Osteopontin Gene Polymorphisms in Spanish Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology characterized by cartilage and bone destruction. The main genetic determinant to RA, the shared epitope, maps to the HLA-DR locus, although this is not the only risk factor. The osteopontin (*OPN*) gene, with pleiotropic functions in inflammatory and immune responses, has been implicated in the pathogenesis of RA. We studied the association of polymorphisms in the *OPN* gene and predisposition to RA.
Methods. Analysis was performed in a case-control study with 263 patients and 478 controls. Four single nucleotide polymorphisms (SNP), 327T/C, 795C/T, 1128A/G, and 1284A/C, of the *OPN* gene were genotyped by primer-specific amplification in the presence of SYBR Green.
Results. Distorted transmission of these polymorphisms was studied in 58 RA trios and 61 affected sibling pairs. These SNP demonstrated strong linkage disequilibrium. No statistically significant association was observed (80% power to exclude a genotypic relative risk of 1.49 at the 5% significance level, with minor allele frequencies of 28%). This lack of association with RA was found after stratification for the shared epitope as well.
Conclusion. Our data suggest that, unlike the reported effect of the *OPN* SNP conferring predisposition to common diseases such as multiple sclerosis or systemic lupus erythematosus, these *OPN* gene polymorphisms do not contribute to RA susceptibility in the Spanish population we studied. (J Rheumatol 2005;32:405–9)

Key Indexing Terms:
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Rheumatoid arthritis (RA) is a common human autoimmune disease with a prevalence of about 1%. Chronic inflammation of multiple joints with accelerated proliferation of synovial cells in RA leads to progressive cartilage and bone destruction, and affects quality of life. Neovascularization and systemic inflammation are also present. A multifactorial etiology, with an underlying genetic susceptibility and undefined environmental causative agents, is well accepted[1]. Various inflammatory cytokines released by activated macrophages, such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and IL-6, have been associated with RA[2]. As well, synovial cells, chondrocytes, and osteoblasts produce abnormal concentrations of cytokines in patients with RA[3].

Data from twin and family studies have shown that a major genetic contribution to disease predisposition resides in the *HLA-DR* locus[4]. More than 80% of Caucasian patients with RA express DR1, DR4, or DR10 subtypes[5,6] sharing an epitope-mapping to amino acids 67–74 of the DRß1 chain. The *HLA* region, however, accounts for only a fraction of the genetic component, at best 50%[7]. Thus evidence indicates that genes outside this *HLA* region also contribute to RA predisposition. A genome-wide screen of multiplex families with RA has been reported, unraveling a number of non-HLA susceptibility loci in chromosomes 1, 4, 12, 16, and 17[8]. The chromosomal region 4q24 showed the most significant linkage with RA outside the *HLA* region. Another screening with 252 sibling pairs also found nominal linkage on chromosome 4q and 11 other non-HLA regions, although none of them reached the genome-wide threshold for significant linkage[9].

The osteopontin (*OPN*) gene maps to 4q23 and presents pleiotropic function[10]. OPN exists both as an extracellular matrix protein whose expression is induced by inflammatory cytokines that are elevated in RA (TNF-α, IL-1, and IL-6) and also as a cytokine in body fluids[11]. This protein, also known as early T-lymphocyte activation-1 (Eta-1) because of its early expression in the course of bacterial infection, is critical for the generation of Th1 immunity enhancing the production of interferon-γ and IL-12 and diminishing that of

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IL-10[12]. OPN is one of the major bone proteins produced by osteoblasts and osteoclasts, with a reciprocally coordinated regulation in both cell types as a mechanism for coupling of bone resorption and formation[13,14]. OPN plays a pivotal role in the pathogenesis of RA in mice. OPN-deficient mice have attenuated joint swelling and cartilage destruction, chondrocyte apoptosis, and synovial angiogenesis compared with arthritic wild-type mice[15]. An OPN epitope was recently reported to be involved in the pathogenesis of a murine model of RA[16]. In RA patients, OPN expression is elevated in joint synovial fluid and also in synovial lining cells[17,18], suggesting involvement of OPN in the pathogenesis of RA. This mounting evidence prompted our study of *OPN* polymorphisms in patients with RA.

