Genes, inflammation, and age-related diseases

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Chapter 3

Genetic variation in the interleukin-10 gene promoter and risk of coronary and cerebrovascular events: the PROSPER study.

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Abstract

Pro-inflammatory cytokines, like interleukin (IL)-6 and tumor necrosis factor-alpha, are implicated in the development of atherosclerosis. The role of anti-inflammatory cytokines, like IL-10, is largely unknown. We investigated the association of four single nucleotide polymorphisms (SNPs) in the promoter region of the IL-10 gene, (4259AG, -1082GA, -592CA and -2849GA), with coronary and cerebrovascular disease in participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial. All associations were assessed with Cox-proportional hazards models adjusted for sex, age, pravastatin use, and country. Haplotype analysis of the four SNPs showed a significant association between haplotype 4 (containing the -592A variant allele) and risk of coronary events (p=0.019). Moreover, analysis of separate SNPs found a significant association between -2849AA carriers with incident stroke (HR (95%CI): 1.50 (1.04-2.17), p-value = 0.02). Our study suggests that not only pro-inflammatory processes contribute to atherosclerosis, but that also anti-inflammatory cytokines may play an important role.

Introduction

Inflammatory stimuli, like the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-alpha (TNFα), are implicated in the development of atherosclerosis (1;2). Besides these pro-inflammatory cytokines, anti-inflammatory cytokines like IL-10 may also play a role in the development of atherosclerosis. In IL-10 knockout mice, the absence of IL-10 leads to a marked increase in susceptibility of atherosclerosis (3). Furthermore, after occlusion of the middle cerebral artery, brain infarcts in IL-10 knockout mice are 30% larger as in wild-type mice (4). These preclinical results indicate a possible role for IL-10 in the atherosclerotic process. However, the contribution of IL-10 to the modulation of the atherosclerotic process in humans remains largely to be elucidated.

IL-10 production levels are under tight genetic control. An extended twin study found that approximately two-thirds of the variance in production level of IL-10 is genetically determined (5).

Moreover, we have previously reported that genotypic variation in the IL-10 gene is associated with significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS)(6).

We performed a genetic association study of four IL-10 promoter SNPs (4259AG, -1082GA, -592CA, and -2849GA) with coronary and cerebrovascular events in participants at risk for vascular disease.

Methods

Study participants come from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial. A detailed description of the protocol and results of the study has been published elsewhere (7). Here a short outline is provided.

Participants

PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. The primary endpoint was the combined endpoint of fatal coronary heart disease (CHD), non-fatal myocardial infarct (MI), and occurrence of clinical stroke, either fatal or non-fatal. Secondary endpoints were the separate coronary and cerebrovascular components of the primary endpoint. All endpoints were adjudicated by a study endpoint committee.

Genotyping

We selected four SNPs in the promoter region of the IL-10 gene, 4259AG (rs3024498), -1082GA (rs1800896), -592CA (rs1800872), and -2849GA (rs6703630) based on the frequency of the minor

allele and possible functionality. All polymorphisms were genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions.

Statistical analysis

The program Haploview (8) was used to estimate allele frequencies, test the consistency of genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium, and estimate and plot pairwise linkage disequilibrium (LD) between the SNPs examined. Haplotypes and haplotype frequencies were calculated using SNPHAP software (http://www-gene.cimr.cam.ac.uk/clayton/software). Haplotypes with a frequency of less than 5 % were combined and included in all analyses, without reporting the results. The posterior probabilities of pairs of haplotypes per subject as estimated by the SNPHAP were used as weights in all analyses. The haplotype analysis approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (9). Hazard ratios with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses were adjusted for sex, age, pravastatin use, and country. The analyses were performed with STATA statistical software, version 9.0 (StataCorp LP, Texas, USA).

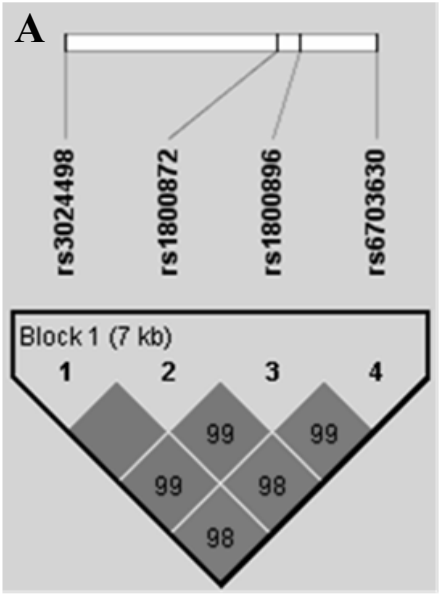
Results

The mean age of the participants was 75.3 years and approximately 50% were female (table 1). There were significant differences in minor allele frequencies between the countries (p-value Chi-square < 0.01, data not shown). The variants 4259G, -1082A and -2849A were more common in the Irish subjects compared with the subjects from Scotland and the Netherlands. Therefore, all analyses were adjusted for country. Mean follow-up of study subjects was 3.2 years (range 2.8-4.0).

