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ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · MARCH 2004

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Analysis of common IL-6 promoter SNP variants and the AnTn tract in humans and primates and effects on plasma IL-6 levels following coronary artery bypass graft surgery

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Received 2 May 2003; received in revised form 10 October 2003; accepted 25 November 2003

Abstract

Interleukin-6 (IL-6) is a pro-inflammatory cytokine and major mediator of the acute phase response. Single nucleotide polymorphisms within the 5′ flanking region (−597G>A, −572G>C and −174G>C) have previously been associated with increased risk of coronary heart disease and influencing transcription of IL-6 both in vitro and in vivo. In addition to these, a polymorphic AnTn tract is also present in the promoter of IL-6. Analysis in five different primate species demonstrated a G allele at −597, −572 and −174 in all species. By contrast, the AnTn tract was polymorphic in at least three species, and was roughly conserved in overall length despite an increase in the relative proportion of A versus T in the evolution of the human sequence from that in the ancestor of the great apes. The effect of the AnTn polymorphism on IL-6 levels was examined following coronary artery bypass graft surgery (CABG), a known inflammatory stimulus for IL-6 production. One hundred and thirty-two patients undergoing CABG were genotyped for the AnTn tract by automated sequencing. Four alleles were identified: A8T12 (allele frequency 0.35, 95% CI 0.29–0.40); A9T11 (0.26, 0.21–0.31); A10T11 (0.21, 0.16–0.26); and A10T10 (0.18, 0.14–0.23). Isolation of the effect of different alleles of the AnTn tract on an identical haplotypic background for the other polymorphisms in the promoter showed that individuals homozygous for A9T11 had significantly higher post-operative IL-6 levels than A10T11 homozygotes (275 ± 46 pg/ml versus 152 ± 29; $P = 0.04$). The effect of the A8T12 allele could not be determined separately due to strong allelic association with −174C. The conserved length of the AnTn tract and the association in vivo with IL-6 levels strongly suggest the functionality of the tract on IL-6 expression, independent of contributions from other polymorphic sites within the promoter.

Keywords: Interleukin-6; Promoter polymorphism; Haplotype analysis; Coronary artery bypass graft surgery; Coronary heart disease

1. Introduction

Inflammatory processes are a key component of the initiation and progression of atherosclerosis, with inflammatory cytokines being candidates as risk factors for coronary heart disease (CHD). The pro-inflammatory cytokine, interleukin-6 (IL-6) is a major mediator of the acute phase response and elevated plasma IL-6 levels are associated with increased risk and severity of CHD [1,2]. Polymorphic variation affecting the expression of IL-6 could therefore have a causal role in influencing individual susceptibility to disease. A number of common polymorphisms have previously been identified in the 5′ flanking region of the IL-6 gene including −597G>A, −572G>C, −174G>C [3–5], and a polymorphic tract of A and T residues (AnTn) at position −373 [3,4]. The −597A and −174C alleles show almost complete allelic association in Caucasian subjects [4,5], while the −572C allele almost always occurs with the −174G allele [5]. These polymorphisms are located adjacent to cis-acting regulatory elements involved in controlling IL-6 expression at the level of transcription, suggesting that they may influence the interaction of proteins with the DNA at these sites [6] (see Fig. 1).

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doi:10.1016/j.bbadis.2003.11.010

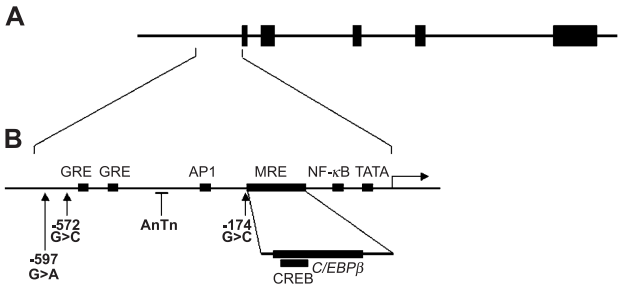

Fig. 1. Diagrammatic representation of the IL-6 gene intron–exon structure (A) and 5′ -flanking region (B). Positions of single nucleotide polymorphisms are indicated by arrows. Black boxes represent known and putative regulatory elements. GRE, glucocorticoid response element; AP1, activator protein 1; MRE, multiple response element; CRE, cAMP response element; C/EBPβ, CCAAT response element binding protein.

