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Role of *IL-10* promoter polymorphisms in the development of severe aorto-iliac occlusive disease

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Summary Aortic severe occlusive disease (ASO) is a peripheral manifestation of atherosclerosis with an inflammatory component. Interleukin (IL)-10 is an anti-inflammatory cytokine that plays a key role in the development of atherosclerosis, promoting the stability of the atherosclerotic plaque. Several polymorphisms within the 5′ region of the *IL-10* gene have been related to altered transcriptional activity and protein levels. We aimed at studying two microsatellites, IL-10R and IL-10G, at -4 and -1.2 Kb, and three single nucleotide polymorphisms at positions -1082A/G, -819C/T and -592C/A in a collection of 94 ASO patients and 519 ethnically matched controls. Our results show that the *IL-10* proximal promoter haplotype IL-10G*11/ -1082G/ -819C/ -592C is more frequent in ASO patients than in controls (28.7% vs 16% p = 0.003; OR = 2.12). Therefore, our data suggest a role of the IL-10 gene on ASO susceptibility.

Introduction

Aortic severe occlusive disease (ASO) is a manifestation of atherosclerosis. During recent years it has been evidenced that atherosclerosis is an inflammatory disease, characterized by the presence of immunocompetent cells and production of mainly pro-inflammatory cytokines [1,2]. Interleukin (IL)-10 is an anti-inflammatory cytokine that modulates the signals between two subpopulations of lymphocytes T helper (Th1/Th2), and that inhibits the production of proinflammatory cytokines released by activated macrophages and T lymphocytes [3]. Studies *in vitro* and *in vivo* have demonstrated that IL-10 plays a key role in the development of atherosclerosis by limiting local inflammatory activity, inhibiting apoptosis and, therefore, promoting the stability of the atherosclerotic lesion [4]. Concordantly, rats genetically manipulated to produce high amounts of IL-10 develop a lesser degree of atherosclerosis than normoproducer rats when both are fed high-lipid diets [5]. Immunohistochemical studies of the human arterial atherosclerotic wall have demonstrated that IL-10 is expressed in the cytoplasm of macrophages and in the connective tissue matrix. Higher levels of IL-10 in atherosclerotic plaques protect against cellular apoptosis [6]. *In vitro* studies show that IL-10 inhibits the synthesis of metalloproteinases and it stimulates the produc-

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ABBREVIATIONS

ASO	aortic severe occlusive disease
IL	interleukin
SNP	single nucleotide polymorphism

tion of its inhibitor, TIMP-1. In this way, IL-10 preserves the extracellular matrix and the fibrous cap, promoting the stability of the atherosclerotic plaque [7]. These results are consistent with the observation of higher levels of IL-10 in patients with stable angina in comparison with those with acute angina [8]. Moreover serum levels of IL-10 are inversely related to future events in patients with myocardial infarction [9].

The *IL-10* gene is located in the long arm of chromosome 1 and three single nucleotide polymorphisms (SNPs) found in its promoter at -1082A/G, -819C/T and -592C/A conform three different haplotypes: ACC, ATA, and GCC [10,11]. In addition, two microsatellites, IL-10R and IL-10G, are located at 4 and 1.2 Kb upstream from the origin of transcription, respectively [12]. These *IL-10* SNPs and microsatellites have been related to cytokine levels [12–14] and they have been associated with susceptibility to different inflammatory conditions. Association studies dealing with the involvement of the *IL-10* gene in atherosclerosis are inconclusive, perhaps because of the incomplete evaluation of the polymorphisms. We aimed at testing the five markers previously mentioned to establish the haplotypes conformed by them and to investigate their distribution in a collection of ASO patients compared with that present in healthy controls. This work aims to fill the gap in terms of the scarce information about peripheral atherosclerosis susceptibility.

Subjects and methods

Patients

A total of 94 patients with aorto-iliac severe occlusive disease were evaluated. All patients were of Spanish descent and belonged to the same healthcare area of Madrid (Area 7, which includes approximately 500,000 inhabitants). Patients with a history of autoimmune or chronic inflammatory disease were excluded. All the participants had undergone revascularization surgery, during which the atherosclerotic nature of their lesions was confirmed and blood samples were drawn.

