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Plasma Soluble IL-6 Receptor Concentration in Rheumatoid Arthritis: Associations with the rs8192284 *IL6R* Polymorphism and with Disease Activity

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ABSTRACT

Background

Soluble interleukin 6 receptor α subunit (sIL-6R) is primarily generated by shedding of the membrane-bound form. This process is influenced by the single nucleotide polymorphism rs8192284 (A>C) resulting in an aspartic acid to alanine substitution (D358A) at the proteolytic cleavage site. The aim of this study was to determine whether plasma levels of sIL6R are influenced by the rs8192284 polymorphism in rheumatoid arthritis patients and to assess the association between plasma sIL-6R levels and disease activity as reflected by anti-CCP status.

Material and Methods

Thirty-nine patients were randomly selected from a cohort of RA patients of Spanish descent. Plasma sIL-6R concentrations were measured using sandwich ELISA. Genotyping of the rs8192284 (A>C) polymorphism was done using a Fast Real-Time PCR System. DAS 28 scores were used to assess disease activity.

Results

Plasma sIL-6R levels were positively associated with the number of C alleles (AA: 35.27 (3.50) ng/ml, AC: 45.50 (4.58) ng/ml, CC: 52.55 (3.18) ng/ml, $p = 0.0001$). DAS28 and plasma sIL-6R levels were positively associated in the anti-CCP positive subgroup ($r^2 = 0.45$, $p = 0.0336$) and negatively associated in the anti-CCP negative subgroup ($r^2 = - 0.45$, $p = 0.0825$). No association between anti-CCP status and sIL-6R level was found.

Discussion

Our findings show that the rs8192284 polymorphism is operative in RA patients. The presence of anti-CCP antibodies determines the relationship between sIL-6R concentration and disease activity.

Introduction

Rheumatoid arthritis (RA) is a systemic immune-mediated disease characterized by chronic inflammation and cellular proliferation of the synovial lining of joints. It has been previously demonstrated that the balance between pro- and anti-inflammatory cytokines plays an essential role in the progression, severity, and morbidity of this condition. Cytokines are critical mediators of synovial cell activation, which leads to joint destruction [1].

Interleukin 6 (IL-6) is a cytokine overproduced in RA, both in serum [2-4] and in the synovial fluid [5]. Although its exact role is unclear, it has been reported to have both pro- [6-8] and anti-inflammatory properties *in vivo* and *in vitro* [9,10].

The IL-6 receptor is formed by two different membrane glycoproteins: IL-6Rα, an 80-kDa type I protein referred to as the ligand-binding subunit, and IL-6Rβ, a 130-kDa (gp130) protein referred to as the signal-transducing subunit [9]. IL-6 and IL-6Rα form a low-affinity complex that must bind with gp130 to form a high affinity complex that can transduce signals to the cytoplasm.

IL-6Rα can be released as a soluble receptor (sIL-6R), bind to IL-6 and then interact with a gp130 moiety located on the surface of any cell [11,12]. This process, referred to as trans-signaling, may be the most prominent mechanism of IL-6 signaling in the human body [12] because the sIL-6R/IL-6 complex can exert an agonist effect on cell types expressing gp130.

The main mechanisms of generating sIL-6R are proteolytic cleavage (PC) of the ectodomain or shedding of the membrane-bound form [11,13,14]. A single nucleotide polymorphism (rs8192284 A>C) located at the proteolytic cleavage site (Gln357/Asp358) [14] has been described that results in an amino acid substitution from aspartic acid to alanine (D358A) [15]. This sequence variation

has a marked effect on the shedding of IL-6R [16], with individuals carrying the minor C allele shedding more membrane-bound receptors than non-C allele carriers.

The aim of this study was to assess the association between the *IL6R* rs8192284 polymorphism and plasma sIL-6R levels in RA patients. We also assessed the correlation between sIL-6R plasma levels and disease activity.

Material and Methods

Patients and blood samples

Thirty-nine RA patients of Spanish descent were randomly selected from the Rheumatology Outpatient Clinic of Hospital Clinico San Carlos (Madrid, Spain). RA diagnoses were established using the 1987 ACR criteria [17]. Written informed consent was obtained from all patients, and the study was approved by the hospital's ethics committee.

Blood samples were obtained after 8–12 h of fasting. The samples were centrifuged at 3,000 rpm for 10 min to obtain plasma that was then aliquoted and stored at -70°C until the day of analysis.

Socio-demographic and clinical data, including age, gender, and anti-citrullinated cyclic peptide antibody (anti-CCP) status (Euro-Diagnostica, Malmö, Sweden, Immunoscan CCPlus®, considered positive if least one ELISA determination yielded a level >25 IU) were obtained from OBDAR (Prospective Rheumatoid Arthritis Clinical Data of Rheumatology Department, Hospital Clinico San Carlos). The median age at the time of plasma extraction was 66.05 years (inter-quartile rank (IQR) 58.05 - 74.87). Thirty-five (89.74%) patients were female and 22 (56.41%) were anti-CCP positive. The tests performed included a 28-joint count for tender and swollen joints, a VAS for the patient's global status, and determination of erythrocyte sedimentation rate (ESR). The tests were performed the same day as the blood extraction, and disease activity scores using 28-joint counts (DAS28) were calculated as previously described [18]. The mean DAS28 was 4.15 (standard deviation (SD) 1.41).

