Interleukin 1B gene polymorphism is associated with baseline C-reactive protein levels in healthy individuals

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ABSTRACT. C-reactive protein (CRP) is a sensitive marker of inflammation induced by both IL-6 and IL-1. Thus, genetic variation in these genes could be associated with the variety in C-reactive protein levels, and therefore with the severity of the entire inflammatory response. Even a subtle elevation in baseline CRP levels in healthy individuals has been found to significantly increase the risk for cardiovascular diseases. Therefore, to find out the possible role of pro-inflammatory cytokines in CRP baseline regulation we conducted a study of 338 healthy blood donors whose CRP levels were determined and whose single nucleotide polymorphisms of IL1A(C/T)-889, IL1B(C/T)-511, IL1B(C/T) + 3954, IL6(G/C)-174 and ILRN (a VNTR) both genotyped and haplotyped. The data revealed an association between CRP levels and the IL1B + 3954 genotype. Also, the bilocus haplotype IL1B-511*1/IL1B + 3954*2 was more frequent in subjects with below median CRP levels (< 0.72 mg/l), and composite genotype analysis of IL1B-511/IL1B + 3954 supported this finding. Our findings suggest that in healthy people, basal CRP levels are regulated by IL1B but not by IL6 genetics.

Keywords: CRP; IL1B; polymorphisms, immunogenetics, inflammation

INTRODUCTION

C-reactive protein (CRP) is a major acute phase protein produced in response to inflammation, infection and trauma. Only a trace amount of CRP is found in the blood of healthy individuals. The baseline level of CRP is less than 1 mg/l, but may rise a thousand-fold in response to various pro-inflammatory cytokines, e.g. interleukin-1 (IL-1) and interleukin-6 (IL-6) [1, 2]. Usually, peak values for CRP concentration disappear within a few days of the inflammatory stimulus [3]. During the acute-phase reaction, CRP is mainly produced by the liver in response to IL-6, which is assumed to be the major regulator of blood CRP [4]. The basal values of CRP appear to be significantly heritable ($\simeq$ 40%) [5, 6], and therefore it is very likely that polymorphisms in genes controlling CRP expression may influence CRP levels.

The blood concentrations of CRP and IL-6 are usually strongly correlated. IL6 promoter region polymorphism have been shown to influence IL6 transcription and, thus, the CRP levels [6]. There is some evidence from in vitro studies that IL-6 is able to induce the transcription of CRP in hepatic (Hep3B) cells, and IL-1β, which alone has no effect, greatly enhances this transcription [7, 8]. Also, the baseline CRP concentrations have been shown to be genetically regulated by CRP gene polymorphisms. A 1059G/C polymorphism in exon 2 as well as a dinucleotide repeat in the CRP gene intron both affect the baseline CRP plasma levels [9, 10]. Other factors associated with CRP blood concentration are e.g. age, smoking and obesity [11, 12].

CRP is a sensitive marker of inflammation. Several studies have shown an association between CRP levels and the risk of coronary heart disease (CHD) [13]. CRP is also a very powerful predictor of future cardiovascular events in healthy adults, in that even a slight elevation in the CRP baseline concentration is enough to significantly increase the risk of cardiovascular disease [14]. Inflammation plays a key role in the progression of atherosclerosis, and high levels of CRP are associated with this disease. Several theories have been proposed whereby CRP might directly influence atherogenesis, rather than being just a sensitive marker of underlying inflammation [15]. Therefore, CRP could be used as a useful marker for atherosclerosis development and may help to identify individuals prone to premature atherosclerosis.

In this study we wanted to examine the effect of IL6 and IL1 complex genetics on the baseline CRP levels. This was examined in healthy, middle-aged people, where no stimulatory effect of inflammation was present and the true baseline CRP level was measured.

PATIENTS AND METHODS

Blood donors

Blood samples were collected from 338, healthy, adult blood donors at the Finnish Red Cross Blood Transfusion Centre, Tampere. The male to female ratio was 1:0.8. The age range was 19-64, mean 44.2 years. All the blood donors were of the same ethnic origin, Finnish caucasians.

Genotype analysis

The intron two polymorphism of IL1RN was amplified by PCR using oligonucleotide primers 5' CTCAGCAACACTCCTAT 3' and 5' TCCTGGTCTGCAGGT 3'. PCR products were analysed on 2% agarose gel as previously described [16].

Two polymorphic regions for the IL1B gene were analysed. The polymorphic site at position – 511 was amplified by PCR and digested with *AvaI* as previously described [17]. The other region at exon 5, position + 3954, was amplified using nucleotides 5' GTTGTCATCAGACTTTGACC 3' and 5' TTCAGTTCATATGGACCAGA 3' as primers. PCR products were digested with *TaqI* restriction enzyme and analyzed on 2% agarose gel [18].

The IL1A gene polymorphic site at position –889 was analysed using oligonucleotides 5' AAGCTTGTTCTACCACCTGAACTAGGC 3' and 5' TTACATATGAGCCTTCCATG 3' as primers in PCR [19]. The products were digested with *NcoI* restriction enzyme and the fragments analyzed on agarose gel.

