Clinical Chemistry 54:5
000–000 (2008)

Association of Serum Interleukin-6 Concentration with a Functional *IL6* −6331T>C Polymorphism

Andrew J. P. Smith,[1*] Francesco D'Aiuto,[2] Jutta Palmen,[1] Jackie A. Cooper,[1] Jane Samuel,[3] Simon Thompson,[1] Julie Sanders,[4] Nikos Donos,[2] Luigi Nibali,[2] David Brull,[5] Pat Woo,[3] and Steve E. Humphries[1]

BACKGROUND: Interleukin-6 (IL-6) concentrations vary substantially among individuals. This study aimed to identify novel genetic markers to explain these differences.

METHODS: We sequenced a region 6-kb upstream of the *IL6* [interleukin 6 (interferon, beta 2)] transcription start site in a search for functional variants and detected 3 common variants: −6331T>C, −6101A>T, and −5617/−5616C/A>T/G. *IL6* −6331T>C (C allele frequency, 0.20; 95% confidence interval, 0.16–0.24) showed strong negative linkage disequilibrium with −174G>C ($D' = -0.97$) and was studied further in 309 individuals who underwent coronary artery bypass grafting.

RESULTS: Patients with the TT genotype had higher IL-6 concentrations 6 h after surgery than those with the CC genotype (mean, 199.4 ng/L vs 114.9 ng/L; $P = 0.02$). A similar association was seen in a cohort of 173 patients who underwent intensive periodontal therapy: Individuals with the CC genotype had significantly lower IL-6 concentrations 24 h after therapy than TT patients (mean, 0.78 ng/L vs 5.00 ng/L; $P < 0.0001$). A similar trend was observed in 203 healthy individuals from northern Europe (1.29 ng/L for the TT genotype vs 0.89 ng/L for the CC genotype; $P = 0.07$). Reporter assays that used a sequence flanking the −6331 single-nucleotide polymorphism spliced upstream to the IL-6 minimal promoter driving luciferase gene expression demonstrated a 1.3-fold increase in promoter activity ($P < 0.01$) for constructs containing −6331T. Electrophoretic mobility shift assays revealed enhanced binding of transcription factor Oct-1 to the T allele.

CONCLUSIONS: *IL6* −6331T is associated with increased IL-6 concentrations in an acute inflammatory state via a mechanism involving binding of the Oct-1 transcription factor. This finding may help resolve conflicting studies based on the *IL6* −174G>C variant.

Interleukin-6 (IL-6),[6] a pleiotropic cytokine of 23.7 kDa produced by both lymphoid and nonlymphoid cells, plays key roles in inflammation, the acute phase response, regulation of immune reactivity, hemopoiesis, and oncogenesis. Within-population differences in IL-6 concentration are due to both genetic and environmental influences *(1)*. This genetic difference is exaggerated during inflammatory events, when plasma IL-6 concentrations increase in response to diverse stimuli. IL-6 synthesis and release are stimulated by the 2 major proinflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor α, but the fact that IL-6 remains in the plasma substantially longer makes this molecule a good marker of inflammation *(2)*.

Increased plasma IL-6 concentrations act as prognostic markers in several clinical conditions, including intra-abdominal sepsis *(3)*, metastatic breast cancer *(4)*, and diffuse large cell lymphoma *(5)*. The development and severity of coronary artery disease are also associated with increased IL-6 concentrations *(6)*, as is the transition to plaque instability *(7)*, possibly because of the action of IL-6 in vascular smooth muscle cell proliferation, leukocyte recruitment, and endothelial activation.

[1] Centre for Cardiovascular Genetics, Department of Medicine, University College London, London, UK; [2] Periodontology Unit, UCL Eastman Dental Institute, London, UK; [3] Centre for Paediatric and Adolescent Rheumatology, Windeyer Institute, London, UK; [4] Department of Surgery, University College London, The Heart Hospital, London, UK; [5] Department of Cardiology, The Whittington Hospital NHS Trust, London, UK.
* Address correspondence to this author at: Centre for Cardiovascular Genetics, Department of Medicine, University College London, 5 University St., London WC1E 6JF, UK. Fax 44-(0)20-7679-6212; e-mail Andrew.J.P.Smith@ucl.ac.uk.
Received October 3, 2007; accepted February 7, 2008.
Previously published online at DOI: 10.1373/clinchem.2007.098608
[6] Nonstandard abbreviations: IL-6, interleukin-6; IL-1β, interleukin-1β; SNP, single-nucleotide polymorphism; CABG, coronary artery bypass graft surgery; EMSA, electrophoretic mobility shift assay; IPT, intensive periodontal therapy; LD, linkage disequilibrium.

IL-6 binds to cell surface complexes consisting of the IL-6 receptor and gp130, thereby activating the JAK/STAT and MAPK cascades. Located on chromosome 7p21, the *IL6*[7] gene [interleukin 6 (interferon, beta 2)] contains 5 exons, and the *IL6* coding sequence contains only a few very rare polymorphisms (http://genome.ucsc.edu/, 2006 assembly). This fact has led to the hypothesis that the observed differences in IL-6 concentration among individuals are influenced by variation in the *IL6* promoter in combination with variation in genes upstream of IL-6 synthesis and environmental stimuli, such as a smoking habit.

IL6 promoter polymorphisms have been well characterized in a number of healthy and disease populations, and 2 single-nucleotide polymorphisms (SNPs) have been studied in detail—the common −174G>C variant *(8)* and the less frequent −572G>C allele *(9)*. The −174C allele has been associated with higher IL-6 serum concentrations in several studies *(10–12)*, with no effect in 1 study *(13)*, and with lower concentrations in another *(14)*. The −572G>C SNP has also been associated with the plasma IL-6 concentration, with the rare C allele associated with higher IL-6 concentrations *(15)*. A recent study also evaluated 2 other promoter variants, −1363G>T and −1480CTdel, and although these variants were not shown to be functional, they did form a haplotype with the other promoter polymorphisms that showed an increased association with systemic-onset juvenile arthritis *(16)*. This finding suggests the presence of functional polymorphisms at this locus that influence IL-6 concentration but that have yet to be identified.