Table 1: Baseline characteristics of the participants of PROSPER per country.

	Scotland (N=2520)	Ireland (N=2184)	The Netherlands (N=1100)
Continous variates (mean, SD)			
Age (years)	75.3 (3.4)	75.5 (3.3)	75.1 (3.3)
Body Mass index, (kg/m2)	26.7 (4.2)	27.0 (4.4)	26.7 (3.8)
Total cholesterol, (mmol/L)	5.7 (1.0)	5.6 (0.9)	5.8 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.7 (0.8)	3.9 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)
Categorical variates (n, %)			
Female	1283 (51)	1197 (55)	520 (47)
Current smoker	708 (28)	583 (27)	267 (24)
History of diabetes	213 (9)	225 (10)	185 (17)
History of hypertension	1446 (57)	1441 (66)	705 (64)
History of angina	811 (32)	523 (24)	225 (21)
History of claudication	229 (9)	114 (5)	47 (4)
History of myocardial infarction	379 (15)	258 (12)	139 (13)
History of vascular disease	1239 (49)	849 (39)	477 (43)
History of stroke or TIA	265 (11)	222 (10)	162 (15)
Genotype, minor allele frequency (%)			
IL-10 4259AG	30	33	28
IL-10 -1082GA	51	55	50
IL-10 -592CA	21	20	21
IL-10 -2849GA	30	33	29

Genotyping of the four IL-10 polymorphisms was complete for 5786 subjects. All four SNPs were in Hardy-Weinberg equilibrium (all $p>0.05$). The four SNPs were in strong linkage disequilibrium (LD) and occurred together in one haploblock (figure 1). Six haplotypes were found in our study population (figure 1B). The four haplotypes with a frequency above 5% were included in analyses. We used haplotype2, with no variants present, as reference haplotype. Haplotype1, the most frequent, had three variant alleles, 4259G, -1082A and -2849A. Haplotype3 carried the -1082A variant and haplotype4 the-592A variant.



B

	4259AG	-592CA	-1082GA	-2849GA	Haplotype frequency
H1	2	1	2	2	0.304
H2	1	1	1	1	0.269
H3	1	1	2	1	0.209
H4	1	2	1	1	0.207
H5	1	1	2	2	0.009
H6	2	1	2	1	0.003

Figure 1: Haplotype information.

Figure A shows the linkage disequilibrium (LD) between the single nucleotide polymorphisms (SNPs) examined. All SNPs are in LD and occur together in one haploblock. Figure B shows the haplotype frequencies. Only the first four haplotypes (frequency> 5%) were included in the analyses.

Haplotype1 was associated with an increased risk for the primary endpoint compared to haplotype2 (HR (95%CI) 1.14 (1.01-1.29), p= 0.035) (table 2). Carriers of haplotype4, with the -592A variant allele, also had a significantly increased risk for the primary endpoint (HR (95%CI) 1.19 (1.04-1.36), p=0.012). To determine whether the significant haplotype associations with the primary endpoint were attributable to coronary events, strokes, or both, we subdivided the primary endpoint into coronary events and strokes. The significant association with haplotype4 was due to a significant relation with coronary events (HR (95%CI) 1.21 (1.03-1.41), p=0.019). The significant association with haplotype1 was not clearly due to coronary events or strokes (HR (95%CI) 1.13 (0.98-1.30), p=0.082 and HR (95%CI) 1.22 (0.96-1.54), p=0.097 respectively).

Therefore, we performed a single SNP analysis with the three SNPs present in haplotype1, 4259AG, -1082GA and -2849GA, to assess the association with coronary events or strokes. No consistent associations were found between the IL-10 4259AG and the -1082GA and any of the endpoints. The

IL-10 -2849AA genotype showed an increased risk of strokes (HR (95%CI): 1.50 (1.04-2.17), p-value = 0.02) (table 3). In each country a comparable trend was observed.