We have recently reported that carriers of the −572C allele and −174CC homozygotes had significantly higher peak IL-6 levels following coronary artery bypass graft surgery (CABG) [5], a defined physiological stimulus for IL-6 production [7]. Furthermore, the −174C allele has been associated with an increased risk of CHD in several studies [8–11]. Experiments have revealed functional effects of both these polymorphisms in vitro in reporter gene assays following stimulation with IL-1 [3,4,12]. This evidence supports the influence of genetic variation on IL-6 expression and susceptibility to CHD.

Initial studies suggested that the AnTn tract in the IL-6 promoter influences transcription in vitro in combination with the other single nucleotide polymorphisms in naturally occurring haplotypes [4]. We have investigated the association between the AnTn tract genotype and baseline and peak IL-6 levels in our previously published cohort of elective CABG cases [5]. Additionally, we have sequenced this region in a variety of primate species to obtain insight into the ancestral sequence of the IL-6 promoter and to gain further clues as to the conservation of this polymorphism and it's potential functional significance on IL-6 regulation.

2. Subjects and methods

2.1. Subjects

DNA samples from 132 elective CABG patients were available, with information on baseline and post-operative serum IL-6 levels at 6, 24, 48, 72 and 96 h after cardiopulmonary bypass (CPB), together with genotype information for IL-6 −597G>A, −572G>C and −174G>C polymorphisms. Patient baseline characteristics, operative details and genotype information for these polymorphisms are described elsewhere [5,13]. All operations were performed by one of only four consultant surgical staff, using a midline sternotomy approach and standard operating procedures. Hypothermic CPB was instituted by cannulation of the right atrium and ascending aorta. Myocardial protection was maintained by cross-clamp fibrillation.

2.2. Genotyping

Genotyping of the AnTn tract was performed by direct automated sequencing (Applied Biosystems) of PCR products generated by primers flanking the polymorphic site. Primer sequences were: forward 5′-CAGAAGAACTCAGATGACTGG-3′ ;reverse 5′CTGATTGGAAACCTTATTAAG-3′. PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s.

2.3. Sequencing of primate species

The IL-6 5′ flanking sequence, from nucleotides −633 to −79, was amplified by PCR from genomic DNA of five non-human primate species (chimpanzee: *Pan troglodytes*; bonobo: *Pan paniscus*; gorilla: *Gorilla gorilla*; orangutan: *Pongo pygmaeus*; and baboon: *Papio papio*) using the primers 5′ -GGAGTCACACACTCCACCTG-3′ and 5′ -TCATGGGAAAATCCCACATT-3′ . The AnTn tract was amplified from an additional four chimpanzees and one bonobo using the same primers as those for genotyping human samples. PCR products were sequenced directly as for genotyping.

2.4. Statistical analysis

Haplotypes were generated for all four IL-6 polymorphisms (−597G>A, −572G>C, AnTn tract and −174G>C) using the PHASE program. This adopts a statistical method based on a Markov chain–Monte Carlo algorithm, to reconstruct haplotypes by exploiting principles of population genetics and coalescent theory that make predictions about the patterns of haplotypes in natural populations [14]. IL-6 values were not normally distributed, therefore, data were log-transformed prior to analysis and, for ease of interpretation, the geometric means and approximate standard error of the mean are presented. To explore the possibility of confounding variables on IL-6 levels by surgical procedure, operative variables (including consultant performing surgery, number of grafts, left internal mammary artery graft, operation duration and CPB and aortic cross-clamp times) were examined to see if they were predictive of changes in IL-6 levels. Differences in these predictors were examined via univariate ANOVA between haplotype groups. Association between difference in IL-6 levels and haplotype groups were assessed by two sample *t*-test and ANOVA. A χ^2 test was used to determine if genotype distribution was in Hardy–Weinburg equilibrium.

2.5. Phylogenetic analysis

The molecular phylogeny of IL-6 flanking sequence and evolutionary distances (excluding the AnTn tract) were

estimated in PAUP [15] using the best-fit maximum likelihood model, which employs unequal equilibrium nucleotide frequencies and unequal rates of transition and transversion substitutions. Rate constancy was tested with a likelihood ratio test.