Our recent work has identified a region between −5202 and −5307 bp upstream of the *IL6* transcription start site that plays an important role in *IL6* gene regulation. In conjunction with the proximal promoter, this region increases gene expression in vitro by up to 20-fold compared with the proximal promoter alone. The aim of the present study was to examine whether any functional genetic variation is present near this region that affects IL-6 concentration.

Materials and Methods

We describe the study population, polymorphism identification and genotyping, cell culture and plasmids, the electrophoretic mobility shift assay (EMSA), the luciferase reporter assay, and the statistical analysis in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue5.

Results

POLYMORPHISM IDENTIFICATION

To determine whether any common variation existed from *IL6* −6391 bp to −5203 bp that could affect gene expression, we sequenced this region for 24 healthy Caucasian individuals. Sequencing confirmed the presence of 4 common variants described in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/): −6331T>C (rs10499563), −6101A>T (rs4719714), −5617C>T (rs2056577), and −5616A>G (rs2056576) (Fig. 1).

GENOTYPING OF *IL6* SNPs AND LINKAGE DISEQUILIBRIUM ANALYSIS

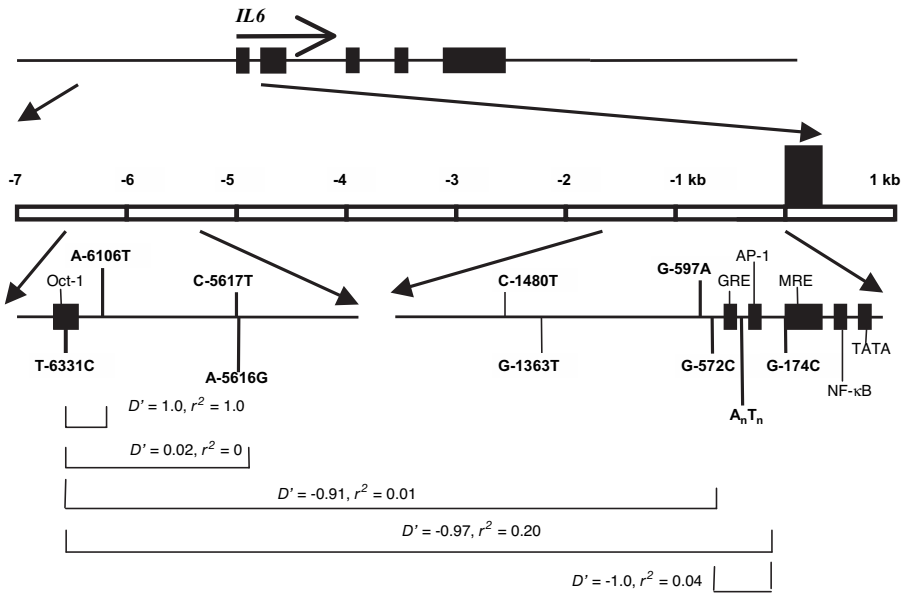
We genotyped these 4 SNPs for a UK cohort of patients who underwent coronary artery bypass grafting surgery (CABG) *(10)*, a cohort of patients who received intensive periodontal therapy (IPT) *(17–20)*, and the control population from a European case-control study of myocardial infarction (HIFMECH) *(21)*. The baseline characteristics of the patients (CABG, n = 321; IPT, n = 173; HIFMECH, n = 421) for whom IL-6 concentrations and at least 1 genotype were measured are shown in Table 1.

We successfully genotyped 309 of the UK CABG patients for all *IL6* SNPs investigated in this study: *IL6* −6331T>C, −6101A>T, −5617C>T, −5616A>G, −572G>C, and −174G>C. All genotype distributions were in Hardy–Weinberg equilibrium at the following allele frequencies: −6331T>C, 0.20; −6101A>T, 0.20; −5617C>T, 0.36; −5616A>G, 0.36; −572G>C, 0.08; and −174G>C, 0.36. These frequencies were similar to those reported in HapMap (http://www.hapmap.org/; data not shown). Genotyping of the contiguous −5617C>T and −5616A>G alleles revealed the presence of 2 naturally occurring diplotypes, C/A and T/G, which were subsequently analyzed as a diallelic SNP. There was complete linkage disequilibrium (LD) between −6331T>C and −6101A>T, and subsequent analysis focused only on −6331T>C (C allele frequency, 0.20; 95% confidence interval, 0.16–0.24), because an in silico analysis revealed that the T>C change eliminates an Oct-1 binding site.

EFFECT OF GENOTYPE ON PLASMA IL-6 CONCENTRATION IN CABG PATIENTS

IL-6 concentrations and the *IL6* genotype at baseline, 6 h, and 24 h before CABG are shown in Table 1A in the online Data Supplement. A previous study examined 127 DNA samples obtained from the same cohort used

[7] Human genes: *IL6*, interleukin 6 (interferon, beta 2); *TNF*, tumor necrosis factor (TNF superfamily, member 2); *IL2*, interleukin 2; *IL5*, interleukin 3 (colony-stimulating factor, multiple); *IL5*, interleukin 5 (colony-stimulating factor, eosinophil); *CSF2*, colony stimulating factor 2 (granulocyte-macrophage); *IL8*, interleukin 8.



Haplotype frequency

			HIFMECH		
-6331	-572	-174	CABG	North	South
T	G	G	0.38	0.30	0.41
T	G	C	0.36	0.45	0.31
C	G	G	0.21	0.20	0.20
T	C	G	0.05	0.05	0.08

Fig. 1. Schematic of the *IL6* gene, with the promoter region highlighted (not to scale).

