sE-Selectin Expression and A561C Polymorphism in Relation to Rheumatoid Arthritis Clinical Activity

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ABSTRACT. ***Objective.*** To investigate the relationship of A561C polymorphism and sE-selectin levels with rheumatoid arthritis (RA) clinical activity.

Methods. In a case-control study, we compared 60 patients with RA and 60 healthy subjects. Patients fulfilled the 1987 American College of Rheumatology criteria. Soluble E-selectin levels were measured from serum samples using the ELISA kit. We investigated E-selectin A561C polymorphism by the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) technique. The disease activity was recorded with Spanish Health Assessment Questionnaire Disability Index (HAQ-DI), Spanish Arthritis Impact Measurement Scales (AIMS), and Disease Activity Score (DAS28) scores. A p value < 0.05 was considered significant.

Results. Patients with RA showed higher sE-selectin levels than controls (mean 91.7 vs 39 ng/ml; p = 0.002). A positive correlation between sE-selectin and rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), Spanish HAQ-DI, and DAS28 scores was found. The E-selectin polymorphism analysis showed diminished frequency in RA of heterozygous A/C genotype and increased frequency of homozygous wild-type A/A genotype (p = 0.043, OR 1.45; 95% CI 1.125-16.167) versus A/C and A/A genotype in healthy subjects. No significant association between A561C polymorphism and clinical activity was present.

Conclusion. The sE-selectin, RF, and ESR, in addition to clinical indices, were associated with clinical activity in RA. We highlighted the presence of A/A genotype A561C polymorphism in our patients with RA. (J Rheumatol 2006;33:1968–72)

Key Indexing Terms:
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Rheumatoid arthritis (RA) is a chronic autoimmune disease with unknown etiology where the synovial membrane is the main target of inflammation. The perpetuation of this process results in transformation of the synovial lining into pannus tissue that invades and destroys joint structures[1]. The major clinical symptoms of RA are joint pain, stiffness, and swelling, with a variable clinical course[2].

The earliest stage of inflammation in RA involves leukocyte migration from the blood vessels and their subsequent rolling along the endothelium of postcapillary venules leading to leukocyte recruitment into the sites of inflammation[3]. The molecules implicated in these interactions are the selectins. There are 3 types: E, L, and P selectin, expressed on the surface of endothelial cells (EC), leukocytes, and platelets, respectively[4,5]. The E-selectin expressed on the surface of EC is considered a marker of EC activation. E-selectin contains a lectin-like N-terminal domain capable of recognizing the tetrasaccharide sialyl-Lewis of monocytes and neutrophils[4]. E-selectin appears to be important in the adhesion of granulocytes, monocytes, and CD4+ T cells in order to activate the EC[6].

Soluble isoforms of E-selectin (sE-selectin) in blood samples from patients with infection, cancer, inflammatory and autoimmune rheumatic diseases including RA, juvenile RA,

systemic lupus erythematosus (SLE), vasculitis, and systemic sclerosis showed elevated levels that probably reflected underlying EC activation[7-10].

The selectin group of cellular adhesion molecule genes were located in the 1q22-25 locus[5]. Several polymorphisms have been described in the E-selectin gene. However, the main polymorphism studied is the transversion from adenine (A) to cytosine (C) at 561 position (A561C), resulting in amino acid exchange from serine (S) to arginine (R) at position 128 (S128R) in the endothelial growth factor-like domain (EGF)[5,11]. We investigated the relationship of A561C polymorphism and the sE-selectin levels with RA clinical activity.

MATERIALS AND METHODS

Study design. This was a case-control study.

Clinical setting. Consecutive patients with RA were recruited from the outpatient Rheumatology Service, Civil Hospital of Guadalajara "Fray Antonio Alcalde," Guadalajara, Jalisco, Mexico.

Patient population. Patients classified as having RA according to the 1987 American College of Rheumatology criteria[12] (n = 60; age range: 22 to 72 yrs) were included from December 2001 to April 2003. The inclusion criteria for the study were: > 16 years of age, no overlapping diseases, and being diagnosed with RA. Sixty healthy subjects (age range: 22 to 72 yrs) were included as a control group. The inclusion criteria for the study were: > 18 years of age and clinically healthy.

Patients with RA and healthy subjects were Mexican Mestizo according to the definition of the National Institute of Anthropology, which states that an individual must be born in Mexico, have a Spanish last name, and a family history of Mexican ancestors at least back to the third generation[13].

