Interleukin-6 gene -174 promoter polymorphism is associated with endothelial dysfunction but not with disease susceptibility in patients with rheumatoid arthritis

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Abstract

Objective

To determine whether the interleukin (IL)6 *-174 gene polymorphism may influence the development of subclinical atherosclerosis manifested by the presence of endothelial dysfunction in RA patients.*

Patients and methods

311 patients (228 [73.3%] women; 243 [78.1%] rheumatoid factor positive) who fulfilled the 1987 ACR classification criteria for RA seen at the Rheumatology outpatient clinic of Hospital Xeral-Calde, Lugo between March 1996 and December 2006 and 226 matched controls were included in this study. Between March and December 2007, a subgroup of 98 patients randomly selected was assessed for the presence of endothelial dysfunction. Patients and controls were genotyped for a single biallelic (G/C) nucleotide polymorphism (rs1800795) in the promoter region at the position -174 of the IL6 *gene using a TaqMan 5´ allele discrimination assay.*

Results

No significant differences in the IL6 *-174 allele or genotype frequency between RA patients and controls were found. However, RA patients homozygous for the* IL6 *-174 GG genotype had more severe endothelial dysfunction (flow-mediated endothelium-dependent vasodilatation-FMD%: 4.2±6.6) than those carrying the* IL6 *-174 GC (FMD%: 6.3±8.1) or* IL6 *-174 CC (FMD%: 6.0±3.3) genotypes. In this regard, significant differences were observed when FMD% values in RA patients carrying the* IL6 *-174 GG genotype were compared with that observed in those carrying the* IL6 *-174 GC and the* IL6 *-174 CC genotypes (FMD%: 6.3±4.6) (p=0.02).*

Conclusions

Our results support a role of IL6 *-174 gene polymorphism in the development of subclinical atherosclerosis in patients with RA.*

Key words

Rheumatoid arthritis, atherosclerosis, cardiovascular disease, gene polymorphism, cytokine, *IL-6*.

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Introduction

Rheumatoid arthritis (RA) is the most common inflammatory disease involving the joints. Proinflammatory cytokines play key roles in driving the inflammation and synovial cell proliferation that characterise joint destruction in patients with RA. In this chronic disease there is an imbalance in the production of cytokines with a relative excess of proinflammatory molecules including interleukin (IL)-1, IL-6 and tumour necrosis factor compared with antiinflammatory mediators such as IL-10 (1). IL-6 is expressed at high levels in RA. This pivotal cytokine is a key mediator of systemic and local inflammation in RA (2). Moreover, IL-6 is directly implicated in the production of serum C-reactive protein (CRP) and a significant correlation between CRP levels at the time of disease diagnosis and the presence of endothelial dysfunction was found in patients with psoriatic arthritis, another chronic inflammatory disease involving the joints (3). CRP was reported to be a predictor of all-cause mortality, and specifically of cardiovascular (CV) mortality, in patients with inflammatory polyarthritis in a 10-year period following the onset of the rheumatic disease (4). Moreover, high grade inflammation is also directly implicated in the accelerated atherogenesis observed in RA (5) and an association between the magnitude and chronicity of the inflammatory response measured by CRP levels and the presence of subclinical atherosclerosis (6) or the development of CV events was found in patients with RA (7).

A functional polymorphism in the 5´flanking region of the *IL6* gene has proved to determine differences in the control of IL-6 expression (8). With respect to this, a single nucleotide change from G to C at position -174 of the *IL6* gene affects the gene transcription rate and is associated with different plasma levels of IL-6 (8). Interestingly, IL-6 plasma levels in individuals carrying the *IL6* GG genotype were found to be twice as high as those found in individuals homozygous for the C allele (8).

RA is a polygenic disease and a number of genes have been implicated in the susceptibility to and severity of the disease (9). In addition to chronic inflammation and traditional CV risk factors (5, 10), the genetic susceptibility plays a pivotal role in the development of accelerated atherosclerosis observed in RA (7). Genes within the major histocompatibility complex, in particular some HLA-DRB1 alleles encoding a common sequence of amino acids corresponding to residues 67-74 within the HLA-DRβ1 chain, called shared epitope (SE) alleles, are associated with disease susceptibility (11) and severity (12), including endothelial dysfunction (13), which is an early step in the atherogenesis process found in patients with RA (14). Also, an additive effect of *NOS2A* and *NOS3* gene polymorphisms and HLA-DRB1*04-SE positive alleles on the increased risk of CV events observed in patients with RA has recently been reported (15).

