterioscler Thromb Vasc Biol. 2001;21:1458-1463.)

Key Words: interleukin-6 - genetic polymorphism - inflammation - coronary artery bypass surgery

Inflammatory processes are known to play a role in atherogenesis, with the presence of chronic gastric, and gum inflammation being associated with the development of coronary disease. The cytokine interleukin-6 (IL-6) is synthesized in response to diverse inflammatory stimuli and as a key orchestrator of the inflammatory response, may play an important role in bridging the inflammatory and atherosclerotic processes. Elevated IL-6 levels are associated with the development and severity of coronary disease, as well as with the transition to plaque instability and subsequent poor outcome. Although such an effect might be mediated through the action of downstream acute-phase proteins, IL-6 may itself be directly pathogenic. IL-6 stimulates endothelial activation, vascular smooth muscle cell proliferation, and leukocyte recruitment, which are effects that may lead to plaque growth or instability. However, despite such mechanistic data, the association of raised IL-6 levels with coronary disease is not a proof of cause. Indeed, coronary lesions may be proinflammatory, causing (rather than being caused by) the elevation of IL-6 levels. One way to clarify the issue of causation is to use a genetic approach. The association of a functional IL-6 polymorphism with coronary disease would suggest a causal, rather than passive, association of the cytokine and disease. Thus, it is of some importance to identify polymorphic variants of the IL-6 gene that are functional in vivo in humans and that might thus prove to be useful investigative tools. Recent experimental work has identified the presence of 4 genetic polymorphisms in the IL-6 gene promoter: $-597G>A$, $-572G>C$, and $-174G>C$, and a fourth locus (position $-373$), which constitutes a tract of adenine and thymidine residues (the AnTn tract) in varying numbers. Initial data suggest that the $-174G>C$ polymorphism may be
functional in vitro. With the use of constructs of the 5′ flanking region of the IL-6 gene in a luciferase reporter vector transiently transfected into HeLa cells, the −174C construct showed lower basal and stimulated (with lipopolysaccharide or interleukin-1) expression than the “wild-type” −174G construct.26 More recently, other experiments have suggested an opposite effect in response to interleukin-1 stimulation and, indeed, possible interaction between differing polymorphic constructs in vitro.27 However, in vitro studies using reporter gene constructs may be confounded by a number of factors. These include the type of cell line and culture conditions used, whether cells are at basal state or “stimulated” by exogenous agents, the nature of the exogenous stimulation, and the presence or absence of additional positive or negative regulatory elements contained within the sequences. Furthermore, varied lengths of DNA are used in the constructs, which are divorced from their normal chromatin context and the influence of distal enhancer or inhibitory regulatory elements that would further influence gene transcription.28 Despite the influence of these factors, in vitro data still provide vital mechanistic insight into the possible influence that genetic polymorphisms might have over the disease process. Nonetheless, further clarification of in vivo human functionality is required for the IL-6 polymorphisms, if data from forthcoming association studies of coronary artery disease are to be fully understood. Such an aim can be achieved by the use of a physiological stressor stimulus to increase gene expression.29,30

We have applied this approach to the 3 IL-6 gene polymorphisms by investigating the genotypic dependence of the circulating IL-6 response to coronary artery bypass surgery (CABG). CABG is a well-characterized inflammatory stimulus that causes a substantial rise in circulating IL-6 levels that peak 6 hours after patients are placed on cardiopulmonary bypass (CPB).31–36 Although a variety of different factors might act as potential stimuli to this inflammatory response, available evidence supports an important role for the process of CPB itself,34 and accordingly, CABG provides a fairly uniform stimulus for IL-6 production. Therefore, the influence of IL-6 genotype on this more homogeneous acute inflammatory stimulus was the primary focus of the present study. In addition, later time points were studied as a model of a more chronic inflammatory process.

**Methods**

**Subjects**

Between October 1999 and September 2000, all patients undergoing elective first-time CABG at the Middlesex Hospital (London, UK) were invited to take part in the Coronary Artery Surgery Inflammation Study (CASIS). Aspirin was routinely omitted 10 days before surgery. Those with evidence of a preexisting inflammatory state (intercurrent infection, active arthritis, or malignancy), active unstable coronary artery disease, or concurrent use of anti-inflammatory agents other than aspirin (including steroids) or those requiring chronic renal replacement therapy were excluded. Subjects were prospectively excluded (before data analysis) if they were suffering from potentially confounding postoperative complications (wound infections requiring antibiotic therapy or renal or circulatory failure requiring inotropic support or intra-aortic balloon pump insertion). The present study had hospital ethics committee approval, and written informed consent was obtained from all participants.