We investigated whether 4 single nucleotide polymorphisms (SNP) in the *OPN* gene were associated with susceptibility to RA. Two of them are located in the coding regions of exon 6 (327T/C; nomenclature is based on the distance from the transcription start site in the *OPN* sequence, listed at NCBI RefSeq NM_000582.1) and exon 7 (795C/T), and the 2 others map to the 3'UTR (1128A/G and 1284A/C). Although more than 40 SNP of the *OPN* gene are available in the NCBI SNP database, those selected have been studied previously, and characterization of their allelic frequency already exists. A report of the association of these polymorphisms with multiple sclerosis has been published[19]. Those exonic polymorphisms code for synonymous changes, which argues against a functional role, but we should keep in mind that they could be in linkage disequilibrium with etiological variants or have noncoding functional relevance. Study of the 3'-UTR polymorphisms is interesting, as the stability of most mRNA has been shown to be regulated by sequences in their 3'-UTR[20].

MATERIALS AND METHODS

We performed a case-control study with 263 consecutively recruited RA patients and 478 unrelated healthy controls. From these RA patients, a set of 56 trios of patients and their parents were included in a transmission disequilibrium test (TDT). Sixty-one affected siblings of those patients were studied as well. All were Caucasian Spanish ethnicity, described in earlier studies[21].

A blood sample was obtained from each subject for DNA extraction, with informed consent. Allelic discrimination was achieved by primer-specific polymerase chain reaction (PCR) amplification of 30 ng DNA in the presence of SYBR Green in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The PCR reaction mixtures were denatured at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, and annealing for 30 s at 61°C, 60°C, 63°C, and 60°C for the following SNP: 327 (rs4754), 795 (rs1126616), 1128 (rs1126772), and 1284 (rs9138), respectively. Two members of our group assessed allelic discrimination, and controls of known genotypes (confirmed by AR familial segregation) were included in each PCR process. Genotyping used the following primers:

327 forward: 5'-TGG ATG ATG AAG ATG ATG A(T/C)-3' and reverse: 5'-AAT GGA GTC CTG GCT GT-3'; 795 forward wild-type and mutant: 5'-TCC AGA TTA TAT AAG CGG AAA GCC-3' and 5'-AGT CCA GAT TAT ATA AGC GGA AAG CT-3'; and reverse: 5'-ATC AAT CAC ATC GGA ATG CTC A-3'; 1128 forward wild-type: 5'-ATG TGT ATC TAT TTG AGT CTG GAA ATA ACT A-3' and mutant 5'-GTG TAT CTA TTT GAG TCT GGA AAT AAC TG-3'; and reverse: 5'-TCC ATG AAG CCA CAA ACT AAA CT-3'; 1284 forward wild-type: 5'-CAT GAA TAG AAA TTT ATG TAG AAG-3' and mutant: 5'-ATG AAT AGA AAT TTA TGT AGA CG-3' and reverse: 5'-TCT TTT TAA GTG GGT AAA AGT-3'.

Two CA-repeat microsatellites, D4S2929 and D4S2460, located at 342 kb and 587 kb from the OPN gene, respectively, were amplified using the following primers: D4S2929 forward primer: 5'-FAM- GGC CAG GAG TTC AAA A-3'; reverse primer: 5-TGC AGC AAG TCC AAC A- 3'; D4S2460 forward primer: 5'-HEX- CCA AAA TCA TGT GAG CCA-3'; reverse primer: 5'-GAG CAG CAG CCA ACT GTA T-3'.

Significance values were obtained by the chi-square statistic for the case-control study and by binomial distribution calculations for the TDT. The number of RA patients and controls yielded a statistical power of 80%, as determined by: http://calculators.stat.ucla.edu [cited October 7, 2004]. The relative risk used in these calculations (1.49) was extrapolated from a study of multiple sclerosis association[19], and the risk factor frequency (0.28) according to information from the SNP database: http://www.ncbi.nlm.nih.gov/SNP [cited October 7, 2004].