Several studies have been conducted to determine the potential influence of functional polymorphisms in the *IL6* gene in the susceptibility and severity of RA (16-20). In assessing the functional *IL6* -174 gene polymorphism some data support a potential role of this biallelic polymorphisms in RA severity. With respect to this, RA patients carrying the GG genotype were found to have more active disease than those carrying CC and GC genotypes (18). Also, an increased risk of having more severe radiographic damage was observed in rheumatoid factor positive RA patients carrying the *IL6* -174 G allele that was also associated with high IL-6 production (20).

Endothelial dysfunction is characteristic of most conditions associated with atherosclerosis and thus is considered an early feature in atherogenesis (21, 22). The endothelium modulates vascular tone by releasing a number of vasoactive substances including nitric oxide in response to mechanical stress (21). Endothelial dysfunction encompasses an imbalance between vasodilating and vasconstricting substances that are produced by endothelial cells or that act on these cells (22). Endothelial dysfunction may be defined as an impaired ability of the artery to dilate in response to physical and chemical stimuli due to a decreased release or increased breakdown of nitric

oxide (21, 23). Endothelial function can be no invasively evaluated by postocclusion flow-mediated vasodilatation (FMD%) of the brachial artery using high-sensitivity brachial ultrasonography (22). However, to the best of our knowledge, studies aimed to establish the potential influence of functional *IL-6* gene polymorphisms in the development of endothelial dysfunction of patients with RA have not been reported.

Taking all these considerations into account, in the present study we assessed whether the *IL6* -174 gene polymorphism might play a role in the increased risk of subclinical atherosclerosis manifested by the presence of endothelial dysfunction in patients with RA.

Methods

Patients and controls

Three-hundred and eleven patients (228 [73.3%] women and 83 [26.7%] men; 243 [78.1%] rheumatoid factor positive) who fulfilled the 1987 American College of Rheumatology classification criteria for RA (24), seen at the Rheumatology outpatient clinic of Hospital Xeral-Calde, Lugo (northwestern Spain) between March 1996 and December 2006 and 226 controls, matched by age, sex and ethnicity, from the same region, were assessed for differences in the *IL6* -174 gene polymorphism. Information on the characteristics of this Caucasian population has previously been reported (25). The mean age±standard deviation (SD) of RA patients at the time of disease diagnosis was 53.0±14.7 years; median 55 years (interquartile range-IQ: 43–64). The mean ± SD disease duration from the onset of the disease until patient's death or until December 2007 was 15.1±9.6 years; median 15 years (IQ range: 8–20). Clinical information on 182 RA patients from this cohort recruited in 1996 and definitions of traditional CV risk factors were also established as recently described (7).

The subject's written consent was obtained and the design of the work was approved by the Ethics Committee of Galicia (Spain).

Brachial artery reactivity study

As discussed above, endothelial dysfunction plays a key role in early atherosclerosis and also contributes to the development of clinical features in the later stages of the vascular disease (26). Since endothelial function in the brachial circulation correlates with that observed in the coronary circulation, vascular ultrasound examination of the brachial artery is now considered a safe noninvasive technique to examine flow-mediated endothelium-dependent vasodilatation (FMD) (26).

To determine the potential association between the *IL6* -174 gene polymorphism and the presence of subclinical atherosclerosis, between March and December 2007, a subgroup of patients randomly selected was assessed for the presence of endothelial dysfunction.

Endothelium-dependent-FMD (post-ischemia) and endothelium independent-NTG (post-nitroglycerin) vasodilatation were measured in 98 patients with RA from this series by brachial ultrasonography as previously reported (3, 13, 27). Since we have observed a rapid but transient effect of the anti-TNF-alpha monoclonal antibody-infliximab that lasted 4 after the infusion of this drug in patients with RA (27), the assessment of the endothelial function was performed in the 14 of the 98 patients undergoing TNF-α blocker therapy (12 of them with infliximab and 2 with adalimumab) 24-48 hours before the administration of the anti-TNF-α blocker. Moreover, subjects were asked to refrain from beverages other than water (especially no caffeine or alcohol), smoking, medication, except acetaminophen if necessary and meals from midnight on the testing day.