**Surgical Procedure**

All operations were performed by 4 experienced senior surgical staff, who used a midline sternotomy approach and standard operating procedures. Hypothermic cardiopulmonary bypass was instituted by cannulation of the right atrium and ascending aorta. Myocardial protection was maintained by cross-clamp fibrillation. Perioperative anticoagulation with heparin was reversed after CPB with the use of protamine sulfate.

**IL-6 Assay**

Citrated 4.5-mL blood samples were initially drawn before surgery and then again at 6, 24, 48, 72, and 96 hours after CPB. These were immediately centrifuged (3500g, 10 minutes), and plasma was separated and frozen at −20°C until analysis. IL-6 concentrations were measured by using a standard commercial assay (R&D Systems) by a staff blinded to all subject data. Interassay and intra-assay coefficients of variation were 5% and 3%, respectively, and assay sensitivity was <0.70 pg/mL.

**Genotyping Protocols**

At recruitment, a 5-mL EDTA sample of peripheral blood was drawn, from which DNA was extracted by use of a salting out method.35 Sequence amplification was performed by using polymerase chain reaction (PCR). The −174G>C genotype was determined by using primers and conditions as previously described27 to yield a PCR fragment of 190 bp after digestion with the restriction enzyme NlaIII. The −597G>A and −572G>C polymorphism genotypes were determined by using primers (5′-GGAGAGCCCTTG-AAGTAACTGC-3′ and 5′-GAGTTTTCCTGACTCCAC-TGAC-3′) to generate a PCR fragment of 163 bp. The reaction volume was 20 μL in each well of a 96-well plate, with final reaction component concentrations of 200 μmol/L for each dNTP, 1.5 mmol/L MgCl2, 10 mmol/L Tris base (pH 8.3), 50 mmol/L KCl, 0.001% gelatin, and 0.06 U Taq polymerase. PCR was performed with an initial denaturation temperature of 94°C for 4 minutes, followed by 35 cycles of 95°C for 40 seconds, 55°C for 40 seconds, and 72°C for 1 minute. Genotypes were resolved by using gel retardation and digestion (the rare −597A allele has a FokI restriction cutting site and the −572C allele lacks the digestion site for MblI). The size of the digestion products was determined by using microtitr array diagonal gel electrophoresis38 by 2 independent observers blinded to clinical details. Positive and negative controls were used to ensure accuracy.

**Statistical Analysis**

IL-6 values were not normally distributed, and data were thus logarithmically transformed before analysis. One-way ANCOVA was performed to test whether the IL-6 genotype was associated with differences in IL-6 levels by using age, sex, body mass index (in kilograms per millimeter squared), smoking, duration of CPB, duration of operation, and aortic cross-clamp time as covariates. Consequently to multivariate analysis, data were adjusted for identified confounding variables. Differences in IL-6 between genotypes were assessed by ANOVA and by Student t tests for unpaired data. Allele frequencies were estimated by gene counting. A χ² test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. Linkage disequilibrium between sites in pairwise combination was estimated by using the method of Chakravarti et al.39 A value of P<0.05 was considered to be statistically significant.

**Results**

In the 12 months of study recruitment, there were 269 elective CABG cases, of which 168 were randomly approached (depending on availability of the recruiting staff). All but 5 agreed to participate. A further 5 declined further blood tests, and 2 suffered unstable coronary syndromes before surgery. Of the remainder, 9 required inotropic or mechanical circulatory support, whereas 12 suffered respiratory tract infections, 2 suffered renal failure requiring ultrafiltration, 2

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suffered postoperative bleeding requiring return to theater, and 4 had wound infections. A total of 5 patients died.

