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E-Selectin S128R Polymorphism and Se-Selectin Levels Influence Severity of Asthma

[1]Mehrdad Hajilooi, [2]Ebrahim Nadi, [3]Alireza Rafiei, [2]Mostafa Ansari,
[2]Sayed Ahmad Hashemi, [2]Sussan Tavana and [2]Fatemeh Zeraati
[1]Department of Immunology, Molecular Medicine Research Center,
Hamadan University of Medical Sciences, Hamadan, Iran
[2]Department of Lung diseases, Ekbatan Hospital,
Hamadan University of Medical Sciences, Hamadan, Iran
[3]Department of Immunology and Microbiology, Sari Medical School,
Mazandaran University of Medical Sciences, Sari, Iran

Abstract: Asthma is a multifactorial disease that its onset and severity are influenced by both genetic and environmental factors. Identification of genetic factors involved in asthma development is required for better managing and controlling the disease. The E-selectin mediates the interaction of activated endothelial cells with leukocytes and plays a fundamental role in the pathogenesis of asthma. Our purpose was to determine whether this Ser128Arg polymorphism influences the risk of asthma and to analyze the possible correlation to disease severity in Iranian patients. We studied the human E-selectin gene polymorphism in a 172 asthmatic patients and 173 healthy volunteers by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). We identified increased levels of sE-selectin in asthmatic patients compared with healthy subjects ($p<0.0001$). Frequencies of the SS, SR and RR genotypes were found as 66.3, 31.4 and 2.3% in the patients and 91.9, 8.1 and 0.0% in control subjects, respectively. The 128Arg allele was more prevalent in patients than controls (OR 5.78; 95% CI, 3.07-10.86, $p<0.0001$). However, in this study the polymorphism was not associated with circulating sE-selectin levels. We found a direct correlation between the level of sE-selectin and severity of asthma ($p=0.001$). On the other hand, there is a close association between 128Arg carriage and disease severity ($p<0.0001$). These results suggest that the Ser128Arg polymorphism of the E-selectin gene is a genetic factor that may be associated with severity of asthma.

Key words: E-selectin, gene polymorphism, asthma, severity, adhesion molecules, inflammation

INTRODUCTION

Asthma and related phenotypes are thought to be complex traits caused by an interaction of multiple disease susceptibility genes and environmental factors. There is pivotal evidence that inflammation is involved in onset and development of asthma. Adhesion molecules like members of selectin family facilitate the recruitment and migration of inflammatory cells from the blood to the airway walls and therefore, have an important role in the pathogenesis of asthma.

Early primate studies showed that blockade of E-selectin reduced allergic pulmonary inflammation (Gundel, 1991). Therefore, increased expression of the E-selectin has been observed in asthmatic patients (Kobayashi *et al*., 1994; Yamashita *et al*., 1997; Symon and Wardlaw, 1999), suggesting that these molecules are up-regulated in human disease and may contribute to the alteration of pathophysiology. On the other hand, the migration of leukocytes into an asthmatic lung is dependent upon multiple mechanisms that are initiated by the binding of leukocytes at the endothelial border to selectins. Blockade of the initial selectin-mediated adhesion event should inhibit subsequent migration into the lung during an allergen challenge.

E-selectin is exclusively expressed on Endothelial Cells (ECs), mostly after their activation (Dong, 1998). It is expressed and proteolytically cleaved from the surface of ECs upon activation by different stimuli, (Wyble *et al*., 1997) including Lnterleukin 1 (IL-1), Tumor Necrosis Factor (TNF). *In vivo*, such a shedding leads to an increase in plasma levels of soluble (s) E-selectin (Kulander and Venge, 2001). Further, sE-selectin levels correlate with its surface expression on ECs *in vitro* (Leeuwenberg *et al*., 1992).

Corresponding Author: Alireza Rafiei, Department of Immunology and Microbiology, Sari Medical School, Mazandaran University of Medical Sciences, Sari, Iran

Thus, sE-selectin serves as an excellent marker for endothelial activation in numerous cardiovascular and inflammatory diseases (Roldan *et al*., 2003). Interestingly, the common Ser128Arg polymorphism has been reported to regulate plasma levels of sE-selectin (Bannan and Grant, 1998; Mlekusch *et al*., 2004). This polymorphism is functional in that it alters ligand affinity. E-selectin has an aminoterminal C-type lectin domain that is thought to possess the carbohydrate binding site that binds the sialylated Lewis×antigen (sLex or CD15s) (Neu5Acalpha 2-3Galbeta1-4(Fucalpha1-3) GlcNAc). Substitution of E-selectin Endothelial Growth Factor (EGF) domain residue Ser128 with an arginine results in E-selectin proteins that have lost the requirement for alpha1-3–linked fucose and are thus able to bind to sialyllactosamine (Revelle and Bech, 1996). Likely as a consequence, this SNP enhances tethering of myeloid cells (Rao *et al*., 2002) and extends the range of lymphocytes recruited by E-selectin (Rao and Landis, 2002). Further, S128R-transduced ECs support significantly more rolling and adhesion of neutrophils and mononuclear cells compared with ECs transduced with wild-type E-selectin. These S128R transduced ECs also exhibit significantly greater levels of phosphorylation of extracellular signal-regulated kinase 1 and 2 and p38 mitogen-activated protein kinase. This suggests that an altered endothelial signaling pathway is associated with this polymorphism (Yoshida *et al*., 2003). In this study, we sought to detect S128R polymorphism in the E-selectin gene to compare the allele and genotype frequencies between asthmatic patients and controls. In addition, we measured serum sE-selectin concentrations to investigate whether a correlation exists between the different genotypes and sE-selectin levels.