3. Results

3.1. Haplotype analysis of IL-6 promoter polymorphisms in humans

Fig. 1B shows a schematic representation of the 5′-flanking region of the human IL-6 gene, indicating the proximity between polymorphic and cis-acting regulatory sites. These include two potential glucocorticoid response elements (GRE) at positions −557 and −466 (relative to the major transcription start site), a recognition site for activator protein-1 (−283), and the multiple regulatory domain (−168 to −153) containing a cAMP response element at position −163, a binding site for the transcription factor C/EBPβ (−158) and an additional GRE.

Genotyping of the AnTn tract was performed by direct automated sequencing of PCR products synthesised from patients using primers flanking this region. Fig. 2 shows a sample of electropherograms to illustrate genotyping of the different alleles. The genotype of homozygous individuals was achieved without difficulty (not shown), and in heterozygous individuals the overlap of peaks could be used to identify the different number of A and T nucleotides present on each chromosome (Fig. 2A). The presence of a 'frameshift' identified by split peaks, following the AnTn tract, indicates a length difference between the two alleles as shown in Fig. 2B. In most cases, genotyping was determined easily from the sequence of one strand only; however, for certain combinations of alleles, sequencing in both forward and reverse directions was required to accurately assign genotypes (Fig. 2C). Only four different alleles were identified in this sample: A8T12 (allele frequency 0.35, 95% CI 0.29–0.40); A9T11 (0.26, 0.21–0.31); A10T11 (0.21, 0.16–0.26); and A10T10 (0.18, 0.14–0.23), with genotype distribution in Hardy–Weinberg equilibrium. Genotype frequencies are listed in Table 1.

Table 1
Results of genotyping the AnTn tract showing genotype frequencies in 132 CABG surgery patients

Genotype	*n*	Frequency
A8T12/A8T12	10	0.08
A8T12/A9T11	29	0.22
A8T12/A10T11	23	0.17
A8T12/A10T10	19	0.14
A9T11/A9T11	10	0.08
A9T11/A10T11	9	0.07
A9T11/A10T10	11	0.08
A10T11/A10T11	6	0.05
A10T11/A10T10	11	0.08
A10T10/A10T10	4	0.03

Analysis of AnTn genotypes with the other IL-6 promoter polymorphisms demonstrated that the −174C allele occurs mainly (95%) with A8T12 ($\Delta = -0.92$, $P < 0.001$), with 5% of −174C occurring with the A9T11 allele. The −572C allele was observed only with A10T10 ($\Delta = -0.53$, $P < 0.001$). Allele frequencies and allelic associations observed were consistent with previous observations [4].

As individual polymorphisms are unlikely to act independently to influence gene expression, haplotypes were generated for all four polymorphisms using a computer algorithm (PHASE) based on principles of coalescent theory [14]. Eight