Indicated are theoretical DNA-binding domains Oct-1 (octamer binding protein-1), GRE (glucocorticoid response element), MRE (multiple response element), and NF-κB (nuclear factor κB). Also indicated are commonly studied promoter SNPs and the upstream promoter SNPs identified by sequencing in this study. *D′* and r^2 values indicate the degree of LD between *IL6* −6331T>C and other important SNPs. Haplotype frequencies for the CABG cohort and for the HIFMECH northern (North) and southern (South) European control cohorts are also shown.

in the present study and identified an association of −572G>C and −174G>C with the plasma IL-6 concentration at 6 h *(10)*. With current IL-6 concentrations and at least one *IL6* genotype measured for 321 patients, we reexamined these 2 SNPs along with the new SNPs described above for an association with IL-6 concentration (variation in sample size reflects missing IL-6 concentration and/or genotype data, because IL-6 concentration data were not available for all patients at every time point). These data show a reduction in the significance of the association of previous −174/−572 genotypic data with IL-6 concentration. The −5617C>T/−5616A>G alleles showed no significant association with IL-6 concentration at any time (all $P > 0.5$; see Table 1A in the online Data Supplement), but a comparison of homozygote genotypes revealed an association with −6331T>C (data not shown in this table). We found a nonsignificant ($P = 0.13$) trend of association between IL-6 concentration and genotype at baseline, with a 34% higher plasma IL-6 concentration for the TT genotype than for the CC genotype [mean (SE), 4.64 (0.23) ng/L vs 3.45 (0.40) ng/L]. The TT genotype was associated with a 73% higher IL-6 plasma concentration at 6 h compared with the CC genotype [199.4 (12.3) ng/L vs 114.9 (36.9) ng/L; $P = 0.02$]. The IL-6 concentrations of the TT and CC genotypes were not significantly different at 24 h after surgery.

EFFECT OF GENOTYPE ON PLASMA IL-6 CONCENTRATION IN IPT PATIENTS

Serum IL-6 concentrations for the IPT cohort showed no association with the *IL6* genotype at the baseline visit (Table 1B in the online Data Supplement). As previously reported, periodontal therapy produced a moderate inflammatory response that lasted 1 week. We have previously shown no effect of −174G>C on IL-6 concentration for a smaller sample of individuals

Table 1. General characteristics and *IL6* genotype frequencies for the CABG, IPT, and HIFMECH control cohorts at baseline.[a]

			HIFMECH	
	CABG (n = 321)	IPT (n = 173)	Northern Europe (n = 221)	Southern Europe (n = 200)
Age, y	64.8 (9.1)	47.2 (8.0)	52.8 (5.0)	50.3 (5.6)
Current smokers, %	15.6		21.3	28.0
BMI[b], kg/m^2[c]	28.2 (4.5)	27.1 (4.9)	25.7 (3.2)	26.0 (3.1)
Cholesterol, mmol/L	4.8 (1.0)	5.3 (0.9)	5.7 (1.0)	5.4 (0.9)
CRP, mg/L[c]	2.2 (2.6)	3.3 (3.1)	0.91 (1.26)	1.60 (1.52)
IL6 genotype, n				
−174G>C, GG/CG/CC	120/152/47	86/62/20	63/108/50	104/79/17
−572G>C, GG/GC/CC	285/30/1	138/20/4	199/22/0	169/29/2
−6331T>C, TT/TC/CC	196/100/13	91/58/4	130/64/9	113/56/8

[a] Age and cholesterol data are presented as the mean (SD).
[b] BMI, body mass index; CRP, C-reactive protein.
[c] Data are presented as the geometric mean (approximate SD).

(18). In the present analysis with a larger sample, we confirmed the lack of an association between the −174/−572 genotypic data and acute increases in IL-6 concentration; however, we did observe a significant association between −6331T>C genotype and serum IL-6 concentration at 24 h after therapy, with CC patients exhibiting significantly lower IL-6 concentrations than TT patients [0.78 (0.55) ng/L vs 5.00 (0.57) ng/L; $P < 0.0001$]. Patients with the CC genotype also had significantly lower IL-6 concentrations at 7 days after therapy than those with the TT genotype [0.40 (0.56) ng/L vs 1.53 (0.24) ng/L; $P = 0.02$].

EFFECT OF GENOTYPE ON PLASMA IL-6 CONCENTRATION IN THE HIFMECH STUDY

To establish whether the findings in this UK cohort could be replicated, we genotyped individuals from another study (HIFMECH) and selected only the control groups to avoid the confounding effects of the medication (421 of the 575 individuals had measurements of IL-6 concentration and at least one genotype). There was no statistically significant association between genotype and plasma IL-6 concentration (see Table 1C in the online Data Supplement); however, the TT and CC genotypes at position −6331 were a strong indicator of IL-6 concentration for the northern European cohort ($P = 0.07$), a result similar to that seen in the baseline data for the CABG cohort. The data for the southern European cohort did not replicate this trend, however; the lowest IL-6 concentrations occurred in heterozygous individuals.

ANALYSIS OF HAPLOTYPE AND IL-6 CONCENTRATION IN CABG PATIENTS

For the haplotype analysis, we included only SNPs for which evidence existed for a functional role [−6331T>C (described here), −572G>C, and −174G>C (Fig. 1)]. There was significant LD between these SNPs, and 4 common haplotypes were observed. The −6331C allele was always observed with −572G and −174G, and −572C was always observed with −174G. After CABG, the patients who carried the haplotype containing −6331C (CGG, 21% of the sample) had IL-6 concentrations that were 34% lower at 6 h than individuals carrying the common haplotype (TGG, 38% of the sample; $P = 0.01$) and had IL-6 concentrations at 24 h that were 22% lower ($P = 0.03$; Table 2A, Fig. 2A).