All subjects were genotyped to determine their *IL-6R* rs8192284 polymorphism status (see below). The genotype distribution was 14 AA, 15 AC, and 10 CC.

Enzyme-linked immunosorbent assay (ELISA) to evaluate plasma sIL-6R levels

A home-made ELISA kit was developed using high binding 96-well plates that had been coated overnight at 4ºC with a monoclonal anti-IL-6R antibody (R&D Minneapolis, MN, USA), #MAB207, 50 µl/well, diluted in PBS). After each well was washed twice with 200 µl/well of PBS-T (PBS 0.05% Tween 20), 50-µl plasma samples that had been diluted 1:200 in PBS with 1% BSA were plated in duplicate. Samples were incubated for two hours at room temperature. Samples were washed five times with PBS-T with 1% BSA. Biotinylated polyclonal antibodies against IL-6R (R&D #BAF227) were used as detection antibodies (50 µl/well, diluted in PBS with 1% BSA at 0.3 µg/ml) and the samples were incubated with these antibodies for two hours at room temperature. The samples were then washed with PBS-T with 1% BSA. Then, 50 µl/well of streptavidin-HRP was added to each well and the samples were incubated for 30 minutes. The samples were then washed five times and developed using TMB (eBioscience, San Diego, CA, USA). After 15 minutes at room temperature, the color reaction was stopped using 1 M H_2SO_4.

Absorbance was measured using a plate reader (Labsystems iEMS Reader MF, Helsinki, Finland) at 450 nm and corrected at 550 nm.

Genotyping

Subjects were genotyped to determine their *IL6R* rs8192284 A>C polymorphism status using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analyzed using the ABI

7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA)

Statistical analysis

The association between plasma sIL-6R levels and the *IL6R* rs8192284 polymorphism was assessed using a Kruskal-Wallis test. The association between both gender and anti-CCP status and plasma sIL-6R levels were assessed using a Mann-Whitney test. The association between DAS28 and sIL-6R plasma levels was assessed using the Spearman correlation. We decided to perform a sub-analysis of the association between DAS28 and plasma sIL-6R levels according to anti-CCP status. Statistical analyses were performed using Stata v.9 software.

Results

Effect of the *IL6R* rs8192284 polymorphism on plasma sIL-6R levels

The mean plasma sIL-6R level of the RA patients was 43.63 ng/ml (SD 7.90). Plasma sIL-6R concentrations according to *IL6R* rs8192284 polymorphism genotype were AA 35.27 (SD 3.50), AC 45.50 (SD 4.58) and CC 52.55 ng/ml (SD 3.18; p = 0.0001) (Figure 1). These results indicate an influence of the rs8192284 polymorphism on sIL-6R plasma levels in RA patients, which is similar to that previously observed in healthy subjects. We also observed a significantly different sIL-6R plasma concentration between the AA and AC genotypes ($p < 0.0001$) and between the AC and CC genotypes (p = 0.0011). No significant difference in plasma levels was found due to gender (females 44.07 (SD 8.17) vs. males 39.85 (SD 3.50), *p* = 0.29) or anti-CCP status (anti-CCP positive 42.56 (SD 8.47) vs. anti-CCP negative 45.02 (SD 7.10), p = 0.34).

Influence of sIL-6R plasma level on disease activity

We analyzed the influence of sIL-6R plasma levels on disease activity, which was measured as DAS28. We found no significant correlation between the two variables (r^2 = 0.03, p = 0.85). However, when we assessed this correlation separately in the anti-CCP positive and anti-CCP negative patients, we observed a significant positive correlation between disease activity and sIL-6R plasma levels in the anti-CCP positive patients (r^2 = 0.45, p = 0.034) and a negative trend in the anti-CCP negative patients (r^2 = - 0.45, p = 0.083) (Figure 2).

Discussion

We observed that the presence or absence of anti-CCP antibodies determines the influence that sIL-6R plasma levels have on RA disease activity. Many authors have previously investigated the association between sIL-6R levels and disease activity, but this is the first study that has used the DAS28 score to estimate disease activity and the first to address the influence of anti-CCP status. We decided to divide our sample according to the presence or absence of anti-CCP owing to evidence gathered in recent years that suggested that this auto-antibody could define two different conditions [19-21].