The IL6 promoter region polymorphic site at the –174 position was amplified by PCR using oligonucleotide primers 5' TGACTTCAGCTTTACTCTTGT 3' and 5' CTGATTGGAAACCTTATTAAG 3' [20]. The amplified DNA was digested with restriction enzyme *NlaIII* and run on 3% agarose gel.

CRP assay

The plasma C-reactive protein concentrations were analyzed by particle enhanced immunonephelometric method using the Dade Behring N High Sensitivity CRP on the Dade Behring Nephelometer II (Dade Behring, Marburg, Germany). The lower detection limit for CRP was 0.16 mg/L (0.016 mg/dL) [21].

Statistical analysis

Statistical calculations were performed using Statistica software (ver, Win.5.1D, StatSoft Inc, Tulsa, OK, USA). Possible deviations of the genotype frequencies from the Hardy-Weinberg equation were detected by a Markov chain algorithm (Arlequin program, ver. 2.0, A software for population genetics data analysis. Schneider S, Roessli D, Excoffer L. Genetics and Biometry Laboratory, Geneva, Switzerland). Haplotype frequency calculations were carried out using the Arlequing program and were based on the expectation-maximization (E-M) algorithm. Differences in the frequencies of haplotypes in subgroups were analyzed using chi-square statistics (Statistica program). Linkage disequilibrium (D' values) between each locus was calculated as described [22].

RESULTS

The median CRP level in healthy blood donors was 0.72 mg/l, ranging from 0.16 to 30 mg/l (only one CRP value was > 10mg/l). The data shown in Table 1 demonstrate the genotype frequencies of IL1A SNP-889, IL1B SNP-511, IL1B SNP + 3954, IL1RN VNTR and IL6 SNP-174. An analysis was computed to identify any genetic associations between different genotypes and CRP levels. The different genotypes of IL1B SNP + 3954 showed significantly different CRP levels (Kruskal-Wallis test, p = 0.038). Other genotypes showed no significant association with CRP levels, although the IL1B SNP-511 genotype 1.1 had somewhat lower CRP levels than other genotypes. The genotype distribution was in accordance with that expected under the Hardy-Weinberg equation (HWE).

In order to investigate the significant association between the IL1B SNP + 3954 genotype and CRP levels more closely, we also compared the allele carrier status of this genotype. The carriers of allele 2 had significantly lower CRP levels than non-carriers (0.57 and 0.87 mg/l, Mann-Whitney test, p = 0.027 respectively, data not shown).

Due the fact that the net effect of the IL-1 complex genes might have an effect on CRP levels, we computed E-M algorithm-based haplotype analysis of this complex. The subjects were divided into two groups based on the median CRP value (≥ 0.72 mg/l [N = 166] and < 0.72 mg/l [N = 168]). The bilocus haplotype analysis of IL1B-511/IL1B + 3954 revealed a significant difference in the overall distribution of the haplotype frequencies between the groups. (Table 2, population differentiation test p = 0.0088, df = 3). When comparing the individual haplotype frequencies between groups, we found the frequency of the third common haplotype IL1B-511*1/IL1B + 3954*2 to be significantly higher in the below-median CRP group compared to the above-median group (0.292 and 0.185, p = 0.049 respectively). The two most common bilocus haplotypes were IL1B-

Table 1
C-reactive protein levels by IL1 genotype

Genotype		N	CRP (mg/l)	P value
IL-1A-889	1.1	161 (48%)	0.81 (0.39-1.62)	0.605
	1.2	148 (44%)	0.67 (0.35-1.52)	
	2.2	26 (8%)	0.80 (0.26-1.59)	
IL-1B-511	1.1	120 (35%)	0.56 (0.31-1.34)	0.101
	1.2	168 (50%)	0.82 (0.39-1.66)	
	2.2	50 (15%)	0.77 (0.37-1.79)	
IL-1B + 3954	1.1	189 (56%)	0.87 (0.39-1.77)	0.038
	1.2	122 (36%)	0.68 (0.34-1.51)	
	2.2	26 (8%)	0.52 (0.24-0.96)	
IL-1-RA	1.1	147 (44%)	0.73 (0.35-1.75)	0.529
	1.2	157 (46%)	0.77 (0.39-1.53)	
	2.2	27 (8%)	0.50 (0.27-1.00)	
	1.3	4 (1%)	0.85 (0.45-2.10)	
	2.3	3 (1%)	1.27 (0.40-1.82)	
IL-6	CC	101 (30%)	0.70 (0.41-1.31)	0.210
	CG	167 (50%)	0.65 (0.33-1.56)	
	GG	69 (20%)	0.69 (0.54-2.01)	

Values for CRP are expressed as medians and interquartile range i.e. values between 25th and 75th percentiles. P-values are based on Kruskal-Wallis analysis of variance.

511*2/IL1B + 3954*1 and IL1B-511*1/IL1B + 3954*1 (respectively), but no significant differences in the haplotype frequencies between the groups were found. Other multiloci haplotype analyses did not show any significant differences.