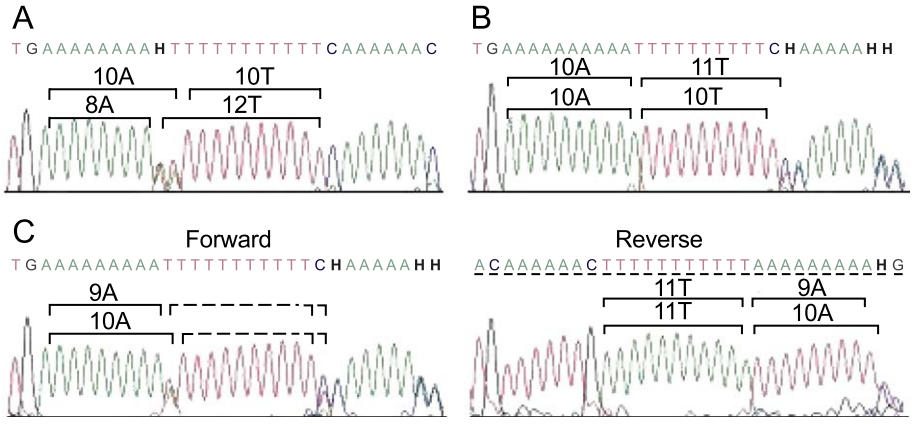

Fig. 2. Sequencing electropherograms illustrating genotyping of the AnTn tract. (A) Genotype A8T12/A10T10: the two overlapping heterozygous peaks at the junction of the AnTn tract can be used to identify the number of A and T nucleotides given that both alleles are the same length. (B) Genotype A10T11/A10T10: the lack of overlapping peaks as in (A) indicates both alleles have the same number of adenosines. Split peaks at the end of the poly d(T) tract indicates a frameshift as a result of one allele being longer than the other by a single T nucleotide. (C) Genotype A9T11/A10T11: heterozygosity for the number of A nucleotides combined with a 'frameshift' indicating an overall size difference of a single nucleotide precludes genotyping from the sequence of a single strand in this case, due to the possible combinations of the number of A and T residues. Sequencing of both forward and reverse strands is required in order to assign the number of A and T nucleotides in each allele given their overall length.

haplotypes were predicted to occur out of a possible 32 and frequency estimates for each haplotype are shown in Table 2. AG[A8T12]C was the most common haplotype (frequency 0.33), with those haplotypes containing the (common) G allele at all three SNP sites being seen with all four different AnTn alleles, A9T11 and A10T11 being the two most common (0.25 and 0.21, respectively). These data raise the issue of which haplotype is the ancestral promoter haplotype in humans and the extent of conservation of this region throughout evolution.

3.2. Sequence analysis of the IL-6 promoter in primates

In an attempt to gain further insight into the potential functionality and molecular ancestry of the AnTn tract, this region of the IL-6 promoter was sequenced in a variety of primate species (from −633 to −79). These included DNA samples from five chimpanzees (four subspecies *verus*, one subspecies *schweinfurthii*), two bonobos, one gorilla, one orangutan and two baboons. Alignment of the sequences shows a considerable degree of conservation between human and primate species within this entire region (not shown). Excluding the AnTn tract, the rate of substitution (Fig. 3A) is approximately one-third the average rate of substitution in non-coding intergenic DNA of great apes [16], consistent with the action of strong purifying selection maintaining cis-regulatory function. In addition, a likelihood ratio test fails to reject evolutionary rate constancy ($P>0.5$), which would be expected when the differences between species are due to the fixation of neutral mutations by genetic drift. The three SNPs present in humans were all homozygous G in these primate samples, suggesting these polymorphisms arose after the divergence of chimpanzee and human and that the common G allele for all three polymorphisms is ancestral. Two other base changes were identified between all five primate species and the human sequence. A substitution of C to T at position −454 and an A to G change at position −239, with no evidence for these sites being polymorphic. Neither of these base changes exist within known cis-regulatory sites in the IL-6 promoter.

Analysis of the sequence demonstrates that the AnTn tract is polymorphic in at least three different primate species. The following alleles were identified: chimpanzee A10T8, A9T9, A8T10; bonobo A8T10; gorilla A5T11, A6T10; orangutan A2T15; and baboon A2T16, A2T13. Alignment of the sequences around this region (Fig. 3B) shows the evolution of the AnTn tract. In the lineage from the common ancestor of the great apes to humans and chimpanzees, the poly d(A) part of the tract appears to be expanding as the poly d(T) component contracts. The overall length has remained fairly constant throughout, expanding by approximately two nucleotides on the human lineage after the split with the chimpanzee lineage. Both within and among species the number of As and Ts are inversely related (Fig. 3C). No common ancestral allele was identified between the human samples in this cohort and any of the primate species studied.

3.3. Effect of the IL-6 promoter AnTn tract polymorphism on plasma IL-6 levels following CABG surgery

The change in IL-6 levels post-CABG was used to examine the in vivo functionality of the AnTn tract. Comparison of specific haplotype combinations enables isolation of the effects of individual polymorphisms on IL-6 levels on a common genetic background. Baseline mean IL-6 levels were independent of haplotype (data not shown) as expected from previous data in this cohort [5]. Peak IL-6 levels, at 6 h following surgery, are presented in Table 3 (mean ± S.E.) for the different haplotype combinations. Haplotype groups containing less than three subjects have been excluded because the low number of individuals precludes any meaningful statistical analysis. At time points beyond 6 h there was no significant effect of haplotype on IL-6 levels.