In previous studies, many variables have been used as measures of disease activity with differing results: while some studies failed to find associations between serum sIL-6R levels and ESR, CRP [5,22,23], a count of tender/swollen joints, or Ritchie's index [23], others were able to find a positive association between these variables and disease activity (measured as a four level scale) [3,4] or Ritchie's index [4]. None of the studies characterized the anti-CCP status of the sample. We found that the presence or absence of these auto-antibodies was essential for determining the association between sIL-6R plasma level and disease activity. Therefore, varying composition of the RA samples included in each study may explain their apparently conflicting results.

As reported by Galicia et al. [16] and Marinou et al. [24], the *IL6R* rs8192284 polymorphism influences the serum level of sIL-6R in healthy individuals, with more copies of the minor C allele causing higher concentrations. In our sample of RA patients, this polymorphism showed a similar influence on plasma sIL-6R levels. We assessed the influence of gender and anti-CCP status on plasma sIL-6R concentration, but none of these factors showed a significant influence.

Many authors [19-21] consider RA with anti-CCP antibodies as a different condition and/or disease than RA without anti-CCP. The fact that the association of sIL-6R concentration with DAS28 depended on anti-CCP status could be a reflection of different pathophysiological processes underlying the presence or absence of this autoantibody. Moreover, as previously mentioned, IL-6 is a pleiotropic cytokine. We hypothesize that the balance between the pro- and anti-inflammatory effects of IL-6 may be tilted towards the pro-inflammatory side in anti-CCP-positive patients and tilted towards the anti-inflammatory side in anti-CCP-negative patients.

Tocilizumab [25] is a novel biological therapy used in the treatment of RA that is composed of antibodies directed against IL-6Rα. Because disease activity only correlated with sIL-6R plasma level in anti-CCP positive subjects, we speculate that this subset of RA patients should show a better response to this treatment. A replication of these observations is needed.

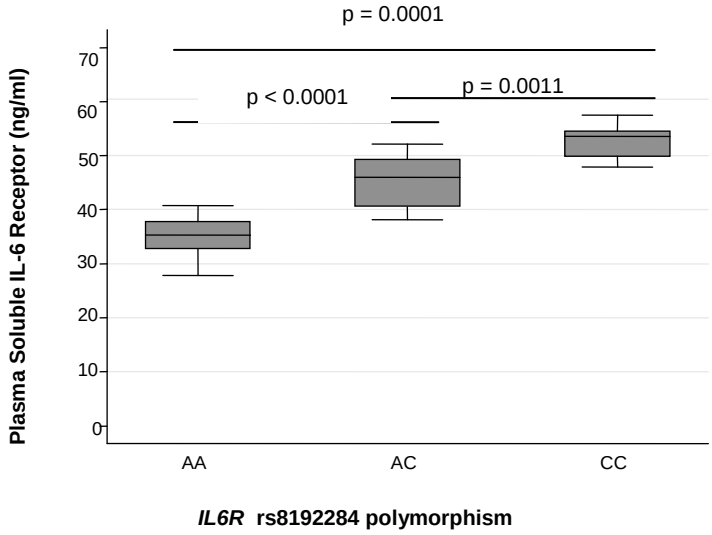

Figure 1. sIL-6R plasma concentration according to the genotype of the *IL-6R* rs8192284 polymorphism. We found significantly different plasma sIL-6R concentrations among the three genotypes ($p = 0.0001$), between the AA and AC genotypes ($p < 0.0001$) and between the AC and CC genotypes ($p = 0.0011$).

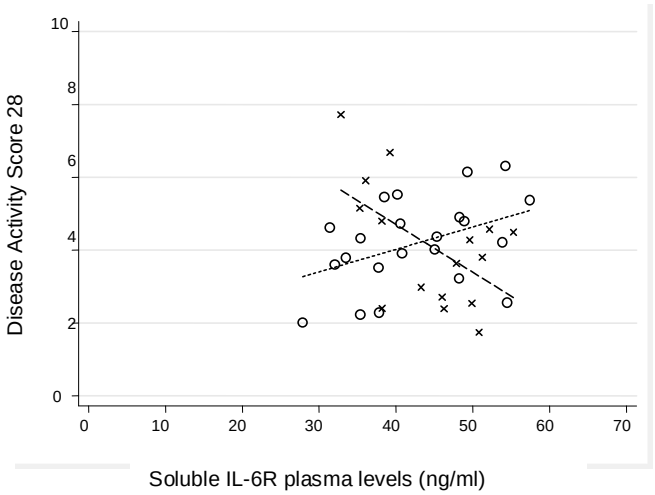

Figure 2. Correlation between disease activity score (DAS28) and sIL-6R plasma concentration with respect to anti-CCP status. Hollow circles represent the anti-CCP positive patients and Xes the anti-CCP negative patients. The dotted line indicates the correlation between DAS28 and sIL-6R levels in the anti-CCP positive subjects (r^2 = 0.45, *p* = 0.034). The dashed line indicates the correlation between DAS28 and sIL-6R level in the anti-CCP negative subjects (r^2 = - 0.45, *p* = 0.083).

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