ANALYSIS OF HAPLOTYPE AND IL-6 CONCENTRATION IN IPT PATIENTS

The haplotype analysis of the IPT cohort confirmed the findings with the CABG cohort, with a lower acute release of IL-6 being associated with the haplotype containing −6331C. Indeed, the CGG haplotype (21% of the sample) was associated with a 51% reduction in IL-6 concentration compared with the common TGG haplotype (39% of the sample) at 24 h after therapy ($P = 0.002$; Table 2B, Fig. 2B). Because the data for the CABG and IPT cohorts are time series, we repeated the analysis by calculating the area under the IL-6 curve and confirmed that IL-6 concentrations for the CGG haplotype were significantly lower than for the com-

Table 2. IL-6 concentrations by haplotype.[a]

A. Analysis of IL-6 concentrations by haplotype at baseline, 6 h, and 24 h after CABG surgery.

	Baseline		6 Hours		24 Hours	
−6331/−572/−174 Haplotype	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]
TGG	3.16 (1.57–4.74)	—	137.95 (116.08–159.82)	—	127.92 (103.75–152.09)	—
TGC	2.70 (1.14–4.26)	0.87	114.94 (86.59–143.28)	0.57	103.66 (78.81–128.50)	0.03
CGG	2.77 (0.98–4.57)	0.44	91.11 (51.67–130.55)	0.01	99.32 (72.37–126.28)	0.03
TCG	3.55 (1.43–5.66)	0.31	190.81 (152.39–229.24)	0.88	110.01 (48.94–171.08)	0.56

B. Analysis of IL-6 concentrations by haplotype at baseline, 24 h, and 7 days after IPT therapy.

	Baseline		24 Hours		7 Days	
−6331/−572/−174 Haplotype	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]
TGG	1.63 (0.87–3.05)	—	5.71 (3.68–8.85)	—	2.16 (1.15–4.12)	—
TGC	1.52 (0.79–2.90)	0.81	4.50 (2.99–6.75)	0.27	2.15 (1.09–4.25)	0.98
CGG	1.25 (0.53–2.93)	0.52	2.81 (1.66–4.76)	0.002	1.06 (0.52–2.18)	0.06
TCG	1.12 (0.43–2.93)	0.45	4.64 (2.29–9.38)	0.54	1.03 (0.41–2.72)	0.16

C. Analysis of IL-6 concentrations by haplotype for the Northern and Southern European control populations of the HIFMECH study.

	Northern Europe		Southern Europe		Combined	
−6331/−572/−174 Haplotype	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]	Expected mean (95% CI), ng/L[b]	*P* (vs TGG)[c]
TGG	0.91 (0.73–1.09)	—	0.87 (0.59–1.15)	—	0.86 (0.66–1.06)	—
TGC	0.81 (0.62–1.00)	0.83	0.91 (0.48–1.34)	0.79	0.81 (0.61–1.02)	0.71
CGG	0.55 (0.08–1.02)	0.31	0.47 (0–1.08)	0.15	0.52 (0.15–0.89)	0.07
TCG	0.43 (0–1.78)	0.45	0.92 (0.56–1.28)	0.74	0.73 (0.44–1.03)	0.37

[a] Haplotype analyses with the putative functional *IL6* SNPs (−6331T>C, −572G>C, and −174G>C).
[b] Expected mean is based on the individual carrying 2 copies of the haplotype. CI, confidence interval.
[c] *P* values are for log-transformed data, and statistical comparisons of IL-6 concentrations are with respect to those for the reference haplotype (TGG).

mon TGG haplotype in both the CABG cohort ($P = 0.03$) and the IPT cohort ($P = 0.02$) (see Table 2 in the online Data Supplement). No effects of any other haplotypes were observed at any of the other times.

ANALYSIS OF HAPLOTYPE AND IL-6 CONCENTRATION IN THE HIFMECH COHORT

The results of our haplotype analysis of the HIFMECH cohort repeated the trend found in the CABG study (Table 2C). IL-6 concentrations were 39% lower in individuals with the CGG haplotype than with the common TGG haplotype, although this difference did not reach statistical significance ($P = 0.07$). We observed a continuation of this trend, however, in both the northern and southern European cohorts, with 45% and 40% reductions, respectively, in IL-6 concentration relative to the reference haplotype, suggesting a genuine haplotypic effect.

LUCIFERASE REPORTER ASSAY

To evaluate whether the *IL6* −6331T>C allele was itself a functional polymorphism or simply a marker for another site elsewhere at this locus, we performed luciferase reporter assays with constructs containing the *IL6* minimal promoter and a 196-bp fragment containing the −6331T>C variant at the 5′ end. All 4 combinations containing −174G/C and −6331T/C alleles were created. The constructs were transfected into HeLa and Huh7 cell lines, both of which express the *IL6* gene (data not shown). Cells were cotransfected with an Oct-1 expression plasmid and stimulated with IL-1β to promote IL-6 production. Fig. 3 shows *IL6*

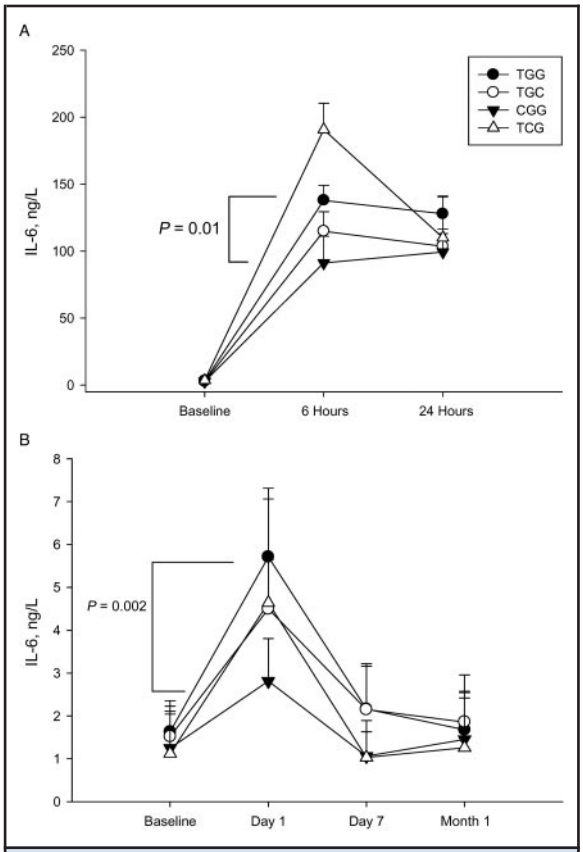


Fig. 2. IL-6 concentrations by haplotype in the CABG cohort.