The control group included 519 Spanish subjects from Healthcare Area 7 in whom cardiovascular or other inflammatory or autoimmune diseases were ruled out. This population is representative of our community and all the participants signed an informed consent. The study was approved by the Ethics Committee of Hospital Clínico San Carlos in Madrid.

IL-10 promoter polymorphisms

IL-10R (111–117 bp) and IL-10G (107–141 bp) microsatellites were amplified under conditions previously reported (14) with the following set of primers: IL-10R: 5′-CCCTCCAAAATCTATTTGCATAAG-3′ 5′-TET-CTCCGCCCAGTAAGTTTCATCAC-3′ IL-10G: 5′-GCAACACTCCTC-GTCGCAAC-3′ 5′-FAM-CCTCCCAAAGAAGCCTTAGTA-3′.

Denatured samples and internal standard sizes were measured on an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA). The results were analyzed using GeneScan (Applied Biosystems) and the local southern size-calling method.

Polymorphisms at positions -1082A/G, -819C/T, and -592C/A were detected by allele-specific polymerase chain reaction with oligonucleotides labeled with distinct fluorochromes essentially as previously described [15]. In short, a combined amplification of the IL-10G microsatellite (using the forward primer previously mentioned, IL-10G: 5′-GCAACACTCCTCGTCGCAAC-3′) and the promoter SNPs were performed, and therefore amplification only proceeds when the markers are located in cis. The reverse primers used for this reaction were:

-1082G: 5′-FAM-CCTATCCCTACTTCCCCC-3′
-1082A: 5′-HEX-CCTATCCCTACTTCCCCT-3′
-819T: 5′-TET-GCAAACTGAGGCACAGAGATA-3′
-819C: 5′-FAM-CAAACTGAGGCACAGAGATG-3′

This typing method allowed to infer the third position (-592C/A) directly, based on the simultaneous detection of the two variants at -1082A/G and -819C/T.

Statistical analysis

Statistical comparisons were performed using $\chi 2$ analysis, applying the Fisher test when one of the expected values was <5 (Epi Info v6.02, World Health Organization, Geneva, Switzerland). A two-tailed value of $p < 0.05$ was considered significant. The method of Cornfield was used to estimate the 95% confidence intervals of the odds ratios.

Results

The study group included 94 patients with ASO disease that underwent revascularization surgery of aorto-iliac sector. According to Fontaine's classification, the surgery indication was: chronic ischemia grade IIB in 59 patients (63.4%), grade III in 23 patients (24.7%), and grade IV in 11 patients (11.8%).

Four possible alleles of the distal promoter microsatellite IL-10R were found in our population: IL-10R*1, IL-10R*2, IL-10R*3, and IL-10R*4, although alleles IL-10R*1 and IL-10R*4 were extremely rare. The phenotypic distribution of these alleles in both control and ASO groups is shown in Table 1. No significant differences in the frequencies of the IL-10R alleles were observed between patients with ASO and control subjects.

Microsatellite IL-10G is more polymorphic than IL-10R, with 15 alleles (IL-10G*1 to IL-10G*15). The first six alleles are rare in our and in other populations, and we only found alleles IL-10G*7 to IL-10G*15. Two promoter polymorphisms at positions -1082A/G and -819C/T were analyzed and another one at -592C/A was inferred because of the linkage disequilibrium displayed with the others. In a given promoter there are only three possible combinations (haplotypes): -1082A, -819T, -592A (haplotype ATA), -1082A, -819C, -592C (haplotype ACC), -1082G, -819C, -592C (haplotype GCC). In combination with the IL-10G alleles, nine haplotypes with a frequency >5% were found in our population. Table 2 shows the distribution of the proximal promoter haplotypes in control and ASO groups. Given that the group of patients is enriched for men as compared with women, control subjects were stratified by gender, and no significant difference in the haplotype distribution between genders was confirmed; therefore, to prevent lack of statistical