B-mode scan of the right brachial artery, in a longitudinal section 2 to 12 cm proximal to the antecubital fossa, was performed in supine subjects using a 7.5-MHz phased-array transducer on a Hewlett Packard SONOS 5500 system (Palo Alto, California). The anterior and posterior media-intima interfaces were used to define the baseline artery diameter, calculated as the average of measurements made during four cardiac cycles at end diastole. The forearm blood pressure cuff was inflated on the ipsilateral wrist to 50 mm Hg above resting systolic blood pressure for 5 minutes, and then released. FMD (an increase in brachial artery diameter) was measured 30 to 60 seconds after cuff release. The value of FMD was calculated as the difference between the maximum diameter post-occlusion and the baseline diameter and it is expressed as percentage (FMD%). Normal values of FMD% vary from a laboratory to another. In general, values of FMD% lower than 5-7% are considered abnormal. In the laboratory of echocardiography of our center adults with FMD% values less than 7% are considered to have endothelial dysfunction (28).

To assess NTG%-endothelium-independent vasodilatation, we used 400 μg of sublingual NTG, which acts directly on vessel smooth muscle to cause vasodilatation. NTG was measured 4 minutes after nitroglycerin intake. In all cases a cardiologist (CG-J) analysed all the ultrasound data offline and he was blind to the clinical information. Based on 32 controls the intra-observer variability showed the following coefficients of variation: FMD (1.3%); NTG (1.9%).

Genotyping

DNA from patients and controls was obtained from peripheral blood, using standard methods. Samples were genotyped for *IL6* rs1800795 variants using a TaqMan 5´ -allele discrimination assay (Applied Biosystems, Foster City, CA, USA). Allele-specific probes were labeled with the fluorescent dyes VIC and FAM, respectively. PCR reaction was carried out in a total reaction volume of 4 μl with the following amplification protocol: denaturation at 95ºC for 10 min, followed by 40 cycles of denaturation at 92ºC for 15 sec and finished with annealing and extension at 60ºC for 1 min. Post-PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI PRIM 7900 Sequence Detection Systems using the SDS 2.3 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA). Duplicate samples and negative controls were included to ensure accuracy of genotyping.

Statistical analysis

Strength of disease association between RA and alleles or genotypes of the IL6

-174 gene polymorphism was estimated using odds ratios and 95% confidence intervals. Levels of significance were determined using contingency tables by Chi-square analysis. The association between genotypes of the IL6 -174 gene polymorphism and FMD%-endothelium dependent vasodilatation, NTG%-endothelium independent vasodilatation was tested using analysis of covariance (ancova) adjusting by gender, age and duration of the disease at the time of the ultrasonographic studies (continuous), and traditional CV risk factors and rheumatoid factor status (presence/absence). CV risk factors were summarised into one variable using the table for countries like Spain with low risk of the Systematic Coronary Risk Evaluation (SCORE) (29). Moreover, corrections were also performed. Statistical significance was defined as $p \leq 0.05$. Calculations were performed using the statistical package Stata 10/SE.

Results

Allele and genotype frequencies of the IL6-174 polymorphism at the promoter region in RA patients and controls

Genotype frequencies were in Hardy-Weinberg equilibrium in patients and controls. Allele and genotype frequencies for the *IL6* -174 gene polymorphism in RA patients and controls were similar in patients and controls (Table I). Therefore, no significant differences in the allele or genotype frequency between RA patients and controls were found ($p>0.05$ for all comparisons). Also, in RA no significant differences in the age at the onset of the disease, age at the time of disease diagnosis or disease duration were observed according to the different *IL6* -174 genotypes (data not shown).

IL 6-174 gene polymorphism and endothelial function

Endothelial function was assessed in 98 RA patients that were stratified according to the *IL6* -174 genotype. The mean value of FMD% in this series of RA patients was lower than 7% (Table II). This result confirms the previously reported presence of endothelial dysfunction in long-standing RA patients from Northwest Spain (13).

Mean values of FMD%- endothelium dependent and NTG%-endothelium independent vasodilatation stratified according to the *IL6* -174 genotypes are shown in Table II.