Firstly we first sought to confirm the effects of −572C and −174CC on pre- and post-operative IL-6 levels previously observed [5]. Examination of only individuals with an AG[A8T12]C haplotype in common, showed that individuals with G$\bar{C}$[A10T10]G had significantly higher IL-6 levels 6 h following surgery than G$\bar{G}$[A10T10]G subjects (Fig. 4A) (378 ± 255 versus 119 ± 28 pg/ml, respectively; $P=0.05$). In a similar analysis to isolate the effect of −174G>C (Fig. 4B), compared to GG[A10T11]$\bar{G}$ homozygotes, AG[A8T12]$\bar{C}$ homozygotes had significantly higher peak IL-6 levels following surgery (152 ± 30 versus 283 ± 41 pg/ml, respectively; $P=0.02$). Individuals heterozygous for these two haplotypes demonstrated intermediate IL-6 levels (179 ± 26 pg/ml) although this difference was not statistically significant.

Comparison between groups with the identical haplotypic background of −597GG, −572GG and −174GG allows investigation of the effects of individual alleles of the AnTn tract on IL-6 levels independent of the three SNPs. Fig. 4C shows that individuals homozygous for A9T11 had 81% higher IL-6 levels than A10T11 homozygotes (275 ± 46 versus 152 ± 29 pg/ml, respectively; $P=0.04$). An intermediate effect was observed in heterozygous individuals carrying A9T11 and A10T11 who had 53% lower

Table 2
Frequency of IL-6 promoter haplotypes for the −597G>A, −572G>C, AnTn tract and −174G>C polymorphisms identified in 132 patients undergoing CABG surgery

Haplotype	AG[A8T12]C	GG[A9T11]G	GG[A10T11]G	GG[A10T10]G	GC[A10T10]G	AG[A9T11]C	AG[A8T12]G	GG[A8T12]G
n	78	59	51	27	12	4	2	1
Frequency	0.33	0.25	0.21	0.12	0.05	0.02	0.008	0.004