Clinical assessment. All patients were evaluated by 2 rheumatologists at the time of the study. Demographic and clinical variables evaluated were age, sex, disease evolution, history of drug use, and current therapy. Disease activity was evaluated using the Spanish version of the Health Assessment Questionnaire Disability Index (Spanish HAQ-DI)[14], Spanish version of the Arthritis Impact Measurement Scales (Spanish-AIMS)[14], and Disease Activity Score (DAS28)[15], using 28 joint counts.

Laboratory assessment. Blood samples were obtained from antecubital venipuncture from all subjects; rheumatoid factor (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelet count (PLT) and white blood cell count (WBC) were determined in all participants by routine methods.

Informed written consent was obtained from all subjects before enrolment in the study meeting the ethical guidelines of the 2000 Declaration of Helsinki.

Sandwich ELISA for sE-selectin. The sE-selectin production (R&D Systems, Minneapolis, MN, USA) was measured on serum samples from patients with RA and controls. The detection range was 0–10 ng/ml and the assay sensitivity was < 0.01 ng/mL. The sE-selectin production was calculated from a standard curve of the corresponding recombinant human sE-selectin.

Molecular analysis of E-selectin A561C polymorphism. Genomic DNA was extracted from 3 ml of whole blood collected in a vacutainer™ tube containing ethylenediaminetetra-acetic acid (EDTA), according to the Miller method[16].

We amplified the 249 bp fragment of the E-selectin gene containing the polymorphic site, using the following primer sequences: forward (5'-CCG TAG CTG CCT GTA CCA AT-3') and reverse (5'-GTC TCA GCT CAC GAT CAC CA-3'). Polymerase chain reaction (PCR) was carried out in 25 μl final volume containing 300 ng of genomic DNA, 10 × KCl buffer, 1 mM $MgCl_2$, 2.5 mM dNTPs, 3 μM of each primer, and 1 U of Taq DNA polymerase (Invitrogen™ Life Technologies). Thermal cycling was done with an initial denaturation of 3 min at 94˚C, followed by 35 cycles of denaturation: 94˚C for 30 s, annealing at 55˚C for 30 s, extension at 72˚C for 30 s, followed by a final extension at 72˚C for 1 min. To identify the gene polymorphism we used the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) technique where 15 μl of PCR products were digested in duplicate in all cases with 5 U of Pst I (Invitrogen™) restriction enzyme 2 h at 37ºC. Digestion products were separated at constant voltage of 80 V, on 3% agarose gel electrophoresis stained with 0.1 μg/ml of ethidium bromide[5].

Electrophoretic E-selectin genotype restriction patterns. The A561C transversion abolishes the restriction site for the Pst I enzyme, therefore in the homozygous A/A genotype, the PCR product is visualized as 2 fragments of 219 and 30 bp. The homozygous polymorphic C/C genotype product appears as one band of 249 bp. The heterozygous A/C genotype produces 3 fragments: 249, 219, and 30 bp.

Sequencing of E-selectin genotype. To confirm the presence of the A561C transversion of the E-selectin genotypes, digested DNA samples eluted from agarose gel were subjected to sequencing using an Abiprism 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS version 10.0. The data were presented as mean and minimum–maximum values. The mean comparison of the 2 groups was performed using the Student t test, if data were normally distributed. Mann-Whitney U-test was applied for data with nonparametric distribution. Pearson and Spearman tests were used to show the correlation of sE-selectin with other laboratory and disease activity variables, respectively. A chi-square test was used to test for Hardy-Weinberg equilibrium. Distribution of E-selectin allele frequencies was tested by Fisher exact analysis using a 2 × 2 table, to find associations between RA and the variant allele. The strength of association between RA and E-selectin alleles or genotypes of E-selectin was estimated using odds ratio (OR) with 95% confidence intervals (CI). In all tests, a probability value of $p < 0.05$ (2-tailed) was considered statistically significant.

RESULTS

Patients. The demographic and clinical characteristics in RA patients are shown in Table 1. Serum RF, CRP and ESR, WBC and PLT were significantly increased in patients with RA compared to controls ($p < 0.05$; Table 2).

