

Original Article

Association between Endothelial Selectin (E-Selectin) Gene Polymorphisms and E-Selectin Level with Visceral Leishmaniasis, in an ARMS-PCR Based Study

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Abstract

Background: In the visceral leishmaniasis (VL), parasites reside in reticuloendothelial system, mainly in macrophages. Endothelial Selectin (E-selectin) might play an important role in leukocyte-endothelium interactions and inflammatory cell recruitment. The aim of this study was determining E-selectin level and its polymorphism in three groups, patients, seropositive and healthy individuals.

Methods: Serum soluble E-selectin levels as well as 2 polymorphisms of E-selectin (Ser128Arg and Leu554Phe) were measured in a cohort of patients with documented VL (n=64), a healthy control group (n=74) and a seropositive for VL but without any symptoms (n=81). Circulation concentration of E-selectin levels was measured by ELIS. The amplification refractory mutation system (ARMS)-PCR procedure was used for detecting polymorphisms.

Results: The mean of E-selectin levels significantly differed between three groups ($P<0.026$), and were increased in patients in comparison with other groups. Difference was more considerable between two groups of patients and healthy ones (patients 92.8 ng/ml; healthy individuals 71.9 ng/ml). Polymorphisms were associated with soluble E-selectin levels and altogether explained 14.4%, 7.2%, and 8.7% in patients, seropositive and seronegative healthy individuals, respectively. Distribution of polymorphisms of 128Ser/Arg and 554Leu/Phe among three groups was not different significantly; however, there was a considerable arrangement in distribution of Ser128Arg polymorphism and 128Arg allele in healthy group was more than two fold of patients (55% against 20%).

Conclusion: The association between soluble E-selectin levels and visceral leishmaniasis suggests that this molecule might have significant role in the inflammatory process in VL. Moreover, frequency of 128Arg allele in healthy group was higher than patients.

Keywords: Soluble E-selectin, Gene Polymorphism, Visceral Leishmaniasis, Human, ARMS-PCR, Ser128Arg, 554Leu/Phe

Introduction

Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1, E-selectin) belongs to the selectin family of adhesion molecules. Together with

LECAM-1 (L-selectin) and GMP-140 (P-selectin), E-selectin mediates the initial interactions of leukocytes and platelets with endothelial cells (1). The individual members of the selectins are mediated by prefixes, which were cho-

sen according to the cell type where the molecules were first identified: L-selectin is expressed on most types of leukocytes, E-selectin is expressed on activated endothelium, and P-selectin was first found in storage granules of platelets and is also expressed by endothelial cells.

As mentioned, together with GMP-140, E-selectin is expressed on cytokine-activated endothelial cells, and contributes to the adhesion of still resting leukocytes to the endothelium (2, 3). The circulating form or soluble (sE-selectin) of this selectin excrete chemo-tactical signals on neutrophils and additionally activates the 2-integrins-sEselectin assists in preparing the migration capacity of this cells (4).

Determination of sE-selectin could provide more detailed insights into the pathological modifications during various diseases (5). The E-selectin mediates the interaction of fundamental role in the pathogenesis of coronary disease (6). Double-knockout mouse experiments suggested that E-selectin plays an essential role in both early and advanced stages of atherosclerotic lesions development and that mutation in cellular adhesion molecules like E-selectin may act as genetic risk factors for coronary atherosclerosis (7). Additionally, the involvement of E-selectin in cardiovascular disease is suggested by the fact that it is expressed only in activated endothelial cells (8).

The E-selectin polymorphism S128R has been associated with the presence of angiographic coronary artery disease (9), myocardial infarction (10), end-stage renal disease in patients with IgA nephropathy and ischemic cerebrovascular disease in different populations and different ethnic groups (11, 12). The role of some intracellular adhesion molecules-1 (ICAM-1) have been studied and confirmed implicated in the cytoadherence process (13, 14). However, no previous studies are available on possible association of this polymorphism with visceral leishmaniasis amongst different ethnic groups, and the correlation of selectins' level with this infection, that is *L. infantum* species prevalent

in this region.

Therefore, the aim of this investigation was to evaluate the potential relevance of E-selectin S128R and Leu554Phe genetic polymorphisms for symptomatic visceral leishmaniasis and asymptomatic individuals, as well as the level of soluble E-selectin in the VL infections, using the Iranian population as a model.