Baseline characteristics of the remaining 127 are shown in Table 1. There was no difference in baseline characteristics or in genotype distribution in patients who were withdrawn or excluded from the study compared with those who participated and completed the protocol (data not shown). A total of 90 (71%) subjects were receiving statin therapy, and 82 (65%) were receiving β-blockers. Genotype distributions were in Hardy-Weinberg equilibrium for all 3 polymorphisms (Table 2) were not significantly different from those reported previously. The −572G>C and −174G>C polymorphisms appear to have an additive effect on peak IL-6 levels after CABG. Carriers of ≥1 rare −572C allele have been combined.

Table 1. IL-6 Promoter Genotype Distributions and Rare Allele Frequencies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rare Allele Frequency (95% CI)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6−174G&gt;C</td>
<td>0.37 (0.31–0.43)</td>
<td>47 (37)</td>
</tr>
<tr>
<td>IL6−572G&gt;C</td>
<td>0.07 (0.04–0.10)</td>
<td>111 (87)</td>
</tr>
<tr>
<td>IL6−597G&gt;A</td>
<td>0.36 (0.30–0.42)</td>
<td>46 (36)</td>
</tr>
</tbody>
</table>

The −174G>C and −597G>A genotypes were in strong allelic association (Δ=0.97, P<0.001). All genotypes were rechecked to confirm accuracy.

Data for the IL-6 response to surgery are shown in the Figure. At baseline, mean cohort IL-6 levels (pg/mL) were independent of the −572G>C genotype (5.2±0.1 for −572GG versus 4.9±0.3 for −572GC+CC, P=0.47). Body mass index was positively correlated with basal IL-6 levels (r=0.31, P=0.004). Before and after adjustment for this confounder, such levels were independent of IL-6 genotype (5.4±0.2 for GG versus 4.9±0.2 for GC versus 5.2±0.5 for CC, P=0.07 and 0.29 for unadjusted and adjusted data, respectively).

Six hours after surgery, mean IL-6 levels rose >45-fold to 232±14 pg/mL in the cohort overall (P<0.001 compared with baseline). The magnitude of this rise was strongly genotype dependent (Figure). Carriers of the −572C allele had IL-6 levels 65% higher than those of −572GG genotype (355±67 versus 215±13 pg/mL, respectively; P=0.02), an effect that persisted after multivariate analysis (P=0.03). In addition, 6-hour IL-6 levels were 26% higher in those homozygous for the −174C allele than among G allele carriers (287±31 versus 227±15 pg/mL, respectively; P=0.07). This difference was statistically significant after adjustment for confounding variables (P=0.04). Although IL-6 levels were lower in those receiving statins, there was no interaction with either IL-6 genotype. The effect of IL-6 genotype persisted after taking these factors into consideration.

The −572G>C and −171G>C polymorphisms appear to have an additive effect on peak IL-6 levels after CABG. The 6 individuals carrying ≥1 C allele of both polymorphisms (combining genotypes −174GC/−572GC and −174GC/−572CC) had significantly higher IL-6 levels than did those 37 patients who were GG homozygous for both poly-
morphisms (527±140 versus 226±22 pg/mL, respectively; \( P<0.04 \)).

At time points beyond 6 hours after CPB, there was no significant effect of the IL-6 –572G>C polymorphism on IL-6 levels. In contrast to C allele carriers, subjects with genotype –174GG reached peak IL-6 levels 24 hours after CPB, demonstrating a statistically significant interaction between genotype and time \(( P<0.04)\). Peak IL-6 level at this time point was higher for those with the GG genotype than for carriers of ≥1 C allele (274.1±22.7 versus 214.4±18.7 pg/mL, respectively; \( P<0.02 \)).

Although the duration of intensive care or of total in-hospital stay was independent of genotype, 3 of the 5 postoperative deaths occurred in –174CC homozygotes, and the other 2 deaths occurred in –174G>C heterozygotes (–174GG versus –174GC+CC, \( P=0.002 \)).

**Discussion**

Patients undergoing coronary artery surgical revascularization are exposed to a range of physiological insults. These evoke a marked inflammatory response, inducing the release of proinflammatory cytokines, such as IL-6. \(^{31-36}\) We describe for the first time the effect of 3 common genetic polymorphisms found in the IL-6 gene promoter on basal and inflammatory IL-6 levels in patients undergoing elective CABG. The use of an in vivo acute inflammatory model, such as bypass surgery, allows more realistic assessment of genotype function than in vitro experimentation and may reflect changes that are clinically relevant.

