Interleukin-12 gene polymorphisim in patients with giant cell arteritis, polymyalgia rheumatica and elderly-onset rheumatoid arthritis

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ABSTRACT

Objective. *The cytokine profile suggests that giant cell arteritis (GCA) is a Th1-driven disease, in which local IFN-γ plays a critical role in the development of a systemic arteritis. IL-12 is a potent inducer of IFN-γ and is critically involved in biasing an immune response towards a Th1 pathway. The purpose of this study was to investigate whether there was an association between an IL-12 gene polymorphism (-1188 A/C 3´UTR) and disease susceptibility for GCA and two other age-related inflammatory conditions, such as polymyalgia rheumatica (PMR) and elderly-onset rheumatoid arthritis (EORA). Furthermore, we attempted to correlate such polymorphism with i*n vitro *IL-12 production.*

Material and methods. *We analyzed genotypes at -1188 in the 3´UTR of the IL-12 promoter by PCR-RFLP in 68 GCA, 138 PMR, and 72 EORA patients as well as in 465 healthy controls (HC). IL-12p70 levels in culture supernatants after stimulation with PMA+Ionomycin was assessed by ELISA.*

Results. *All groups were in Hardy-Weinberg equilibrium. Allelic and genomic distribution was not significantly different among the study groups. None of the genetic variants was associated with disease severity. Although the differences were not statistically significant, HC genotypes were associated with distinct IL-12 p70 production.*

Conclusions. *The IL-12 (-1188 A/C 3´UTR) gene polymorphism is not associated with disease susceptibility or severity in three age-related chronic inflammatory syndromes. The production of IL-12 p70 is dependent on the genetic background in HC, although in patients such association may be biased by other unknown factors.*

Introduction

The aging process is accompanied by qualitative and quantitative changes in the immune system, which are grouped by the term of immunosenescence (1). As a consequence, elderly individuals show an increased susceptibility to neoplasias, infections and autoimmune disorders (2). In this regard, aging is accompanied by the appearance of some age-restricted disorders, and one of the best examples is giant cell arteritis (GCA), a systemic granulomatous vasculitis with a preference for the medium- and large-sized arteries that affects only elderly individuals (3). The clinical manifestations of GCA range from the classic symptoms result of the involvement of the carotid artery branches to aortic arch syndrome or less specific manifestations such as fever, malaise, weight loss, or a polymyalgic syndrome (4-9). Polymyalgia rheumatica (PMR) is a clinical syndrome characterized by pain and stiffness in the neck, shoulder and pelvic girdles that also affect only aged individuals (10). Although PMR usually occurs in the absence of GCA, it is well recognized that some patients may also have or develop clinical arteritis on follow-up (11). On the other hand, it has also been suggested that PMR and elderly onset rheumatoid arthritis (EORA), especially the seronegative forms, have much in common (12).

The profile of T-cell-derived cytokines in GCA suggests that it is a Th1-driven disease, in which local interferon (IFN)-γ plays a critical role in the development of a systemic arteritis (13). Interleukin-12 (IL-12) is a potent inducer of IFN-γ and is believed to be critically involved in biasing an immune response towards a Th1 pathway (14). Although IL-12 mRNA transcripts are expressed in arterial tissue from GCA

patients, they are not correlated with local production of IFN-γ or with specific clinical patterns (15). However, there is no information about the circulating levels of IL-12 or the influence of genetic polymorphisms of this cytokine in GCA. It has been demonstrated that a polymorphism within the IL-12 p40 gene is associated with susceptibility to other Th1 disorders (16, 17) and that this polymorphism also affects protein expression of IL-12 p70 (18), providing a rationale to explore its influence on susceptibility and severity of other Th1 disorders like GCA.

The purpose of this study was to investigate whether there is an association between an IL-12 (-1188 A/C 3´UTR) gene polymorphism and the disease susceptibility for GCA and two other age-related inflammatory conditions, such as PMR and EORA. Furthermore, we attempted to correlate such polymorphism with IL-12 production after *in vitro* stimulation.