Molecular analysis of E-selectin A561C polymorphism. Sixty patients with RA were compared to 60 controls with respect to their E-selectin A561C genotype. Allele and genotype frequencies of A561C polymorphism in patients and controls are shown in Table 3. Frequencies of the A/A, A/C, and C/C genotypes did not deviate significantly from the predicted frequencies according to the Hardy-Weinberg equilibrium (chi-square, $p = 0.936$). The A561C polymorphism analysis showed a diminished frequency in RA of heterozygous A/C genotype and an increased frequency of homozygous A/A genotype ($p = 0.043$, OR 1.45; 95% CI 1.125–16.167) versus A/C and A/A genotype in controls. The allele frequency did not show statistical differences between groups (Table 3). In addition, we observed that the A/C genotype carriers developed RA at an early age in comparison with A/A genotype carriers ($p = 0.03$; Table 4).

Comparison of sE-selectin levels. The sE-selectin levels in sera were significantly higher in patients with RA (91.7 ng/ml) versus controls (39 ng/ml) ($p = 0.002$; Table 2).

Correlations of sE-selectin with RF, ESR, and Spanish HAQ-DI and DAS28 indices. Correlation between sE-selectin level and clinical variables was assessed by Pearson and Spearman correlation coefficients as shown in Table 5, where sE-selectin

Table 1. Demographics and clinical characteristics of patients with RA.

	All Patients (n = 60)	Women (n = 55)	Men (n = 5)
Age, yrs (range)	42 (22–72)	46 (22–72)	43 (30–67)
Drug treatment			
Prednisone < 8.5 mg/day	11/60	—	—
DMARD	26/60	—	—
NSAID	39/60	—	—
Disease duration, yrs (range)	9.63 (0.2–30)	11 (0.2–30)	1.75 (1–3)
Clinical assessment			
Swollen joints count 28	9.84 (0–24)	11 (0–24)	14.6 (7–21)
Painful joints count 28	20.8 (0–28)	20 (0–28)	21.6 (2–19)
Morning stiffness, min	76.70 (5–480)	70 (5–480)	10 (8–10)
Patient's global assessment of disease status (0–10 VAS)	6.76 (1–10)	6.6 (1–10)	6.5 (5–8)
Spanish HAQ-DI (scale 0–3)	1.20 (0.08–2.56)	1.19 (0–2.8)	0.8 (0–1.3)
Spanish-AIMS (scale 1–7)	3.98 (2.21–5.76)	3.9 (2.1–6.1)	4.7 (3.4–5.7)
DAS28 (scale 0–10)	6.23 (3.58–8.15)	6.23 (3.58–8.15)	6.4 (4.8–7.2)

RA: rheumatoid arthritis; DMARD: disease modifying antirheumatic drug; NSAID: nonsteroidal antiinflammatory drugs; VAS: visual analog scale; HAQ-DI: Health Assessment Questionnaire Disability Index; AIMS: Arthritis Impact Measurement Scales; DAS28: disease activity score, using 28 joint counts. Data presented as mean (min–max).

Table 2. Laboratorial assessment in patients with RA and healthy subjects.

Laboratorial Assessment	Patients with RA (n = 60)	Controls (n = 60)	p
sE-selectin, ng/ml	91.7 (± 71.0)	39 (± 17.8)	0.002
RF, IU/ml	656.2 (± 1134.75)	25.9 (± 55.85)	0.001
ESR, mm/h	40.3 (± 11.87)	19.48 (± 12.00)	0.002
Serum CRP level, mg/dl	2.9 (± 3.85)	0.33 (± 0.25)	0.001
PLT, κ/μl	337.3 (± 93.83)	257.3 (± 63.1)	0.001
WBC, κ/μl	7.4 (± 2.25)	6.3 (± 1.73)	0.001

RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; PLT: platelet count; WBC: white blood cell count. Data are mean (± SD).

Table 3. Genotypes and allele frequencies of E-selectin A561C polymorphism.

	Frequency of E-selectin A561C	
	Patients with RA (n = 60) n (%)	Controls (n = 60) n (%)
Genotypes		
A/A	57 (95)	49 (82)*
A/C	3 (5)	11 (18)*#
C/C	0 (0)	0 (0)
Alleles		
A	117 (97.5)	109 (90.8)
C	3 (2.5)	11 (9.2)

* p = 0.043 vs RA subjects (Fisher exact test). # OR = 1.45 (95% CI 1.12–16.16), p = 0.03 (Mantel-Haenszel common OR estimate).

showed a positive correlation with RF, ESR, Spanish HAQ-DI, and DAS28.