These in vivo data for the –572G>C polymorphism suggest, for the first time, that this polymorphism is indeed functional, with 65% higher 6-hour IL-6 levels found in carriers of the –572C allele. Although the borderline statistical significance of higher basal (unstimulated) IL-6 levels among those with the GG genotype \(( P<0.07)\) was annulled by correction for body mass index \(( P<0.26)\), a slight elevation would be consistent with data from in vitro constructs. \(^{27}\)

However, investigation of any association with basal levels will require further study, and the observation that patients of genotype –174CC showed a trend toward higher IL-6 levels is in direct contrast to the initial published data. \(^{27}\) There are a number of possible explanations for this apparent contradiction. First, no in vitro model of gene transcription is perfect. Each depends on the presence or absence of vital regulatory elements to exert their effect; thus, the addition or absence of a number of bases might have a significant impact on their response. In addition, the effects of 1 genetic construct may differ between the cell lines used and indeed may not be reproducible in another model. \(^{26}\) For these reasons, one model may reflect the clinical scenario better than another.

The association between the –174C allele and increased IL-6 levels has recently been reported in a sample of patients with aortic aneurysms who have evidence of chronic low-grade systemic inflammatory responses. \(^{46}\) In this group, the IL-6 genotype –174CC was also associated with increased cardiovascular risk. Thus, the IL-6 –174 genotype appears to influence not only the dramatic response to a profound physiological stress (namely, CABG) but also the more indolent response associated with aortic aneurysm. Furthermore, –174C allele carriage was associated with increased cardiovascular risk in a large prospective study of previously healthy men. \(^{51}\) Together, these observations highlight the potential difficulty in comparing in vitro and in vivo assessments of functionality.

Intriguingly, subjects of genotype –174GG appeared to reach peak IL-6 levels at a later time than did C allele carriers, perhaps reflecting a more damped response to the inflammatory stimulus. Although absolute peak IL-6 levels were not significantly different from those of genotype –174CC, the later time-to-peak IL-6 for genotype –174GG might partly explain some of the differences seen between different genotypes in vitro. This is the first suggestion that genotype might influence the dynamic profile of the IL-6 response, although a similar genotype effect has been observed on post-CABG fibrinogen levels. \(^{32,43}\) However, these observations require further and more detailed investigation in this and other clinical models. More detailed in vitro studies of the time-course response are also required.

CABG as an inflammatory model has been well characterized. \(^{31-38}\) In the early phase, a severe acute inflammatory response occurs, resulting in a peak IL-6 response at the 6-hour time point. \(^{32,34-36}\) Evidently, a variety of different stimuli (such as sternal incision and general anesthesia) might be thought to play a role in the genesis of this response. However, available evidence suggests that it is the mechanical process of extracorporeal circulation and CPB itself that plays a major role. \(^{34}\) It was for this reason that only patients managed in this way were included in the present study, that those undergoing other procedures (such as off-bypass coronary revascularization) were excluded, and that the 6-hour time-point was the chief focus of our analysis. Thus, the primary acute inflammatory stimulus was uniform. Nonetheless, we would concur that other variables (such as those mentioned) might also have acted as inflammatory stimuli, albeit subsumed by a potentially overwhelming bypass-associated effect. The role for such other factors (and variation in their nature and scale) is likely to increase as the effect of CPB wanes, as a more chronic inflammatory phase is entered (associated, for instance, with wound healing, subclinical infective processes, and postoperative atelectasis), and as drugs such as nonsteroidal anti-inflammatory agents are introduced in later days. However, the study design was rigorous in ensuring that no other concurrent surgical procedures were undertaken and that any differences in surgical procedure involved were minimized. By maintaining these stringent criteria and by restricting our primary analysis to the acute-phase 6-hour time point, homogeneity of the inflammatory stimulus was maintained. Finally, any undetermined heterogeneity in the nature of the acute inflammatory stimulus only strengthens the robustness of these results. In any gene-environment interaction, increasing diversity in the environmental challenge will weaken the power to identify genetic variation in response. The fact that we have identified such differences in the face of any unidentified potential variation adds scientific validity to the results as reported. For this not to be so, one would have to suggest a (blind) genotype dependence on the nature of (occult) variation in the surgical procedure, which would seem unlikely.