Patients and methods

Patients

The present study included 68 patients with GCA, 138 with PMR, 72 with EORA and 465 healthy controls. All patients with GCA fulfilled the 1990 American College of Rheumatology classification criteria for GCA (19). Patients with PMR were diagnosed according to the criteria proposed by Chuang *et al*. (10). Patients with PMR and ESR <40 mm/1 hr but who satisfied other clinical criteria were also included in the study (20). Patients with RA had to satisfy the ACR 1987 revised criteria for RA (21). Patients with RA were considered as elderly-onset if disease symptoms started after the age of 60 (22). As control group, we included 465 healthy unrelated blood donors. Both patients and controls were Caucasians of Spanish ancestry and lived in the same geographic area of north Spain, Cantabria. All the patients and controls gave informed consent, and the study was approved by the regional Ethics Committee.

The clinical findings at diagnosis and during follow-up, the ESR and CRP values at diagnosis, as well as the initial prednisone dosage, were ascertained by reviewing the patients' medical records. Patients were subgrouped according to the presence or absence of PMR and the presence or absence of ischemic complications. Ischemic complications were defined as the presence of visual loss, jaw claudication, cerebrovascular accidents, or/and aortic arch syndrome (4, 7). Relapse was defined as the new appearance of typical clinical symptoms with increased acute-phase reactants. In the case of the new appearance of typical clinical symptoms without increased acute-phase reactants, a relapse was considered if symptoms disappeared with the increase of steroid dose. For the analysis of some variables such as relapses/recurrences, duration of corticosteroid therapy, and accumulated dose of prednisone, only patients with a follow-up ≥2 years were included. In patients with EORA, disease severity was not assessed.

Genotyping

Genomic DNA was extracted from 5ml of whole blood, using a DNA isolation kit (GENTRA, GENERATION® DNA Purification kits; Minneapolis, MN, USA). Haplotype at -1188 in the 3´UTR of the IL-12 promoter was determined as previously described (23). Briefly, DNA samples were amplified using the forward primer 5´-TTCTATCTGATTTGCTTTA´ and the reverse primer 5´-TGAAACATTCCATACATCC-3´. Genomic DNA was amplified, 94ºC for 4 minutes, 49ºC for 1 minute and 72ºC for 2 minutes, then for 35 cycles of 94ºC for 30s, 47ºC for 45s and 72ºC for 1 min, with a final extension at 72ºC for 15 min. The PCR mixture contained 1.25 units of Taq polymerase in PCR buffer 10X (100 mM Tris HCl, 500 mM KCl and 15 mM $MgCl_2$), 2μM of each primer, 200μM dNTP, and 2mM $MgCl_2$. All PCR products were digested with 2 units of the restriction enzyme TaqIα, for 3 hours at 65ºC. The digested products were run out on a 2.5% agarose gel containing ethidium bromide and an appropriate size ladder. Bands were visualized under UV light. Samples showing one 233 bp band were typed as homozygous AA, samples displaying 165 bp and 69 bp bands were typed as homozygous CC, and samples exhibiting 233 bp, 165 bp and 69 bp bands were typed as heterozygous.

Detection of IL-12p70 in culture supernatant

PBMCs from heparinized blood were obtained by Ficoll Histopaque 1077 (Sigma Aldrich; St Louis, MO) gradient centrifugation. PBMCs from 10 patients of each disease group and age-matched controls were cultured in RPMI 1640 and 10% FCS with Phorbol 12-Myristate 13-Acetate (PMA) (100 ng/ml; Sigma-Aldrich) and Ionomycin (4 μg/ml; Calbiochem; Gibbstown, NJ) for 24 hours. Supernatants were obtained and stored at –80ºC until analysis. The determination of IL-12p70 was performed by ELISA (DIACLONE; Besançon, France). The sensitivity of the Elisa Kit for IL-12p70 was 0.75 pg/ml.

Statistical analysis

All the statistical analysis of data was carried out using SPSS 12.0 software (Chicago, IL). The strength of the association between PMR, GCA or EORA and alleles or genotypes of the IL-12 gene was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher exact test analysis. For supernatant studies, the statistical comparisons of data between different pathologies and controls were performed using the Mann-Whitney U-test. Differences were considered significant when *p*-values were <0.05.