The composite genotype analysis of IL1B-511/IL1B+3954 was also analyzed to back up the computational haplotyping (Table 3). The analysis revealed a significant down-regulating effect of IL1B-511 1.1. /IL1B+3954 2.2. composite genotype on CRP levels [odds ratio 0.206 (95% CI 0.07-0.62)].

The determination of linkage disequilibrium (LD) coefficients (D') between loci revealed significant linkage between every locus, expect for those between IL1A-889 and IL1RN. Importantly, the linkage between loci IL1B SNP-511 and IL1B SNP + 3954 in subjects with a CRP value < 0.72 mg/l was relatively weak, but by contrast, very strong in subjects with CRP value ≥ 0.72 mg/l (D' = – 0.026 and – 0.920, p = 0.0128 respectively).

DISCUSSION

The findings of this study show that the IL1B gene is involved in the regulation of plasma CRP levels in healthy blood donors. As is very well known, the IL1B gene product is a major inducer of systemic inflammation in its early phases, and therefore the impact of this gene on acute phase proteins such as CRP is entirely logical. The genetics of the IL1 complex are known to affect the strength and course of the immunological and inflammatory responses to various inducing agents. The IL-1α and IL-1β cytokines in this complex are clearly proinflammatory molecules, while IL1RN has a role as a natural antagonist molecule. The balance of these cytokines in blood during inflammation is the key element for either the induction or suppression the whole inflammation reaction. The present study shows that haplotype IL-1B–511*1/ + 3954*2 is associated with low CRP levels and this result was also confirmed with composite genotype analysis. We have previously shown that alleles – 511*1 and + 3954*2 are both associated with low *in vitro* IL-1β production in healthy people [23]. Logically, low IL-1β levels are now connected to low baseline CRP levels.

Table 2
Bilocus IL1 beta –511/ + 3954 haplotypes and their frequences

Haplotypes	IL1B-511	IL1B+3954	CRP > 0.72	CRP < 0.72	P value
h1	2	1	0.405	0.357	n.s
h2	1	1	0.385	0.342	n.s
h3	1	2	0.185	0.292	0.0486
h4	2	2	0.003	0.009	n.s

Population differentiation test: p = 0.0088
P value is calculated in 2 × 2 table in Chi^2 -test

The same IL1B gene SNP + 3954 was recently associated with the CRP levels associated with cardiac symptom patients in a study by Berger *et al.* [24]. In that study, allele 2 of this locus was associated with higher CRP levels. The reason for this inconsistency between these studies is unknown and additional studies are needed in diseased people to investigate this issue. Based on the results of our present study, IL-6 genetics do not seem to be involved in the CRP levels found in healthy blood donors.

The IL-1 complex is 300 Kb in size, and therefore ideal for haplotype analyses, because linkage disequilibrium can still be seen at this distance [25]. The net effect of the genes in this complex has been shown to be important or causative in certain diseases, for example in cardiovascular diseases, and in periodontitis there is evidence of a genetic pattern for each of these diseases [26]. However, the haplotype analysis showed that the only gene that seems to be related to CRP levels is IL1B. We did not detect any association between the net effect of all four genes and CRP levels, nor the effect of three genes.

Furthermore, the strong linkage (D' = – 0.92) between IL1B-511 and IL1B + 3954 loci in the low CRP group indicates the importance of this haplotype in baseline CRP level determination. This analysis emphasises the significance of IL-1B + 3954 allele 2 in CRP levels, together with allele 1 of – 511 SNP. A genetic mask of this kind favours lower CRP values, which could mean that these people may have a milder inflammatory response than others to the same stimulus. The understanding of the baseline regulation of CRP is fundamental to the further investigation of this issue. Inflammation itself proceeds like a cascade, and therefore only minor adjustments at the beginning of this process could have a major outcome at the end of the process. IL-1 acts early in the cascade of inflammatory response, inducing the reaction, and it could be that such patients are protected from excessively strong inflammatory reactions by this genotype. Such informa-

Table 3
Composite genotypes of IL1B SNP –511 and SNP + 3954 and odds ratios (95% confidence interval)

Genotype no.	IL1B-511	IL1B + 3954	CRP > 0.72	Freq.	CRP < 0.72	Freq.	OR (95% CI)
1.	1.1	1.1	23	0.139	19	0.113	1.260 (0.66-2.41)
2.	1.1	1.2	23	0.139	32	0.190	0.680 (0.38-1.23)
3.	1.1	2.2	4	0.024	18	0.107	0.206 (0.07-0.62)
4.	1.2	1.1	56	0.337	44	0.262	1.430 (0.90-2.30)
5.	1.2	1.2	32	0.193	33	0.196	0.977 (0.57-1.68)
6.	1.2	2.2	3	0.018	0	0.000	–
7.	2.2	1.1	23	0.139	21	0.125	1.130 (0.60-2.12)
8.	2.2	1.2	2	0.012	0	0.000	–
9.	2.2	2.2	0	0.000	1	0.006	–
Total			166		168		

tion should be taken into consideration when designing therapeutic drugs for use in inflammatory reactions.

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