MATERIALS AND METHODS

Study population: One hundred seventy-two patients with asthma (Table 1) before treatment were recruited from the out-patient clinics of Hamadan University Hospital, Southwest of Iran, during 2004-2006. Asthma was diagnosed in subjects according to presence of the clinical symptoms and the physical examinations. Each patient showed airway reversibility as documented by an inhalant bronchodilator-induced improvement of >15% of Forced Expiratory Volume In one second (FEV1) and/or an airway hyperreactivity of <10 mg mL^{-1} of methacholine. Patients who previous used inhaled steroid or systemic steroid within the past four weeks, were excluded from the study. The categorization of asthma severity was done according to method has been described previously; Mild intermediate, mild persistent, moderate persistent and severe persistent asthma (National Asthma Education and Prevention Program).

Table 1: Characteristics of patients and controls

Characteristic	Asthmatic patients n = 172	Controls n = 173
Age (year)	56.56±12.69	54.43±10.11
Gender (M/F)-%	99/73 (57.6/42.4)	114/59 (65.9/34.1)
Smoking (current)-n (%)	54 (31.4)	62 (36)
FEV1% predicted	54.3±22.4	102.2±14.32*
PEF% predicted	49.6±20.75	ND
PEF Variability%	26.42±8.1	ND
Reversibility%	19±6.24	ND

* $p<0.001$ for differences between patients with asthma and healthy controls by T test. ND: Not Done

One hundred seventy-three healthy, non asthmatic, adult unrelated control subjects with no past history of wheeze, no previous diagnosis of asthma or a prior prescription of an asthma medication were recruited from the same geographical area through blood donor clinics. The normal controls had FEV1 >75% predicted and normal findings on a simple chest radiogram. The Ethics Committee of Hamadan University of Medical Sciences was approved the study protecols.

E-selectin genotype: Venous blood from each subject was collected in tubes containing 50 mmol of EDTA per liter. Genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting out method (Miller and Poleski, 1988). A Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to detect the substitution responsible for E-selectin polymorphism. Amplification was carried out using a PCR Techne Flexigene apparatus (Roche, Germany) in a total volume of 50 μL containing 0.2 ng of genomic DNA. The primers used and their specificities were shown in Table 1. PCR performed without DNA template represented the negative controls. Each pair of primer consisting of 10 pmol of each allele-specific primer, 200 mol L^{-1} each dNTP, 10 mol L^{-1} Tris-HCl (PH 8.3), 50 nmol L^{-1} KCl, 1.5 mol L^{-1} MgCl2 and 0.5 IU Taq DNA polymerase. PCR performed without DNA template represented the negative controls. The reaction was carried out as follow: Initial denaturation was at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 15 sec and annealing at 60°C for 45 sec, with final extension for 2 min at 72°C. The PCR product of 186 bp was then digested by the restriction enzyme *Pst I* (10-μL PCR product and 5 U of *Pst I* were digested for 3 h at 37°C), separated in 3% agarose gel electrophoresis and visualized by ultra-violet transillumination. Genotype SS yielded two fragments of 123 and 63 bp, genotype RR yielded one fragment of 186 bp (not detected), SR yielded three digestion fragments of 186, 123 and 63 bp (Wenzel and Spear, 1994). To prevent observer's bias, the investigator was unaware of sample origin and a different individual cross-checked all the gels.

Soluble E-selectin measurement: Whole blood was drawn in tubes and allowed to coagulate at room temperature for 1h. The serum was then separate by centrifugation and stored at -80°C before analyzed. Serum levels of sE-selectin were measured in duplicated by commercially available ELISA kits (sE-Selectin; Bender Med Systems, BMS 205, Vienna, Austria) following manufacturers instructions. The standard curve was generated with human recombinant sE-selectin provided and the lower limit of detection sensitivity was 1.6 ng mL^{-1}.

Statistical analysis: The consistency of genotype frequencies with Hardy-Weinberg equilibrium was checked using Chi-square test. Results of the gene polymorphism studies were analyzed by comparison of allele frequencies (ratio of test allele to total alleles). Frequencies of allele and genotype distribution were analyzed using Fisher's exact test or $\div^2$ test as appropriate. Multiple linear regression analysis was applied to assess the association between E-selectin genotype (independent variable) and plasma levels (dependent variable). Differences in the serum sE-selectin levels between groups were confirmed by Kruskal-Wallis test. Odds Ratios (OR) were calculated for disease susceptibility or severity in carriers of specific alleles. The 95% Confidence Intervals (CI) for the OR were also calculated. P-values of less than 0.05 (two-tailed) were considered significant.