Effect of homozygous 3-allele haplotype (*inset*) on IL-6 concentrations in the CABG cohort (A) and the IPT cohort (B). Data are presented as the mean (SE).

expression for each plasmid construct in both stimulated and unstimulated HeLa and Huh7 cell lines, relative to that of an unstimulated wild-type vector (−6331T/−174G) without Oct-1 cotransfection. All constructs that were cotransfected with the Oct-1 expression vector produced significantly higher luciferase activity than those cotransfected with the empty vector (data not shown). There was no significant difference in luciferase activity between any of the constructs in either unstimulated cell line. Compared with the −6331C/−174G construct, IL-1β stimulation significantly increased the expression by the −6331T/−174G construct by 1.2-fold ($P = 0.013$) and 1.1-fold ($P = 0.01$) in the HeLa and Huh7 cell lines, respectively (Fig. 3). Similarly, the −6331T/−174C construct had 1.3-fold ($P = 0.0014$) and 1.3-fold ($P = 0.001$) higher expression in HeLa and Huh7 cells, respectively, than the −6331C/−174C construct. Performing the luciferase reporter assay with IL-1β stimulation for 3 h or 6 h did not alter the observed relative differences in luciferase expression (data not shown).

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Our in silico analysis of the wild-type and variant sequences with MatInspector (Genomatix Software) indicated that transcription factor Oct-1 binds to the T allele but not to the C allele at position −6331. To investigate the effect of this SNP on transcription factor binding, we performed an EMSA analysis on a 22-bp sequence surrounding the SNP. We used a known Oct-1 consensus sequence as a positive control and carried out supershift EMSA assays with an Oct-1–specific monoclonal antibody to confirm binding specificity. As shown in Fig. 4, both the Oct-1 consensus sequence and the −6331T allele probe were shifted after incubation with nuclear extract from IL-1β–stimulated HeLa cells, and we observed a supershift after adding Oct-1–specific monoclonal antibody. The −6331C allele probe was not shifted after incubation with HeLa nuclear extract.

Discussion

The IL-6 signaling pathway plays a critical role in a number of acute and chronic diseases *(26)*. IL-6 concentrations vary widely within populations *(27)*, and much research has focused on identifying the causal factors that underlie this diversity. Genetic factors are thought to play a major role, along with environmental factors, particularly inflammation *(28)*. This study sought to investigate a region upstream of the proximal promoter that has been shown to influence *IL6* gene expression (unpublished data).

We examined a UK CABG cohort, hypothesizing that the increase in plasma IL-6 concentration following this proinflammatory insult would increase our power to detect an association with SNPs that may play a role in producing the documented variation in IL-6 concentration. This study identified the −6331T>C SNP to be an important predictor of IL-6 concentration at 6 h after surgery and demonstrated the TT genotype to be associated with a 74% higher IL-6 concentration than the CC genotype. The genotype ranking of IL-6 concentrations at baseline was similar to that at 6 h after surgery, but the differences did not reach statistical significance. It is notable that the patients with the CC genotype eventually reached the same IL-6 concentrations by 24 h after surgery as the TT and CT genotypes had reached by 6 h. Therefore, the −6331 genotype may be not only a predictor of IL-6 concentration after acute stimulation but also a predictor of the rate at which the IL-6 concentration increases after stimulation. To confirm these findings, we repeated this analysis with a cohort of individuals who under-

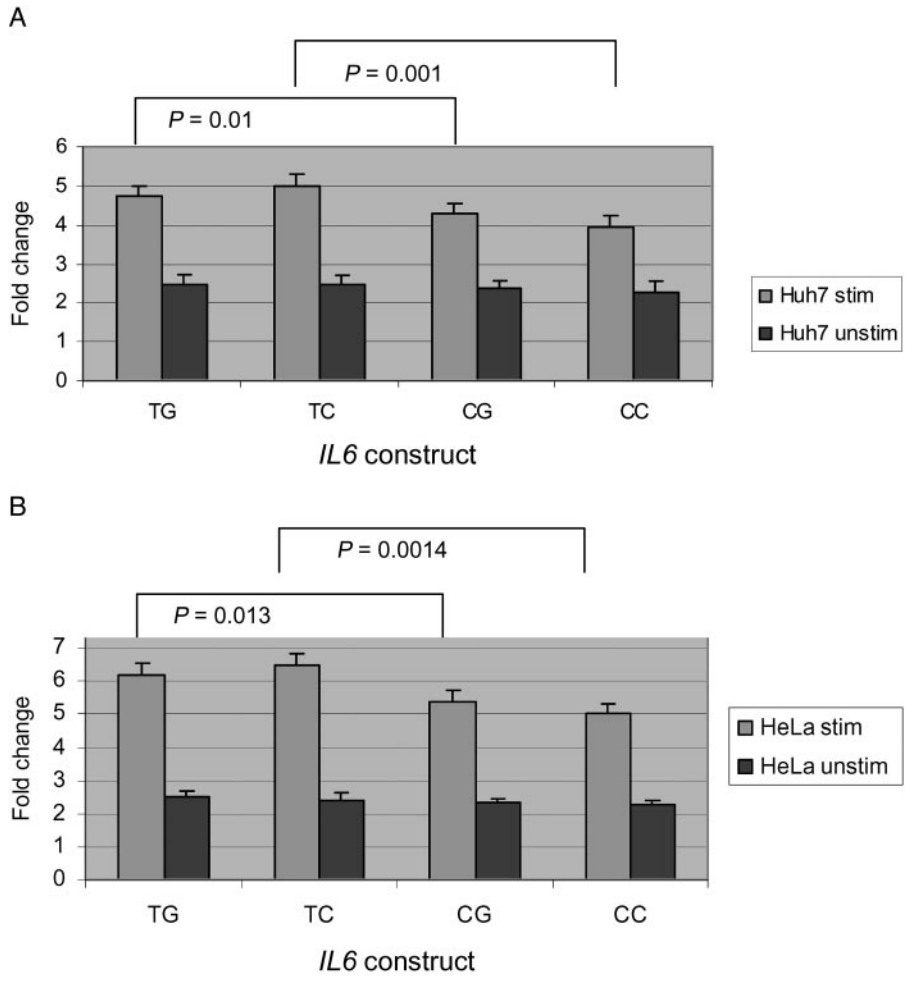


Fig. 3. Results of the luciferase reporter assay with *IL6*/pGL3 plasmids (see text and online Data Supplement) for the effect of *IL6* construct on IL-6 concentration.