RA patients homozygous for the *IL6* -174 GG genotype were found to experience more severe endothelial dysfunction (FMD%: 4.2±6.6) than those carrying the *IL6* -174 GC (FMD%: 6.3±8.1) or *IL6* -174 CC (FMD%: 6.0±3.3) genotypes. In this regard, when endothelial function in RA patients carrying the *IL6* -174 GG genotype (FMD%: 4.2±3.3) was compared with that observed in RA patients carrying the *IL6* -174GC plus the *IL6* -174 CC genotype (FMD%: 6.3± 4.6) significant differences were observed ($p=0.02$) (Fig. 1).

Furthermore, as shown in Table II, the *IL6* -174 gene polymorphism was also associated with NTG%-endothelium-independent vasodilatation. In this regard, RA patients carrying the *IL6* -174 GG genotype had lower NTG% (13.9±5.8) as compared to the remaining patients carrying the *IL6* -174GC plus the *IL6* -174 CC genotype (NTG%: 16.8±6.6) ($p=0.04$).

Table I. Allele and genotype frequencies of the *IL6* –174 polymorphism at the promoter region in RA patients and controls*.

	RA patients	Controls
N. of individuals	311 (%)	226 (%)
Genotype		
GG	127 (41)	103 (45)
GC	144 (46)	94 (42)
CC	40 (13)	29 (13)
Allele (2N)	622 (%)	452 (%)
G	398 (64)	300 (66)
C	224 (36)	152 (33)

*No statistically significant differences between RA patients and controls were found.

Table II. Association between *IL6* -174 *GG* genotype and endothelial dysfunction in RA patients.

	n. of patients	FMD%	*p*-value	NTG %	*p*-value
Genotype					
GG	40	4.2 ± 6.6	Reference	13.9 ± 5.8	Reference
GC	47	6.3 ± 8.1	0.07	16.8 ± 7.3	0.03
CC	11	6.0 ± 3.3	0.05	16.6 ± 5.4	0.14
GC+CC	58	6.3 ± 4.6	0.02	16.8 ± 6.6	0.04

Discussion

Inflammation is known to play a key role in the development of CV disease in the general population and in the accelerated atherogenesis observed in RA. However, in patients with RA there is limited information aimed to establish the association between genes implicated in the development of inflammatory response and the presence of subclinical atherosclerosis determined by functional tests such as those assessing the presence of endothelial dysfunction.

IL-6 is a proinflammatory cytokine implicated in the pathogenesis of different rheumatic diseases (30, 31). IL-6 also acts as an important mediator of systemic and local manifestations of RA (2). Circulating IL-6 concentrations are associated with endothelial activation in RA. Interestingly, Dessein *et al*. disclosed a relationship between circulating IL-6 concentrations and endothelial dysfunction as assessed by serum adhesion molecule concentrations, and the amelioration of IL-6 related endothelial dysfunction upon disease activity suppression in RA (32, 33). Moreover, the successful therapeutic use of inhibitors of this cytokine underlines the importance of this molecule in driving RA inflammation and tissue damage (34).

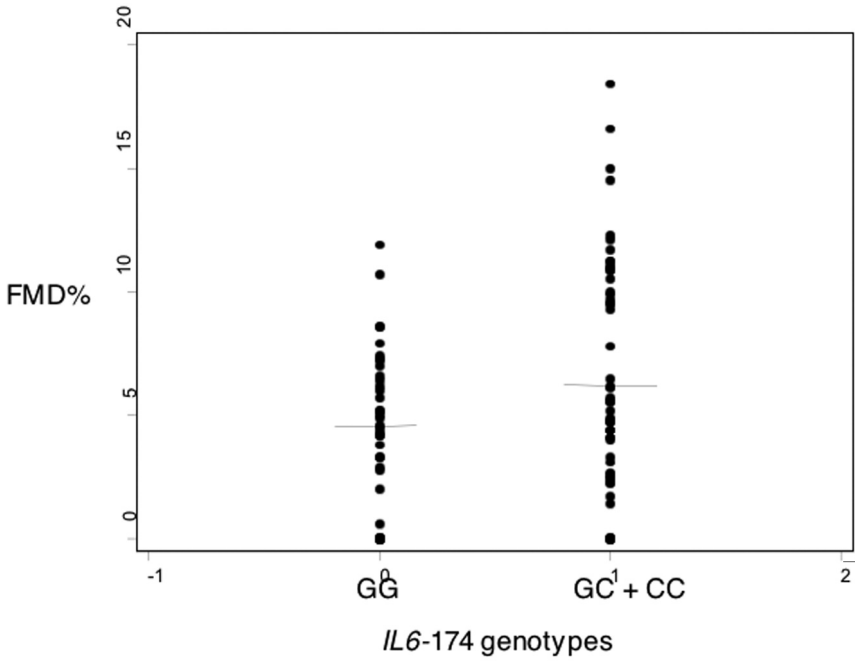

Fig. 1. Study of flow-mediated vasodilatation (FMD%) in RA patients according to the *IL6*-174 genotype. Individual and mean values (horizontal lines) expressed as FMD% are shown.