Results

Characteristics of study patients

The main demographic, clinical and laboratory characteristics of the study population are shown in Table I. The study comprised 206 patients with PMR/GCA, 72 patients with EORA and 465 healthy controls. Forty-one percent of the GCA patients had also polymyalgic symptoms and forty-seven percent of the GCA patients had ischemic manifestations. In patients with PMR, GCA was excluded because of a negative temporal artery biopsy (TAB) and/or a complete resolution of symptoms with low-dose corticosteroids (10-15 mg/d of prednisone) and the absence of char-

acteristic GCA manifestations. Eighty-five percent of the patients with GCA had signs of arteritis in the TAB (24), and the remaining patients fulfilled the 1990 ACR classification criteria (19). As expected, acute phase reactants were higher in GCA patients compared with PMR and EORA populations.

Seventy-nine percent of the EORA patients had polymyalgic symptoms. Forty-three and twenty-nine percent of the EORA patients were seropositive for rheumatoid factor and anti-cyclic cytrullinated peptide antibodies, respectively.

Association between IL-12 (-1188 A/C) gene polymorphism with disease susceptibility and severity in patients with age-related chronic inflammatory conditions

The distribution of cytokine allele frequencies and genotypes in patients and controls is shown in Table II. The study population was found to be in Hardy-Weinberg equilibrium. The distribution of the alleles and genotypes for this IL-12 gene polymorphism was not significantly different between patients and controls. Furthermore, no significant differences were found between the three disorders.

The severity of PMR and GCA was addressed by analyzing the presence of at least one relapse/recurrence, number of relapses, duration of corticosteroid treatment and cumulative prednisone dose. As patients had different follow-up times and the average of corticosteroid treatment duration in both diseases is around 2 years (5, 10, 25), only patients with at least two years of follow-up were included in this analysis. For GCA patients, the presence of ischemic manifestations at any time was also analyzed. No significant differences in the allele or genotype distribution between GCA patients with or without ischemic complications or between PMR with or without relapses/recurrences were found (data not shown).

Relation between IL-12 gene polymorphism (-1188 A/C) alleles and in vitro production of IL-12 p70

We also analyzed the *in vitro* production of IL-12 p70 according to the IL-12 allele distribution. In agreement with previous studies (17, 26) we found that healthy individuals that carried the C allele had an increased production of IL-12 p70 compared with non-carriers (Fig. 1). Such an influence of the IL-12 haplotype on *in vitro* IL-12 production was observed only in healthy subjects but not in any patient group.

Table I. Demographic and clinical data of patients with giant cell arteritis (GCA), polymyalgia rheumatica (PMR) and elderly onset rheumatoid arthritis (EORA).

	GCA	PMR	EORA
Number	68	138	72
Female (%)	60.3	57.2	63.9
Age at diagnosis (mean±SD, years)	73 ± 7	72.3 ± 7.2	69.1 ± 7
PMR symptoms (%)	41.2	100	78.9
Temporal artery biopsy positive (%)	84.8	0	-
Ischemic manifestations (%)	47.1	0	-
ESR at diagnosis (mean±SD, mm/1° hr)	86.9 ± 31.3	56.7 ± 28	58.5 ± 39
CRP at diagnosis (mean±SD, mg/dl)	9.0 ± 5.7	4.8 ± 4.3	5.8 ± 4.2

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

Table II. Allele and genotype distribution in controls and patients with giant cell arteritis (GCA), polymyalgia rheumatica (PMR) and elderly onset rheumatoid arthritis (EORA).

Gene	Controls (%)	GCA (%)	PMR (%)	EORA (%)
Alleles				
A	764 (82.2)	115 (84.6)	232 (84.1)	117 (81.3)
C	166 (17.8)	21 (15.4)	44 (15.9)	27 (18.7)
Genotypes				
AA	316 (68)	50 (73.5)	102 (73.9)	47 (65.3)
AC	132 (28.3)	15 (22.1)	28 (20.3)	23 (31.9)
CC	17 (3.7)	3 (4.4)	8 (5.8)	2 (2.8)

Discussion

IL-12 is a pro-inflammatory cytokine that modulates the immune response by favouring the generation of Th1 cells (14). As GCA is considered the best example of a Th1 vasculitis, the study of the IL-12 gene may be of relevance in this disorder and other close-related diseases that are the result of imbalance in immune regulation. Only a few studies have addressed the levels of circulating Th1 cytokines (27, 28) or studied the influence of related genes in susceptibility or severity of the disease (29, 30). Therefore, and taking into consideration the lack of previous studies that examined the influence of IL-12 p40 polymorphism in these disorders, we conducted the present study in a large sample of patients with GCA and two other age-restricted conditions.