It is unlikely that our data report the sole functional variant of the IL-6 promoter region. In particular, the –373 AnTn site generates variable combinations of A and T residues (eg, A8T12, A10T11, A9T11, and A10T10) with a variable...
linkage association to the other polymorphic sites. Data suggest that different haplotypes may affect transcriptional regulation in vitro, making them potential powerful tools worthy of investigation. Such investigation was beyond the scope of the present study: analysis of multiple polymorphic variants and haplotypes requires very large sample numbers for which studies should be appropriately powered to allow for the statistical influence of multiple comparisons. Furthermore, the present study was focused on a site with a single polymorphic variant that is currently being used as an investigative tool in association studies by a number of centers.

The identification of functional variants of the gene encoding the proinflammatory cytokine IL-6 may prove clinically useful. IL-6 plays a pivotal role in generating the inflammatory response, regulating the hepatic synthesis of acute-phase proteins such as C-reactive protein and fibrinogen, as well as having a wide range of effects relating to inflammation and tissue injury. IL-6 mRNA is found at high levels in atheromatous arteries and colocalizes with macrophages in areas of plaque rupture. In addition, there is already a wealth of data supporting a role for IL-6 as a useful marker of cardiovascular risk. Elevated levels of IL-6 and C-reactive protein are predictive of future cardiovascular events in healthy men and women, and these elevated levels are markers of poor prognosis in chronic angina and after acute coronary syndromes. Despite this, it is still not known whether this association is causal or merely an epiphenomenon, a subject that remains a topic of hot debate. Our results will aid the interpretation of data from association studies of IL-6 genotype and coronary artery disease, which should, in turn, help to resolve this issue.

One potential criticism that might be levied is that data for IL-6 concentration were confounded by unrecognized dilutional differences between subjects. Relative differences in plasma volume could influence IL-6 concentration and give rise to spurious associations. Although accurate data relating to the changes in intravascular volume associated with surgery were not available, estimates of blood volume loss are also notoriously difficult. Furthermore, the use of combinations of crystalloid, colloid, and packed cells makes the hematocrit an equally unreliable guide. Although it has not been possible to account for the effects of hemodilution on the measurements of IL-6 levels, such a confounding effect is likely to be small. IL-6 levels rose by a mean of 46-fold; thus, even a 20% difference in intravascular blood plasma volume (or a 30% change in plasma volume) would have a negligible fractional effect on IL-6 levels. In any event, such changes are likely to be random across genotypes and would weaken, rather than strengthen, the likelihood of identifying any genetic associations with change. Even if genotype were to alter transfusion requirements (which we consider unlikely), it is biologically implausible that a spurious association between IL-6 levels and genotype could be generated in this manner.

It is tempting to speculate that the excess mortality seen in this group of patients for carriers of the IL-6 −174G allele might relate directly to the more exuberant acute inflammatory response that they mount. Evidence suggests that the severity of the inflammatory response is strongly correlated with poorer outcome in the critically ill. This association seems causal; in other words, it is not just that those with the greatest pathogenic insult will have the worst outcome but that those who mount the most extreme inflammatory response to any given stimulus are at greatest risk. Such an effect is manifested in death from a variety of modes, whether it be multiorgan failure or respiratory or circulatory failure. However, the primary aim of the present study was not to address this issue, and the small number of deaths that were incidentally noted necessarily limits our conclusions. Indeed, the aim was to provide evidence for the use of these genetic markers as tools in just such prospective studies.

Nonetheless, our results might suggest that these IL-6 genotypes have a greater influence on acute IL-6 response to injury incurred at the time of bypass rather than the IL-6 levels determined by more chronic inflammation. Confirmation is required in other inflammatory states. In particular, studies in lower grades of inflammatory response and in chronic inflammation are needed.

In conclusion, we demonstrate for the first time that the IL-6 gene promoter −572G>C and −174G>C polymorphisms influence the IL-6 response to the inflammatory stimulus after CABG by a magnitude that is likely to be clinically relevant. Although the present study is insufficiently powered to examine the effects of genetic haplotype, our results suggest that these polymorphisms are functional with additive in vivo effects. These data set the framework for exploring the role of IL-6 genotypes in other inflammatory situations, such as in acute coronary syndromes.

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References


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