Materials and Methods

Samples collection

This investigation was conducted from 2005-2006 in East Azerbaijan, North West of Iran. In a comparative case-control study, three groups of individuals were recruited for present study by convenience sampling from East Azerbaijan health centers. The patient group comprised 64 clinically and paraclinically confirmed visceral leishmaniasis, majority were <10 years old. The inclusion criteria for VL were the presence of main signs and symptoms, including fever, hepatomegaly, splenomegaly, anemia and weight loss; serologically positive by Direct Agglutination Test (DAT) and Indirect Fluorescent Antibody (IFA) with agreement together and detecting Leishman bodies in bone marrow aspiration by direct smear. All bone marrows had been done in hospitals and those results obtained from patients' files.

The parasite strain used for diagnostic tests was Iranian strain of *L. infantum* LON-49 that obtained from Drug Applied Research Center, Tabriz University of Medical Science. Antigens prepared according to methods described previously (15-19). A titer $\geq 1:3200$ considered as positive in DAT (17) and, a titer of $\geq 1:160$ considered as positive in IFA technique (15). Second group comprised 81 serologically positive for VL by IFA and DA tests, but without any signs and symptoms and history of confirmed VL disease as well. Third group comprised also 74 healthy individuals without any history of VL and negative for VL serologically. This group was selected from patient's relatives.

Two procedures, DAT and IFA were done as methods has described elsewhere (15, 16).

The E-selectin level determined by ELISA procedure using Human sE-selectin ELISA (BMS205 Version 2, Bender Medsystems, Vienna, Austria), as described by manufacturer. Ten ml of peripheral blood for serum components was collected into EDTA glass tubes from all participants after obtaining their written informed consent and allowed to coagulate overnight at 4 °C. Serum level of sE-selectin were estimated by a two-site ELISA. DNA was extracted by using standard salting-out procedure using the Fermentas Co kit, and stored at -20 in aliquots until required. The proposal was approved by Ethics Committee of Chancellor for Investigation, Hamadan University of Medical Sciences.

Measurement of E-selectin level

Serum level E-selectin concentration were measured quantitatively and is reported an ng/ml using a commercially ELISA kit. Briefly, micro wells coated by monoclonal antibody (anti E-selectin monoclonal antibody). Thereafter, micro wells filled by 100 ul diluted antigen (80µl solution+20µl serum) and properly mixed. In next step, 50µl prepared appropriate dilution of the horseradish peroxidase (HRP)-conjugated with monoclonal anti E-selectin antibody were added to wells (including sample, standard and blank). Plate covered by an adhesive plastic cover and incubated for 2h in room temperature. Then, removed cover and also removed coating solution by washing the plate (3 times) by filling and empty the wells by 300µl PBS. The solutions or washes were removed completely by flicking the plate over a sink and the remaining drops were removed by patting the plate on a paper towel. Then, 100µl of the fresh TMB substrate per well dispensed with a multichannel pipette and covered with an adhesive plastic and incubated for 15 min at room temperature (in a dark place). Reaction was stopped adding 100 µl of stop solution to the wells. The absorption measurement was

carried out at 450 nm using a micro titer ELISA Reader. All samples and standards were run in duplicate.

Detection of the S128R polymorphism

This was carried out by the amplification refractory mutation system (ARMS)-PCR.

The amplification refractory mutation system (ARMS)-PCR or allele-specific PCR assay has been widely used for HLA typing at the DNA level as well as in other applications. This assay can detect up to two cis-location motifs per reactions, using forward and reverse sequence specific primers (20).

For DNA amplification we used the forward primer 5-CTGTACCAATACATCCTGCA-3 and reverse primer

5-TCTGACTTCATAGTCTCAGCT-3

designed to amplify a 230 bp fragment of the E-selectin gene for S128R, and the forward primer 5-TCCTGACATTAGCACCATTTC-3 and reverse primer

5-CCACACTGAGTTGTACTACTA-3

for F554L polymorphisms (15). Each 25 µl of 10X reaction buffer with MgCl₂, 0.4 nM of each primer, 200 µM each of deoxynucleoside triphosphate in Tris HCl buffer, 0.2 unit Taq DNA polymerase and 0.1 µg genetic DNA template. The mixture was denature at 96 °C for 1 min, and the PCR reaction was carried out for 35 cycles, in a DNA-Technology MCT91 PCR system (4), under the following conditions: denaturation at 96 °C for 1 min, annealing at 54 °C for 45 sec, extension at 72 °C for 1 min, and final extension cycle of 72 °C for 5 min. The PCR products were electrophoresed on a 1% agarose gel and detected with 0.5 µg/ml Ethidium bromide to confirm the correct amplicon size. The sizes of the amplicons were determined using the 50-bp ladder (2).