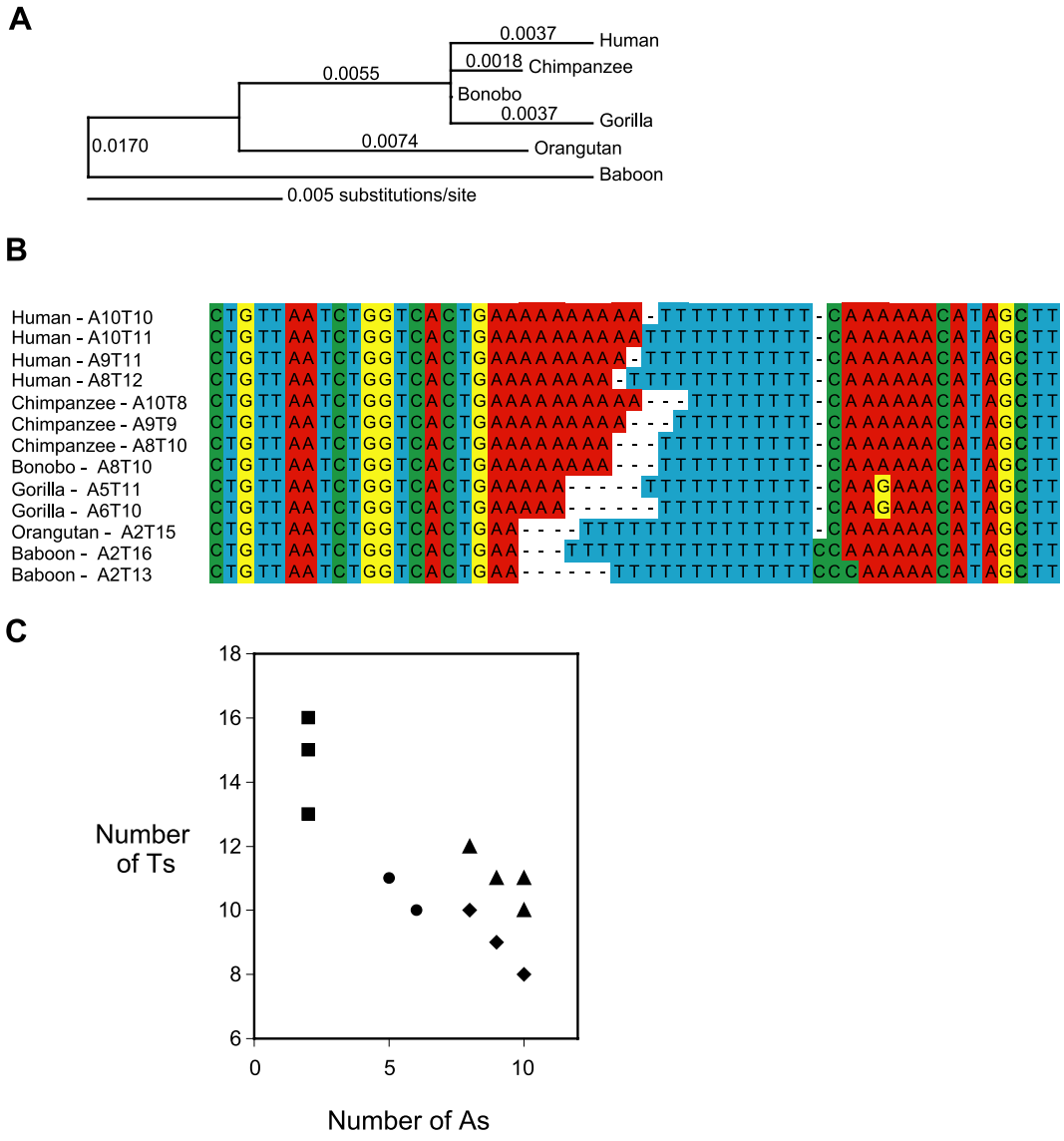

Fig. 3. (A) Phylogeny of primate IL-6 promoter (excluding the AnTn tract) shows branch lengths proportional to the estimated number of nucleotide substitutions per site. The bonobo branch has length zero. (B) Alignment of the region surrounding the AnTn tract of the IL-6 promoter in various primate species compared to the human sequence. The genotype of the AnTn tract for each sequence is indicated at the left. Dashes indicate gaps in the alignment. (C) Graph showing that within and between species the number of A and T nucleotides are inversely related. Symbols represent alleles from human (triangle), chimpanzee and bonobo (diamond), gorilla (circle), and orangutan and baboon (square).

IL-6 levels (179 ± 36 pg/ml) than A9T11 homozygotes and 17% higher than A10T11 homozygotes, but this difference did not reach statistical significance. In the presence of the common GG[A10T11]G haplotype, a modest stepwise increase was observed in mean IL-6 levels between individuals carrying the A9T11 and the A10T10 alleles (Fig. 4D), although this difference was not significant (179 ± 36 versus 183 ± 28 pg/ml, respectively; $P = 0.35$). Of all variables measured on the operating procedure, only operation duration and CPB time correlated with peak IL-6 levels.

Table 3
Serum IL-6 concentration (pg/ml) 6 h following surgery for IL-6 promoter haplotypes

Haplotype 1	Haplotype 2				
	GG[A10T11]G	AG[A8T12]C	GC[A10T10]G	GG[A9T11]G	GG[A10T10]G
GG[A10T11]G	152 ± 29 ($n = 6$)	179 ± 25 ($n = 20$)	207 ± 32 ($n = 5$)	179 ± 36 ($n = 9$)	183 ± 28 ($n = 4$)
AG[A8T12]C		283 ± 41 ($n = 9$)	378 ± 255 ($n = 4$)	179 ± 17 ($n = 23$)	119 ± 28 ($n = 12$)
GG[A9T11]G				275 ± 46 ($n = 8$)	221 ± 58 ($n = 7$)

Data are presented as mean ± S.E.