The present study analyzed the IL-12 (-1188 A/C 3´UTR) polymorphism in GCA, PMR and EORA, and provide no evidence for the association of this polymorphism with disease susceptibility or severity. These results are in agreement with the genotype and allele frequencies for IL-12 p40 polymorphism in previous studies (23, 31). Although in this study we did not observe a significant association between the polymorphism examined and these three disorders, we cannot exclude that other polymorphic variations within this gene or its receptor may denote susceptibility to these conditions (32).

The reasoning behind the proposed involvement of cytokine gene polymorphisms in susceptibility or disease severity is that they may influence *in vivo* cytokine levels (33). Therefore, it is reasonable to study the relationship between the genotype and the phenotype for a given polymorphism. These differences are in general difficult to detect *in vivo*, and may be more apparent after *in vitro* culture with different stimuli (33). For this reason, we studied *in vitro* levels of IL-12 p70 in relation to this polymorphism. In agree-

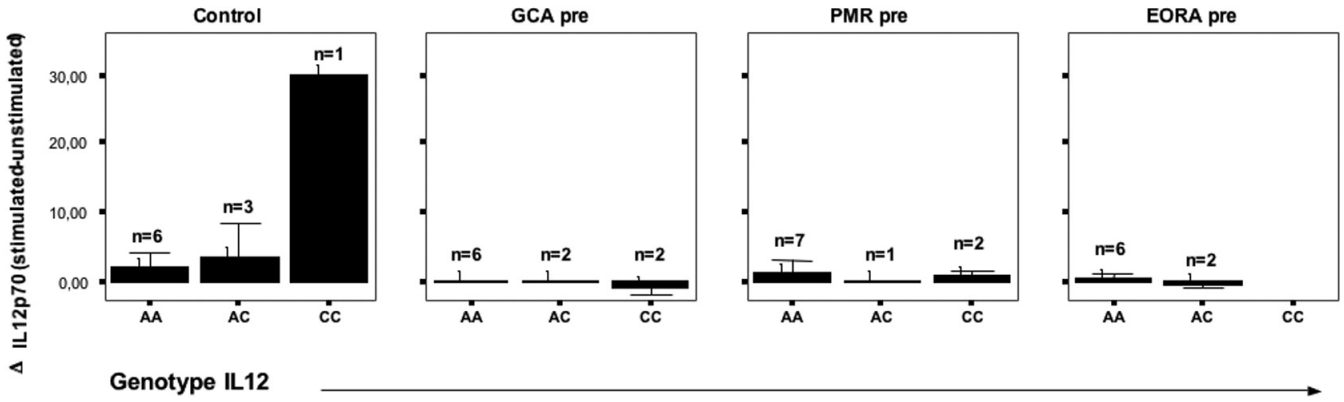

Fig. 1. Detection of IL-12p70 in culture supernatant.
PBMCs from 10 patients of each disease group and controls were stimulated in culture with PMA and Ionomycin for 24 hours. The determination of IL-12p70 in supernatants was performed by ELISA.

ment with previous studies (18, 26) we found that healthy individuals with C allele had an increased production of IL-12 p70 compared with non-carriers. By contrast, no clear association was found in any of the inflammatory conditions.

Although initial studies suggested a possible association of the IL-12 (-1188 A/C 3´UTR) polymorphism with type 1 diabetes (17) and multiple sclerosis (16), these findings were not confirmed in other populations (31) and also no significant association was found with other Th1 autoimmune diseases such as RA (31, 34) or Crohn's disease (18). However, this polymorphism was associated to susceptibility to Takayasu's arteritis (35). Takayasu's arteritis is also a large-vessel granulomatous vasculitis that, in contrast with GCA, mainly affects young individuals (36). There are a number of studies addressing circulating levels of IL-12 in Takayasu's arteritis but not in GCA. Whereas some authors have found increased circulating levels of IL-12 and a relationship with disease activity (37), these findings have not been confirmed by others (38).

In summary, the IL-12 (-1188 A/C 3´UTR) gene polymorphism was not associated with disease susceptibility or severity in three age-related chronic inflammatory syndromes. In agreement with previous reports, the production of circulating IL-12 is dependent of the genetic background in healthy subjects, although in patients such association is biased by other unknown factors.

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