DISCUSSION

We showed that sE-selectin levels were correlated with disease activity evaluated by Spanish-HAQ-DI and DAS28 indices in patients with RA. Several reports have evaluated sE-selectin in RA, early RA, juvenile arthritis, psoriatic arthritis, Sjögren's syndrome, gout, and osteoarthritis[4,6,8,10,16-19]. In our study the sE-selectin concentration was significantly raised in all patients with RA. These results are in accord with reports describing high sE-selectin levels in patients with RA[4,10,17,20]. sE-selectin has been described as a molecule capable of triggering a proinflammatory environment, increasing angiogenesis and the migration of mononuclear cells into the synovial tissue. On the other hand, sE-selectin might compete for binding with the ligands on the surfaces of leukocytes. We can speculate that the balance between the level of E-selectin on the cell surface and sE-selectin may provide an additional signal that critically regulates the inflammatory response[8,21].

Many reports[5,11,22-24] have identified the E-selectin A561C polymorphism with different frequencies. We studied the A561C polymorphism, which showed a higher frequency of A/A genotype in patients with RA than healthy controls (Table 3). Moreover, the A/C genotype was higher in controls. A possible explanation for these results is the genetic background

Table 4. Demographics and clinical characteristics in RA patients.

	All Patients (n = 60)	E-selectin A561C Polymorphism A/A Genotype (n = 57)	A/C Genotype (n = 3)	p
Age, yrs	42 (22–72)	46.6 (22–72)	31.3 (28–36)	0.037
Sex (F:M)	55:5	53:4	2:1	NS
Laboratory assessment				
sE-selectin level (ng/ml)	91.7 (19–467)	93.9 (21–467)	57.0 (19–109)	NS
RF (IU/ml)	607.9 (20–4240)	619.6 (20–4240)	409.2 (68.2–583.4)	NS
ESR (mm/h)	33.9 (10–55)	34.1 (10–55)	30.6 (28–36)	NS
Disease status				
Disease duration, yrs	9.63 (0.2–30)	11.0 (0.2–30)	3.0 (2–4)	0.002
Spanish HAQ-DI (scale 0–3)	1.20 (0.08–2.56)	1.16 (0.08–2.56)	0.59 (0.42–1.58)	NS
Spanish-AIMS (scale 1–7)	3.98 (2.21–5.76)	3.97 (2.14–6.14)	4.09 (3.93–4.25)	NS
DAS28 (scale 0–10)	6.23 (3.58–8.15)	6.30 (3.58–8.15)	5.80 (4.5–7.21)	NS

Definitions as in Table 1. NS: not significant.

Table 5. Correlation of sE-selectin levels with clinical data in patients with RA.

Clinical Data	sE-selectin Correlations	p
Disease duration, yrs	0.202	NS
RF, IU/ml	0.588*	0.001
ESR, mm/h	0.325*	0.023
CRP, mg/dl	0.108	NS
PLT κ/μl	0.107	NS
Spanish HAQ-DI (scale 0–3)	0.331#	0.019
Spanish-AIMS (scale 0–7)	–0.167	NS
DAS 28 (scale 0–10)	0.383#	0.011

Definitions as in Table 1. * Pearson and # Spearman correlations, $p < 0.05$; NS: not significant.

that influences the interpopulation variability of the Mexican population. One limitation of our study is the low frequency found in A/C carriers. Even though the number of A/C genotype carriers among patients with RA is small (n = 3), it is important that these patients were younger and had shorter disease duration (Table 4). We propose that the A/A genotype might be a probable marker of susceptibility for development of RA in Mexican Mestizos versus a potential protective role of being a carrier of the A/C genotype.

These patients expressed low levels of sE-selectin, but we cannot conclusively state that the A/C genotype would offer protection for an uncontrolled production related to sE-selectin[25,26]. In contrast, RA A/A carriers produced the highest levels of sE-selectin as well as major clinical activity. Even if this was not statistically significant, we suggest that this finding must be reevaluated on a larger sample size.

Our main focus was the correlation of sE-selectin with clinical activity in RA. This correlation was found in our patients (Table 5), and also by other authors[4,8,16,17]. In conclusion, sE-selectin levels were associated with the clinical activity evaluated by Spanish-HAQ-DI and DAS28 indices in patients with RA, as well as RF and ESR. We highlighted the presence of A/A genotype A561C polymorphism in the Mexican Mestizo patients with RA. The A/A genotype might be a marker of susceptibility for development of RA in Mexican Mestizos versus a possible protective role for RA expression of the A/C genotype.

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