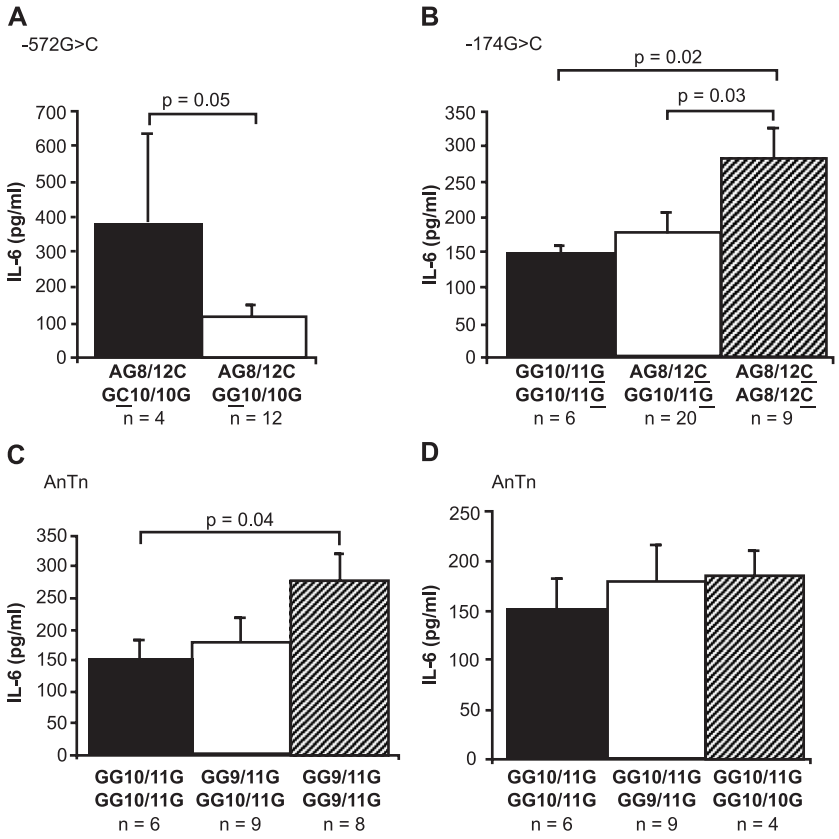

Fig. 4. Effect of IL-6 promoter haplotypes for −597G>A, −572G>C, AnTn and −174G>C polymorphisms on serum IL-6 levels 6 h following surgery. Results presented as mean ± S.E., significant differences in IL-6 levels between haplotypes are indicated. A value of $P < 0.05$ was taken as significant.

However, no significant differences were demonstrated between any of the haplotype groups and these variables, therefore it is not possible for them to confound the observed relationship between haplotype and peak IL-6 levels post-surgery.

As a result of strong allelic association, the A8T12 allele could not be analysed in isolation from −174C in this study. Of note, a single individual in the cohort was homozygous for the haplotype GG[A10T10]G with serum IL-6 of 324 pg/ml 6 h post-operatively, however, no significant conclusions can be drawn from this observation due to insufficient sample size.

4. Discussion

In order to investigate whether the AnTn tract polymorphism in the promoter of the IL-6 gene has any association with IL-6 levels in response to an inflammatory stimulus in vivo, we genotyped a cohort of patients undergoing CABG surgery to assess the effect of genotype on pre- and post-operative serum IL-6 levels. Only four different alleles were identified in this group, but subjects in this study were of Caucasian origin from the UK and it is possible that additional alleles may be identified in more diverse human populations. After taking account of the effects on IL-6 levels associated with the −174C and −572C haplotypes previously reported [5], it appears that certain alleles of the AnTn tract do influence IL-6 expression in response to a defined physiological inflammatory stimulus (CABG), independent of the other polymorphisms in the promoter. Compared to the GG[A10T11]G haplotype, the GG[A9T11]G haplotype was associated with higher IL-6 levels, such that A9T11 homozygotes had 81% higher post-operative IL-6 levels than A10T11 homozygotes, with heterozygotes having approximately intermediate levels. Published in vitro reporter assays showing that constructs containing GG[A9T11]G had significantly higher promoter activity than GG[A10T11]G in cells stimulated with IL-1 support this finding [4]. The GG[A10T10]G haplotype may also be associated with higher levels than GG[A10T11]G homozygotes, but the effect was smaller and the A10T10 allele is less frequent, so the differences were not statistically significant. These data suggest that either the presence of A9T11 acts to increase transcription, or that A10T11 has a repressive effect. Due to strong allelic association between the −174C and the A8T12 allele, the effects of this allele could not be analysed in isolation. Therefore, it remains unclear whether the A8T12 allele has a significant influence on IL-6 expression or whether the observed effect originated exclusively from the −174C allele, shown to be functional in vitro [3,4].