Due to this, in the present study we investigated for first time the potential implication of the functional biallelic *IL6* -174 gene polymorphism in the development of the endothelial dysfunction that is present in RA patients with severe disease (13).

In keeping with former studies that did not find an influence of the *IL6* -174 gene polymorphism in the susceptibility to different diseases associated with high inflammatory response such as giant cell arteritis (35, 36) or Henoch-Schönlein purpura (37), in the present study we did not observe an association of this biallelic polymorphism with the susceptibility to RA in patients from northwestern Spain. However, our data support an association of the *IL6* -174 gene polymorphism with subclinical atherosclerosis manifested by severe endothelial dysfunction in *IL6* -174G homozygous patients with RA. In this regard, individuals carrying the *IL6* -174 GG genotype had significantly lower values of FMD% and NTG% than the remaining RA patients assessed in our study.

The G>C at -174 (rs1800795) is a common SNP in Caucasians and according to our results *IL6* -174G allele is a risk factor for subclinical atherosclerosis in patients with RA. The *IL6* -174GG genotype was found to promote peripheral artery disease development among individuals with type 2 diabetes by inducing increased release of IL-6 (38). This was a consequence of the higher gene expression that had previously been associated with the *IL6*-174G allele (8) as higher concentrations of IL-6 among patients with type 2 diabetes with the *IL6*-174 GG genotype were associated with increased plasma concentrations of fibrinogen and CRP (38). Moreover, in accordance with our results, in a study aimed to determine whether the *IL-6-174* polymorphism might be linked to the number of severely occluded coronary arteries in patients with advanced coronary heart disease, Myśliwska *et al*. found significantly more patients with triple vessel disease within the -174GG group as compared to the -174GC and CC genotype carriers (39). In addition, the highest IL-6 serum levels were also found in the group of -174GG and the lowest in the -174CC genotype patients (39). These investigators also found significantly higher spontaneous in vitro IL-6 secretion in the -174GG as compared to the GC and CC genotype carriers (39). Also, in assessing consecutive patients with history of ischemic stroke, Flex *et al*. reported that the *IL6*-174 GG genotype was significantly increased in cases with strokes compared to controls (40). The potential influence of the *IL6*-174 G allele was also supported by Balding *et al*. who found an increased frequency of this allele in stroke patients without hypertension compared to stroke patients with hypertension and controls (41). However, other studies have found no differences in the *IL6* genotype or allele frequencies between cases and controls (42). Moreover, unlike previous reports, Berg *et al*. found an association of the *IL6*-174 CC genotype with significant angiographic coronary artery disease (43). More recently, as described in our present study, Panoulas *et al*. did not observe significant differences in the allele or genotype frequencies of the *IL6* -174 *G/C* gene polymorphism between UK patients with RA and controls (44). These authors did not observe differences in RA activity/severity or in CV disease risk factors between *IL6-174* genotypes (44). However, *IL6-* 174 C-allele carrier status was found to be independently associated with the presence of CV disease in this series of UK patients with RA (44).

Taken together all these observations, a plausible explanation for these contradictory results may be the expression of the complex physiology of the IL-6. RA appears to be a polygenic disease and different genes may influence its phenotype and outcome (9, 12). As a result, it is possible that environmental factors, probably infectious agents, may upregulate gene expression, which may be different according to the specific genetic background of the population, leading to the development and different grade of the severity of the disease.

In conclusion, our results support, for first time, a role of *IL6*-174 gene polymorphism in the development of subclinical atherosclerosis in patients with RA. However, further studies in individuals with different genetic backgrounds are warranted to confirm the potential role of this *IL6* gene polymorphism as well as the influence of the genetic polymorphisms of other inflammatory factors implicated in the development of the accelerated atherosclerosis and the increased incidence of CV events observed in RA.

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