Huh7 cells (A) and HeLa cells (B) were cotransfected with the Oct-1 expression plasmid and were either stimulated (stim) or not stimulated (unstim) with IL-1β. Results are presented as the mean (SE) fold difference in *IL6* expression relative to that of the wild-type (GT) construct without Oct-1 transfection or IL-1β stimulation. Data are for 3 separate assays with 8 samples/assay. The dinucleotides on the *abscissa* indicate the nucleotides at *IL6* positions −6331T/C and −174G/C, respectively.

went IPT. Although IPT patients do not constitute such a severe model of acute inflammation, our group had previously characterized the IPT model to be associated with a significant inflammatory response at 1 week. We previously had shown in a smaller IPT cohort a positive association between common SNPs and the acute release of other inflammatory mediators *(29)*. In the IPT model, we confirmed a strong association of the −6331C allele and haplotypes containing this allele with the release of substantially lower serum IL-6 concentrations after a moderate inflammatory stimulus. As in the CABG cohort, we observed no association between any of the SNPs and baseline IL-6 values.

We carried out the same analysis with the control group of a case-control study of myocardial infarction, in which IL-6 was measured for 6 months following myocardial infarction. We confirmed the −6331T>C SNP as a predictor of IL-6 concentration in the northern Europe cohort but not in southern Europeans, possibly because of diet, lifestyle differences, and genetic interactions. Southern Europeans have significantly lower systolic blood pressures and cholesterol and triglyceride concentrations (Table 1A) and are ge-

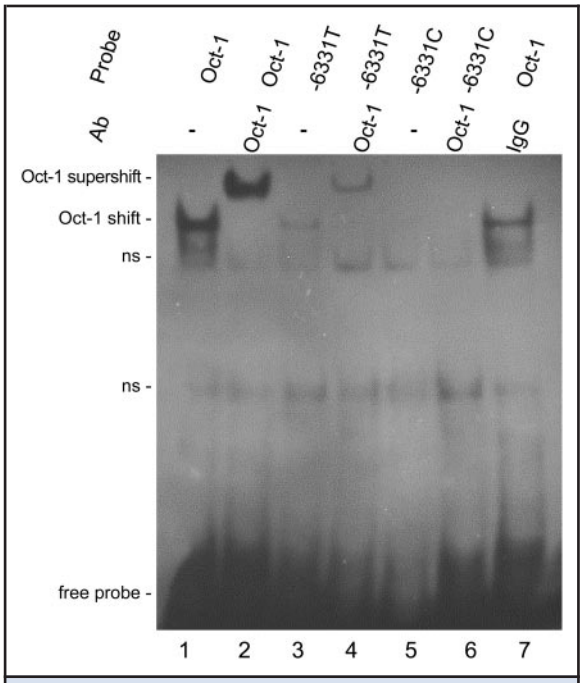


Fig. 4. EMSA of the binding abilities of the *IL6* −6331T>C variants.

See text and the online Data Supplement for details of probes and binding conditions. Lane 1, Oct-1 consensus oligonucleotide; lane 2, consensus oligonucleotide with Oct-1 monoclonal antibody (Ab); lane 3, T allele oligonucleotide; lane 4, T allele oligonucleotide with Oct-1 Ab; lane 5, C allele oligonucleotide; lane 6, C allele oligonucleotide with Oct-1 Ab; lane 7, Oct-1 consensus oligonucleotide with mouse IgG. ns, nonspecific band.

netically distinct. As with the CABG study at baseline, the genotype/phenotype correlation did not reach statistical significance, indicating that significant concentrations of phenotypic variation are dependent on acute inflammatory stimuli. The chronic inflammatory response to smoking had little effect on −6331T>C and IL-6 concentrations in all of the cohorts, and we also noted no association between any *IL6* polymorphism studied and the concentration of C-reactive protein (data not shown). Because the numbers in some groups were small, we assessed differences in IL-6 concentration with the Kruskal–Wallis test. We chose a significance level of $P < 0.05$. We made no adjustments for multiple comparisons, because such adjustments have been suggested to lead to more errors in interpretation *(30)*. The results should therefore be interpreted in the context of the number of tests conducted.

We carried out a haplotype analysis with the 2 *IL6* SNPs that have previously been associated with IL-6 concentration [−174G>C *(10–12, 14)* and −572G>C *(15)*] and the functional −6331T>C SNP, because if the genotypes are truly functional, they should show a greater degree of genetic association with the phenotype. In all 3 of the cohorts examined, haplotype was not strongly associated with IL-6 concentration, although it was evident that the haplotype containing −6331C was consistently associated with lower IL-6 concentrations. This result indicates that the −174G>C and −572G>C variants are of less importance than previously thought *(14, 15)*. A recent study by Malarstig et al. identified an association between −572 and IL-6 concentration ($P = 0.07$), the significance of which increased when acute coronary syndrome patients with higher IL-6 concentrations were selected ($P = 0.01$) *(15)*. The −572G>C variant does not alter a transcription factor–binding site in vitro (unpublished data), and it is likely that the SNP is simply in strong LD with a functional SNP. Indeed, there is high LD between −572 and −6331, with −572C always being associated with −6331T.

A recent study by Müller-Steinhardt et al. examined *IL6* haplotypes and IL-6 concentrations in healthy blood donors after stimulating their monocytes and T lymphocytes with lipopolysaccharide for 4 h *(31)*. In an analysis of *IL6* −572/−174 haplotypes, the investigators observed an association of significantly lower concentrations of IL-6 secreted with the G/G haplotype than with all other haplotypes combined; however, the SNPs used by Müller-Steinhardt et al. do not distinguish the low IL-6–secreting haplotype containing −6331C that we have described.