Several other examples are known where a polymorphic tract of poly d(N) residues has been reported to affect the regulation of transcription. These include a run of five or six adenosines in the promoter of the matrix metalloproteinase 3 gene (*MMP3*; Stromelysin-1), and a polymorphic run of guanosine residues in the promoter of the Kallikrein gene, both of which have been shown to influence gene expression in an allele-specific manner [17,18]. A possible mechanism for differential effects on expression may be as a result of an altered binding affinity for a transcriptional activator, or repressor, between particular alleles, as is the case for the *MMP3* promoter polymorphism [17]. Alternatively, distinct alleles may bind different transcription factors each with a particular effect on gene expression. This switching of transcription factor specificity has been observed for single nucleotide variation in the promoters of numerous genes [19]. However, variation in the molecular architecture of the DNA at this site may directly play a role in influencing gene expression. Variation in the helical structure resulting in bending of the DNA, or the formation of secondary structure, dependant on the composition of the AnTn tract, may result in steric hindrance of transcription factors or other regulatory proteins to the DNA. In this way, certain alleles of a VNTR-like repeat located in the promoter region of the insulin gene (*INS*) form unusual DNA structures which affect transcription factor binding and influence expression of *INS* [20].

Although it is clear that the ancestral allele of the three SNPs in the promoter is G at all sites, no clear insights as to the functionality of the AnTn tract can be gained from the sequence comparison of this region in other primate species. The failure to identify any ancestral AnTn allele across species suggests that this site has been subject to continual evolution. However, the strong linkage disequilibrium observed between the A8T12 and −174C allele suggests that the A8T12 allele is of recent origin. This inference is supported by the geographically restricted range of −174C, while common among Europeans, the allele is exceedingly rare or absent among East Asians, native Americans and African-Americans [21–24]. The observation that the length of the poly d(T) component of the tract contracts as the poly d(A) expands through divergence between the different primate species suggests a selection pressure on the overall length of the tract. The inverse relationship between the number of A and the number of T nucleotides is not expected if the two components of the tract are evolving independently. An unusual mutation mechanism cannot be ruled out, but the apparent overall length restriction of the AnTn tract suggests it may have some functional role on influencing IL-6 expression, and supports the hypothesis that this effect is mediated through differences in the conformation of the DNA at this site, or transcription factor binding affinity.

One limitation of this study is that the sample size is small and we are thus unable to rule out small differences on IL-6 levels associated with certain haplotypes. However, the sample is large enough to detect the raising effects on IL-6 levels associated with the AG[A8T12]C and GC[A10T10]G haplotypes, and the lowering effects associated with the GG[A10T11]G haplotype with reasonable statistical significance. Further studies in larger samples, particularly for the GG[A9T11]G and GG[A10T11]G haplotypes are warranted. It is possible that individual AnTn alleles make differing contributions to IL-6 expression, acting to either enhance or repress transcription. This may explain why only significant effects were observed in this study in individuals homozygous for the AnTn tract in which these effects would be greatest. Further in vitro studies are currently underway in order to test this hypothesis.

It is clear that regulation of IL-6 expression is complex and subject to subtle variation by independent contributions from combinations of factors at several polymorphic sites in the promoter. Furthermore, differences in the effects of these polymorphisms on IL-6 expression between different cell types in vitro [3,4] suggests tissue specific regulation of the gene adding further complexity. Investigation of the interaction between proteins and components of the transcription machinery at these sites will lead to a better understanding of transcriptional control and mechanisms by which these polymorphisms influence susceptibility of disease.

Acknowledgements

We would like to thank Emma Hawe for assistance with the statistical analysis, and Anne Stone and Dagan Loisel for the primate DNA samples. This research is supported by the British Heart Foundation (grants RG 2000/015 and FS99025) and the Arthritis Research Campaign (AR47363).

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