Table 2: Haplotype analysis with various endpoints in the overall group (n=5786)

	Haplotype 2 (1.1.1.1)	Haplotype 1 (2.1.2.2)		Haplotype 3 (1.1.2.1)		Haplotype 4 (1.2.1.1)	
	HR (95%CI)	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
Primary endpoint	1.0 (ref)	**1.14 (1.01-1.29)**	**0.035**	1.14 (0.99-1.30)	0.065	**1.19 (1.04-1.36)**	**0.012**
Coronary events	1.0 (ref)	1.13 (0.98-1.30)	0.082	1.10 (0.94-1.29)	0.236	**1.21 (1.03-1.41)**	**0.019**
Clinical stroke	1.0 (ref)	1.22 (0.96-1.54)	0.097	1.23 (0.95-1.59)	0.114	1.14 (0.89-1.47)	0.291

Hazard ratios are assessed with Cox-proportional hazards model adjusted for sex, age, treatment, and country. The primary endpoint included coronary heart disease death, non-fatal myocardial infarct, and fatal/non-fatal stroke

Table 3: Association between IL-10 -2849GA genotype and stroke in separate countries (n=5786)

Clinical stroke	Genotypes			HR 2 vs 1	HR 3 vs 1	p-value
	Wt/Wt (1) n/N (%)	Wt/Var (2) n/N (%)	Var/Var (3) n/N (%)	(95% CI)	(95% CI)	
Scotland	51/1165 (4)	37/997 (4)	15/217 (7)	0.83 (0.55-1.27)	1.57 (0.88-2.80)	0.12
Ireland	41/926 (4)	38/887 (4)	15/234 (6)	0.96 (0.62-1.49)	1.45 (0.80-2.63)	0.37
The Netherlands	27/531 (5)	19/415 (5)	7/92 (8)	0.89 (0.49-1.60)	1.53 (0.66-3.51)	0.47
Overall	119/2622 (5)	94/2299 (4)	37/543 (7)	0.88 (0.67-1.16)	1.50 (1.04-2.17)	**0.02**

Hazard ratios (HR) are assessed with the Cox-proportional hazards model adjusted for sex, age, treatment, and country

Discussion

This study investigates the association between functional polymorphisms in the promoter region of the IL-10 gene with coronary and cerebrovascular events. The haplotype analysis showed an association of two haplotypes with the primary endpoint. When we looked at this association in more detail haplotype 4, with the -592A variant, was associated primarily with coronary events. The -2849AA in haplotype1 was found to be associated with an increased risk of clinical strokes.

Production of IL-10 is under tight genetic control, with heritability estimates between 50-70% (10). Part of this genetic variation comes from polymorphisms in its own promoter sequence (11). We have previously reported that carriers of the IL-10 -2849AA genotype have significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS)(6). Although it is unknown whether the -592GA SNP is functional by itself or that it is in linkage disequilibrium with another variant, the -592A variant has been associated with low IL-10 production rates (12).

We found that the -592GA polymorphism in the promoter region of the IL-10 gene was associated with coronary events. In IL-10 knock-out mice the absence of IL-10 leads to an increased susceptibility of atherosclerosis (3). Furthermore, a study with an overexpressing transgenic mice model and IL-10 null mice showed a marked difference in lesion size between the groups (13). IL-10 transgenic mice displayed significantly less atherosclerotic lesion formation compared to wild-type whereas IL-10 null mice had increased lesion formation (13). Moreover, in patients with acute coronary syndromes it was demonstrated that elevated IL-10 serum levels are associated with a significantly improved outcome (14).

We also showed that the -2849AA genotype was associated with an increased risk of incident stroke. The role of IL-10 in ischaemic brain damage has been evaluated before. Brain infarcts produced by occlusion of the middle cerebral artery were 30% larger in IL-10 knockout mice as compared with wild-type mice (4). Furthermore, exogenous administration of IL-10 induces neuroprotection in rat models of cerebral focal ischaemia (15). Moreover, we have earlier demonstrated that elderly subjects with low IL-10 production capacity have an increased risk of incident stroke (16).

The reported associations are relatively small. However, due to our large study population they are significant and consistent. The strength of our study is that we could replicate the increased risk for strokes for -2849AA carriers in three separate study populations. Because we randomized participants from three countries, each study group could be used separately. Although the separate

associations were not significant due to small numbers, similar trends were observed. Another strength of our study is that all subjects were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes, therefore genetic markers of coronary and cerebrovascular events may be identified easily.

In conclusion, genetic variation in the promoter region of the IL-10 gene is associated with vascular events. This provides evidence that not only pro-inflammatory processes contribute to atherosclerosis but that also anti-inflammatory cytokines are implicated. These findings support the hypothesis that genetic programming of the inflammatory response may be relevant to the pathogenesis of atherosclerosis. If these findings are confirmed and adequately explained on the basis of independent studies, screening patients for the IL-10 promoter polymorphisms may contribute to a better risk stratification of patients at increased risk for atherosclerosis and may improve individual treatment.

Acknowledgements

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