To assess the in vitro ability of the −6331T>C SNP to confer alterations in *IL6* expression patterns, we designed a reporter assay with constructs consisting of a region 100 bp on either side of −6331T>C spliced to the minimal *IL6* promoter. The results indicated no difference between T and C constructs in unstimulated cells, but IL-1β stimulation produced a noticeable difference for the 2 constructs containing −6331T, compared with the 2 constructs containing the −6331C variant. Interestingly, there was no significant difference in *IL6* expression between the −174 G and C alleles when spliced to this upstream element, suggesting that the previously reported effects of this SNP are diluted when larger constructs are used. The reporter assay has limitations as a vehicle for testing the effects of SNPs on the concentrations of products of expressed genes because of the restriction on insert size, the large numbers of possible SNP combinations, and the role that long-range chromatin structure may play in this situation (data not shown); however, the effects of IL-1β stimulation on the reporter constructs seem to reflect a pattern similar to what is seen in patients with acute inflammation in that the −6331T allele confers higher *IL6* expression than the C allele.

The EMSA analysis revealed that transcription factor Oct-1 bound to −6331T to a much greater degree than to −6331C. Oct-1 plays a major role in controlling housekeeping genes and tissue-specific genes, including genes encoding several other cytokines: *TNF-* [tumor necrosis factor (TNF superfamily, member 2)] *(32)*, *IL2* (interleukin 2) *(33)*, *IL3* [interleukin 3 (colony-stimulating factor, multiple)], *IL5* [interleukin 5 (colony-stimulating factor, eosinophil)], *CSF2* [colony stimulating factor 2 (granulocyte-macrophage)] *(34)*, and *IL8* (interleukin 8) *(35)*. Oct-1 now appears to play a role in *IL6* expression. Binding to the −6331T allele was increased when we used nuclear extract from IL-1β–stimulated HeLa cells compared with the use of extract from unstimulated cells (data not shown), and this finding correlates with both in vitro expression data and data on the effect of acute inflammation on plasma IL-6 concentrations in individuals with the −6331T allele. This result may be explained by several mechanisms. Oct-1 may be up-regulated by inflammatory stimuli, DNA/Oct-1 complexes at the −6331T/C position may require the presence of inflammation-induced cofactors such as AP-1 or nuclear factor κB for increased DNA-binding affinity, or Oct-1 binding requires these or other cofactors to activate the *IL6* transcriptional machinery.

Variation in inflammatory responses between individuals can have considerable effects on disease outcomes for a number of conditions, and such variation may play a role in the outcomes of cardiac surgery *(36, 37)*, in which IL-6 concentrations remain high in patients with postoperative complications. Indeed, preoperative IL-6 concentrations can act as a prognostic marker for postoperative complications (data not shown). Lowering the IL-6 concentration with medication may be a useful way to reduce adverse outcomes in such patients, and knowledge of the genetic contribution to IL-6 variation may facilitate this process.

The data we have presented show that the effect of the −6331T>C polymorphism on IL-6 concentration is at the transcriptional level following acute stimulation. Numerous studies have examined the −174G>C variant as an indicator of IL-6 concentration and its association with disease. The rare alleles −6331C and −174C never occur on the same chromosome, and studies that associate −174G>C with a particular phenotype are in fact using this SNP as a marker for a SNP with greater functionality, such as −6331T>C. Alternatively, both the −174G>C and −6331T>C variants may act in concert to affect gene expression. Fully addressing which SNPs are functional requires a haplotypic analysis of the entire *IL6* gene and its regulatory region, and this investigation is currently under way.

Grant/Funding Support: This research was supported by the British Heart Foundation (RG2005/015 and FS99025). The core HIFMECH study was supported by grants from the European Commission (BMH4-CT96-0272). F.D. was supported by a fellowship from the European Social Fund and by a Scholarship of the association "Il Circolo." The funding bodies had no role in study design, data collection, analysis, or interpretation and were not involved in the preparation of the manuscript. They also had no rights in approving, delaying, or disapproving of the publication of this work.
Financial Disclosures: None declared.
Acknowledgments: We thank Winship Herr for his kind gift of the Oct-1 expression plasmid. The key investigators in the HIFMECH study are Anders Hamsten, Steve E. Humphries, Irène Juhan-Vague, Maurizio Margaglione, Giovanni di Minno, John Yudkin, and Elena Tremoli. We acknowledge all of the staff of the Periodontology Unit at the UCL Eastman Dental Institute for their help and support.

References

1. Pantsulaia I, Trofimov S, Kobyliansky E, Livshits G. Genetic and environmental influences on IL-6 and TNF-alpha plasma concentrations in apparently healthy general population. Cytokine 2002; 19:138–46.
2. Song M, Kellum JA. Interleukin-6. Crit Care Med 2005;33(Suppl):S463–5.
3. Patel RT, Deen KI, Youngs D, Warwick J, Keighley MR. Interleukin 6 is a prognostic indicator of outcome in severe intra-abdominal sepsis. Br J Surg 1994;81:1306–8.
4. Zhang GJ, Adachi I. Serum interleukin-6 concentrations correlate to tumor progression and prognosis in metastatic breast carcinoma. Anticancer Res 1999;19:1427–32.
5. Preti HA, Cabanillas F, Talpaz M, Tucker SL, Seymour JF, Kurzrock R. Prognostic value of serum interleukin-6 in diffuse large-cell lymphoma. Ann Intern Med 1997;127:186–94.
6. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 2000;101: 1767–72.
7. Biasucci LM, Vitelli A, Liuzzo G, Altamura S, Caligiuri G, Monaco C, et al. Elevated concentrations of interleukin-6 in unstable angina. Circulation 1996;94:874–7.
8. Olomolaiye O, Wood NAP, Bidwell JL. A novel *Nla*III polymorphism in the human IL-6 promoter. Eur J Immunogenet 1998;25:267.
9. Terry CF, Loukacis V, Green FR. Cooperative influence of genetic polymorphisms on interleukin-6 transcriptional regulation. J Biol Chem 2000;275:18138–44.
10. Brull DJ, Montgomery HE, Sanders J, Dhamrait S, Luong L, Rumley A, et al. Interleukin-6 gene −174G>C and −572G>C promoter polymorphisms are strong predictors of plasma interleukin-6 concentrations after coronary artery bypass surgery. Arterioscler Thromb Vasc Biol 2001;21: 1458–63.
11. Boiardi L, Casali B, Farnetti E, Pipitone N, Nicoli D, Cantini F, et al. Relationship between interleukin 6 promoter polymorphism at position −174, IL-6 serum concentrations, and the risk of relapse/recurrence in polymyalgia rheumatica. J Rheumatol 2006;33:703–8.
12. Ravaglia G, Forti P, Maioli F, Chiappelli M, Dolzani P, Martelli M, et al. Associations of the −174 G/C interleukin-6 gene promoter polymor-