Genotype frequencies in three groups were compared by chi-square test multivariable logistic regression was used to study the effect of E-selectin F554L allele (S128R allele) on VL. All analysis was performed using SPSS V.13 statistical analysis software. A two-tailed *P* value

<0.05 was considered statistically significant.

Results

In a screening program, 96 confirmed patients of VL detected amongst 768 individuals. One hundred forty two out of 672 samples were negative and the rest of samples were positive for VL serologically by IFAT with the titers 1:10 to 1:160. A total of 64 samples of confirmed VL, 81 samples seropositive for VL without history of clinical disease, and 74 samples seronegative also without history of VL disease were eligible and enrolled for study and DNA successfully extracted for amplification. The mean of soluble E-selectin in the patients, seropositive and seronegative individuals were 92.8, 69.2, and 74.6 ng/ml, respectively. The

difference of means between three groups were significant statistically by ANOVA test ($P < 0.026$). Moreover, the mean of sE-selectin between two groups of patients and healthy individuals also were significant statistically (OR= 2.04, $P < 0.008$). E-selectin polymorphisms were associated with soluble E-selectin levels and altogether explained 14.4%, 7.2%, and 8.7% in patients, sero-positive and sero-negative healthy groups respectively. Distribution of polymorphisms of 128Ser/Arg and 554Leu/Phe among three groups was not different significantly; however, there was a considerable arrangement in distribution of Ser128Arg polymorphism and, 128Arg allele in healthy group was more than two fold of patient's group (55% against 20%). The PCR product related to S128R and 554F/P alleles showed in the Fig. 1 and 2.