RESULTS

One could expect, taking into account the involvement of osteopontin in RA, that genomic variants in the *OPN* gene would be at least partially responsible for the arthritic genetic background. We investigated the genetic susceptibility to RA conferred by the *OPN* gene, and we genotyped 4 SNP of this candidate gene. No evidence for deviations from Hardy-Weinberg equilibrium for any of the markers was found. The haplotypes formed by those SNP were deduced from the analysis of linkage disequilibrium between them in our control population. These haplotypes were also confirmed by direct visual inspection of segregation within the RA families. As described[19], strong linkage disequilibrium was found among alleles at the 4 different polymorphic sites of the *OPN* gene. Apart from these experimental methods, a statistical method estimates haplotype frequencies through phenotypes in unrelated individuals. A computer program implementing the expectation-maximization algorithm was used for this purpose (Arlequin ver. 2.000: Software for Population Genetics Data Analysis. Schneider S, Roessli D, Excoffier L, University of Geneva).

Several analytical approaches were performed to test whether *OPN* may be a susceptibility gene in human RA. First, a case-control study to compare the haplotypic frequencies between 263 consecutively recruited RA patients and 478 ethnically matched controls. No apparent differences were observed between the groups in the distribution of the 2 more abundant haplotypes: one comprising all 4 SNP wild-type alleles (haplotype 1, frequency 63–64%) and another with all the mutant alleles (haplotype 2, frequency 25–28%). The other haplotypes together were present at a frequency of around 10% (Table 1). The wild-type haplotype has recently been related to about 5-fold lesser expression of the OPN protein compared to the mutant haplotype[22]. The error in typing the whole haplotype is influenced by the error affecting the allelic genotyping of each individual SNP, which in most cases will lead to the apparent dis-

Table 1. Osteopontin haplotypic frequencies in RA patients and controls.

Haplotype*	RA (%) 2n = 526	Controls (%) 2n = 956	OR	p
1	324 (62)	617 (64)	0.88	NS
2	150 (29)	283 (30)	1.06	NS

* Haplotype 1: wild-type alleles present in all 4 SNP. Haplotype 2: mutant alleles present in all 4 SNP. NS: nonsignificant.

ruption of one of the 2 more frequent haplotypes. An efficiency of 99% in individual SNP allelic discrimination will lend 4% error in haplotypic typing. Consequently, our data may even underestimate the frequency of the 2 most common haplotypes. The lack of association with the disease seen in the study sample was again reproduced when the sample was previously stratified by the presence of the shared epitope, the main susceptibility determinant for RA. The carrier rates of the wild-type and mutant haplotypes were 89% and 48% in either the RA or control groups. No difference was observed between patients and controls in the *OPN* genotypic distribution of the heterozygous (37%) and homozygous wild-type (40.4%) or mutant (7.5%) individuals.

We then applied a family-based design, the Transmission Disequilibrium Test[23], with 56 trios, to evaluate whether the parental haplotypes transmitted from the heterozygous parents to the affected proband differed statistically from those not transmitted. The TDT analysis of the heterozygous progenitors yielded no preferential transmission of any of the 2 major haplotypes found — wild-type and mutant (Table 2). The mutant haplotype was transmitted to the patients 22 times from their heterozygous progenitors and was not transmitted 24 times.

To further test the involvement of *OPN* in RA, we typed 61 affected sibling pairs (ASP) for markers on chromosome 4q23, where *OPN* was mapped. Two additional microsatellites located in the vicinity of the *OPN* gene were analyzed to determine the identity by state. In this way we extended the initial haplotype formed by the 4 SNP, increasing its variability up to 91% heterozygosity. The haplotypes were established manually and, although occasionally there is uncertainty in the phase, we were able to analyze the distribution of the shared haplotypes. We found lack of bias in such distribution with respect to that expected theoretically (Table 3). When the haplotypes shared by the 2 siblings (32 haplotypes that would be expected to show influence of the disease allele) were compared with those haplotypes present in siblings that did not share any of the 4 haplotypes (36 haplotypes that would therefore predominantly include siblings who had RA due to other susceptibility loci), no difference was found among them. Moreover, when the sibling of the indexed case was included in a case-control study instead of the indexed patient and compared to the control group, we again found no association of the *OPN* gene to RA.