phism with serum interleukin 6 and mortality in the elderly. Biogerontology 2005;6:415–23.
13. Walston J, Arking DE, Fallin D, Li T, Beamer B, Xue Q, et al. IL-6 gene variation is not associated with increased serum concentrations of IL-6, muscle, weakness, or frailty in older women. Exp Gerontol 2005;40:344–52.
14. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 concentrations, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest 1998; 102:1369–76.
15. Malarstig A, Wallentin L, Siegbahn A. Genetic variation in the interleukin-6 gene in relation to risk and outcomes in acute coronary syndrome. Thromb Res 2007;119:467–73.
16. Fife MS, Ogilvie EM, Kelberman D, Samuel J, Gutierrez A, Humphries SE, Woo P. Novel IL-6 haplotypes and disease association. Genes Immun 2005;6:367–70.
17. D'Aiuto F, Nibali L, Mohamed-Ali V, Vallance P, Tonetti MS. Periodontal therapy: a novel non-drug-induced experimental model to study human inflammation. J Periodontal Res 2004;39: 294–9.
18. D'Aiuto F, Nibali L, Parkar M, Suvan J, Tonetti MS. Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. J Dent Res 2005;84:269–73.
19. D'Aiuto F, Parkar M, Nibali L, Suvan J, Lessem J, Tonetti MS. Periodontal infections cause changes in traditional and novel cardiovascular risk factors: results from a randomized controlled clinical trial. Am Heart J 2006;151:977–84.
20. Tonetti MS, D'Aiuto F, Nibali L, Donald A, Storry C, Parkar M, et al. Treatment of periodontitis and endothelial function. N Engl J Med 2007;356: 911–20.
21. Juhan-Vague I, Morange PE, Aubert H, Henry M, Aillaud MF, Alessi MC, et al., on behalf of the HIFMECH Study Group. Plasma thrombin-activatable fibrinolysis inhibitor antigen concentration and genotype in relation to myocardial infarction in the north and south of Europe. Arterioscler Thromb Vasc Biol 2002;22:867–73.
22. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.
23. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Vols. 1–3. New York: Cold Spring Harbor Laboratory Press, 1989.
24. Tregouet DA, Escolano S, Tiret L, Mallet A, Golmard JL. A new algorithm for haplotype-based association analysis: the Stochastic-EM algorithm. Ann Hum Genet 2004;68(Pt 2):165–77.
25. Stephens M, Donnelly P. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003;73:1162–9.
26. Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: Is interleukin-6 the link? Atherosclerosis 2000;148:209–14.
27. Haddy N, Sass C, Droesch S, Zaiou M, Siest G, Ponthieux A, et al. IL-6, TNF-alpha and atherosclerosis risk indicators in a healthy family population: the STANISLAS cohort. Atherosclerosis 2003;170:277–83.
28. Worns MA, Victor A, Galle PR, Hohler T. Genetic and environmental contributions to plasma C-reactive protein and interleukin-6 concentrations—a study in twins. Genes Immun 2006;7:600–5.
29. D'Aiuto F, Casas JP, Shah T, Humphries SE, Hingorani AD, Tonetti MS. C-reactive protein (+1444C>T) polymorphism influences CRP response following a moderate inflammatory stimulus. Atherosclerosis 2005;179:413–7.
30. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology 1990;1:43–6.
31. Müller-Steinhardt M, Ebel B, Hartel C. The impact of interleukin-6 promoter(−597/−572/−174) genotype on interleukin-6 production after lipopolysaccharide stimulation. Clin Exp Immunol 2007;147:339–45.
32. Hohjoh H, Tokunaga K. Allele-specific binding of the ubiquitous transcription factor OCT-1 to the functional single nucleotide polymorphism (SNP) sites in the tumor necrosis factor-alpha gene (TNFA) promoter. Genes Immun 2001;2: 105–9.
33. Ullman KS, Flanagan WM, Edwards CA, Crabtree GR. Activation of early gene expression in T lymphocytes by Oct-1 and an inducible protein, OAP40. Science 1991;254:558–62.
34. Kaushansky K, Shoemaker SG, O'Rork CA, McCarty JM. Coordinate regulation of multiple human lymphokine genes by Oct-1 and potentially novel 45 and 43 kDa polypeptides. J Immunol 1994;152:1812–20.
35. Wu GD, Lai EJ, Huang N, Wen X. Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to overlapping elements within the interleukin-8 promoter. The role of Oct-1 as a transcriptional repressor. J Biol Chem 1997;272:2396–403.
36. Kilger E, Weis F, Briegel J, Frey L, Goetz AE, Reuter D, et al. Stress doses of hydrocortisone reduce severe systemic inflammatory response syndrome and improve early outcome in a risk group of patients after cardiac surgery. Crit Care Med 2003;31:1068–74.
37. Hedman A, Larsson PT, Alam M, Wallen NH, Nordlander R, Samad BA. CRP, IL-6 and endothelin-1 concentrations in patients undergoing coronary artery bypass grafting. Do preoperative inflammatory parameters predict early graft occlusion and late cardiovascular events? Int J Cardiol 2007;120:108–14.