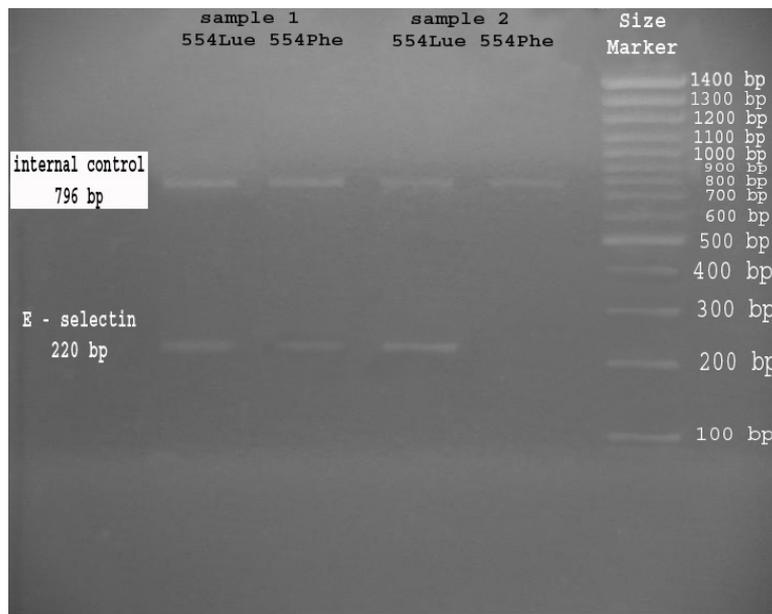


Fig. 1: Gel electrophoresis of PCR product related to S128R allele

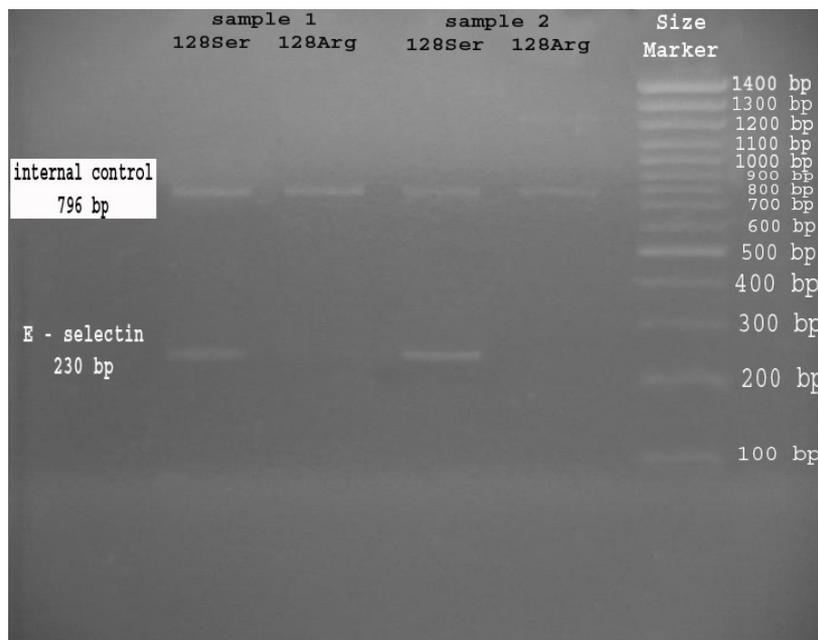


Fig. 2: Gel electrophoresis of PCR product related to 554L/P allele

Discussion

This study showed high amount of E-selectin in VL patients. However, distribution of different alleles had not considerable differences between patients and health individuals. Different studies have demonstrated markedly elevated levels of the soluble E-selectin during the course of severe cerebral *Plasmodium falciparum* infection (21-23), and some other protozoan infections (24, 25), or cardiovascular problems such as coronary heart disease (26), cardiovascular damages in central obese subjects (27), myocardial infarction (10), and also in the SLE (28). It also have studied in different population and ethnic groups, such as Arabs (5), Chinese (7), Japanese (12, 29), and indicated relatively controversial results (30). Genetic mutations and polymorphisms are known to be risk factors for various infectious diseases and especially cardiovascular diseases, such as malaria, atherosclerosis, coronary angioplasty, and have been extensively studied for their potential association with atherogenic vascular

diseases (31, 32). Similarly, mutations in adhesion molecules and their association with cardiovascular disease, in some cases as a side effect of some helminthic infections such as schistosomiasis and onchocerciasis, have been recently investigated (33-35). In the present study, we demonstrated the concentration of E-selectin in the confirmed visceral leishmaniasis in an endemic area for this disease.

Visceral leishmaniasis could assume as an inflammatory infection, with a chronic process. The importance of E-and P-selectin in recruitment of T lymphocytes to inflamed tissues is less well understood. E-selectin may be especially important for T-lymphocyte migration to cutaneous inflammation, such as cutaneous leishmaniasis, because a subset of T cells found enriched in human inflammatory dermatoses express a specific ligand for E-selectin (36). Although many studies suggest a role for these selectins in T lymphocytes or lymphoblast recruitment, the contribution of E-and P-selectin to resting T lymphocyte migration to sites of inflammation such as the skin and or different

visceral organs, such as a process that occur for visceral disseminated infection by *Leishmania*, had not been documented till now (37). Although, some workers demonstrated that, CD4 (+) T cells recruited to the cutaneous compartment during infection with *Leishmania* major express P-and E-selectin ligands and, expression of P-and E-selectin ligands correlates with activated *Leishmania*-specific Th1 cells and is depend upon IL-12 (38).

Recent attentions has focused mainly on the inflammatory components, such as TNF α , TNF β and those polymorphism, TGF α , TGF β and those polymorphism, interleukin 1 system polymorphism, CD 14 polymorphism and different adhesion proteins in the various infectious diseases (30). Several of the polymorphisms (for example, those for TNF α and β , TGF β 1, IL 1, CD 14, and E selectin) have been found to be functional-that is, to have direct effects on gene transcription and protein function. Additionally, the small contribution of a single novel polymorphism to the overall risk of a multifactorial disorder, such as IHD (and not simple infections) may be obscured by the presence of one or more dominant classical risk factors. Several reports support this possibility (for example, association between CD 14 variants and myocardial infarction, in normotensive and non-smoking patients or in the generally low risk Japanese population, or between E selectin or PECAM 1 polymorphisms and coronary atherosclerosis in young, non-diabetic, normotensive groups). Prospectively designed studies and the analysis of haplotypes may overcome some of limitations of population studies. Another way to confront these limitations is through careful phenotypic characterization of patients, since a new genetic risk factor is more likely to emerge within homogeneous groups of patients in whom it has a similar role. Identifying such homogeneous groups may be difficult and may require rigorous control not only of age, sex, race, and ethnic grouping, but also of clinical features and

biochemical markers linked to specific pathogenic mechanisms.

In spite of such a limitations, this study confirmed different distribution of some alleles in patients group in comparison to healthy ones, as well as higher level of soluble E-selectin in patients.

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