Table 2. Haplotype transmission from heterozygous parents.

Haplotype*	T	NT	p
1	28	21	NS
2	22	24	NS

* Haplotype 1: wild-type alleles present in all 4 SNP. Haplotype 2: mutant alleles present in all 4 SNP. NS: nonsignificant.

Table 3. Affected sibling pairs, distributed by number of haplotypes shared.

No. of Shared Haplotypes	Sib Pairs
0	9
1	36
2	16

We conclude from all this evidence that osteopontin is not an etiological gene in RA (80% power to exclude a genotypic relative risk of 1.49 at the 5% significance level, with minor allele frequencies of 28%).

DISCUSSION

OPN is a protein with diverse immunological activities and its role in different autoimmune diseases has been described. Overexpression of OPN in patients with systemic lupus erythematosus (SLE)[24,25] as well as in the lupus murine, MRL/lpr mouse, model[26,27] suggests its participation in the pathogenesis of lupus. Indeed, a significant association of the 795 SNP of *OPN* with SLE in a Caucasian population has been reported[28]. Differential expression of genes, when brain lesions of patients with multiple sclerosis (MS) and control brains were compared, showed abundance of *OPN* transcripts, and *OPN* deficient mice were resistant to progressive experimental autoimmune encephalomyelitis, a disease model of MS[29]. Recently, 2 association studies have been published: one was performed with Japanese MS patients and the results suggest that the 327 and 1128 SNP might be associated with susceptibility and with age of onset, respectively[30]. The allelic frequencies of the *OPN* polymorphisms in Asian countries are significantly different from the ones described for Caucasian populations, which could indicate a lesser linkage disequilibrium among them. A report investigating the 4 SNP in the *OPN* gene in a Caucasian MS cohort detected genetic association with the course of the disease, but not with overall MS susceptibility[19]. These apparently contradictory results could reflect ethnic differences, as the prevalence of MS in Asian populations is much lower than in Caucasians[31]. Another complex multifactorial disease with an increased immune activity, ulcerative colitis, was found to have a more than 10-fold increased expression of both macrophage metalloelastase and *OPN* genes, compared to normal levels[32]. The mutant haplotypes formed by the 4 SNP we studied have also

recently been associated with Dianzani autoimmune/lymphoproliferative disease[22]. Thus, all this evidence of OPN functions in the pathogenesis of autoimmune diseases led us to test the effect of *OPN* polymorphisms in the etiology of RA. Unexpectedly, when association of the 4 SNP in the *OPN* gene with RA was analyzed, we found no involvement of this gene in either the case-control study or the family-based test. Despite the experimental data supporting a role for this cytokine in arthritis, analysis of the haplotypes defined by the 4 *OPN* polymorphisms did not identify any significant association with RA in our population. The possibility could not be discarded that another *OPN* etiological polymorphism or haplotype that has not yet been studied may be responsible for the association with RA. For instance, Mochida, *et al*[33] studied 4 polymorphisms in the promoter region of the *OPN* gene and observed that 3 of them are in linkage disequilibrium, and the fourth acts as a marker in chronic hepatitis C. Unfortunately, they did not check for the disequilibrium present with the 4 SNP that we studied. With all this evidence, one could speculate that probably most of the 47 SNP described in the OPN gene are in linkage disequilibrium, although, to our knowledge, formal proof is still lacking.

Our results would suggest that the increased levels of OPN protein seen in RA lesions are more a consequence than the cause of the pathology. A scenario can be envisioned where the functional differences in the *OPN* gene existing in the population do not lead to an increase in the OPN protein level prior to disease onset, due to the compensation provided by physiological modulation. Unregulated *OPN* expression only arises when the pathogenic cascade unfolds and consequently the synthesis of OPN is uncontrolled. Therefore, a gene of crucial importance in the symptomatology of the disease would contribute nothing to the susceptibility to RA, and pathological changes in a potential regulator of *OPN* gene expression would lead to the accumulation of OPN protein ultimately observed in patients with RA.

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