RESULTS

Patient data: The characteristics of the subjects with asthma and the normal controls are presented in Table 1. The mean age of the patients and control were 56.56±12.69 years and 54.43±10.11 and 99 (57.6%) patients and 114 (65.9%) control individuals were male. There were no age and sex significant differences between the patients and controls (p = 0.08 and p = 0.12, respectively). The FEV1% predicted values were significantly decreased in the patients compared with healthy controls.

E-selectin genotypes: DNA samples from 172 subjects with asthma and 173 healthy individuals were analyzed for Ser128Arg polymorphism of E-selectin gene. The frequencies of E-selectin genotypes in the patients and control individuals were found in accordance with those expected by the Hardy-Weinberg equilibrium (p = 0.41 and p = 0.57, respectively). Homozygous Arg128Arg genotype was found in 4 (2.3%) patients, heterozygous Ser128Arg genotype was found in 54 (31.4%) patients and 114 (66.3%) asthmatic patients were wild type (Ser128Ser)

Table 2: Ser128RArg allele and genotype frequencies in asthmatic patients and healthy controls

E-selectin *polymorphism*	Asthmatics n = 172	Controls n = 173	p-value
Allele -n/total (%)			
128 Ser (S)	282 (82)	332 (96)	
128 Arg (R)	62 (18)	14 (4)	<0.0001
Genotype-n/total (%)			
Ser/Ser	114 (66.3)	159 (91.9)	
S/R	54 (31.4)	14 (8.1)	0.000
R/R	4 (2.3)	0	

Table 3: Comparison of demographic data and lung function of asthmatic patients according to E-selectin Ser128Arg genotype

	Asthmatics (n=172)		
	R/R +S/R (n = 58)	S/S (n = 114)	p-value
Age (year)	55.6±9.76	57.05±13.95	0.48
Sex (M/F) (%)	28.3/41.1	71.7/58.9	0.103
sE-selectin	110.84±71.36	101.6±47.84	0.31
FEV1% (predicted)	48.88±21.22	57.05±22.95	0.02
PEF%	45.53±20.91	51.74±20.44	0.06
PEF variability (%)	27.67±7.93	25.76±8.12	0.156
Reversibility	19.12±7.55	18.95±5.49	0.86

ND: Not Done, FEV1: Forced Expiratory Volume at one second, PEF: Peak Expiratory Flow

(Table 2). The allele and genotype frequencies of the polymorphism were significantly differed between patients and controls. The 128Arg allele was more prevalent in patients than controls (OR 5.78; 95% CI, 3.07-10.86, p<0.0001). These findings suggested that the E-selectin polymorphism might be a risk factor of bronchial asthma in the Iranian population. There was found no homozygous mutant (Ser128Ser) genotype in the control subjects. Since the mutant Arg128Arg homozygous genotype was too low in the patients for releasing any statistical analysis, further analysis proved dominance for the arginine allele, i.e. Arg/Arg +Ser/Arg vs. Ser/Ser.

Soluble e-selectin levels: The serum levels of sE-selectin were analyzed in 172 patients and 173 control individuals. We found that the patients expressed significantly higher levels of sE-selectin compared with the controls (104.71+56.83 vs. 47.51+18.01, p<0.0001). However, analyzing the levels of sE-selectin in herozygous or homozygous asthmatic patients harboring 128Arg allele with homozygous individuals for Ser128 allele showed no significantly differences regarding the age, sex (Table 3). Meanwhile, mean initial percentage of predicted FEV1 and approximately PEF were significantly lower in the patients harboring Arg allele compared with homozygous Ser128Ser (p = 0.02 and p = 0.08, respectively). No significant differences were seen between Arg and Ser carried patients regarding PEF variability and reversibility (p = 0.06 and p = 0.156, respectively). However, in this study the polymorphism was not associated with circulating sE-selectin levels in the patients with asthma (p = 0.31).

Table 4: sE-selectin levels, genotypes and lung function activity according to severity of asthma

	Mild intermittent (n=27)	Mild persistent (n=31)	Moderate persistent (n=46)	Severe persistent (n=68)	p-value
S128R polymorphism-n (%)					
R/R+S/R	5 (18.5)	3 (9.7)	16 (34.8)	34 (50)	0.000
S/S	22 (81.5)	28 (90.3)	30 (65.2)	34 (50)	NS
sE-selectin (pg mL^{-1})	72.46±32.79	93.78±44.58	107.87±54.07	125.1±65.8	0.001
Age (year)	47.58±12.97	47.84±13.54	60.80±9.73	61.69±9.81	0.001
Lung function					
FEV1% predicted	78.08±9.24	73.04±9.2	52.33±15.64	36.87±17.53	0.0001
PEF	76.17±5.34	62.76±7.91	47.52±13.14	35.42±19.78	0.0001
PEF variability	17.92±3.56	20.52±4.58	27.56±6.66	31.09±7.41	0.001
Reversibility	14.92±4.23	17.12±5.43	19.0±5.13	21.25±7.23	0.0001

NS: Not Significant

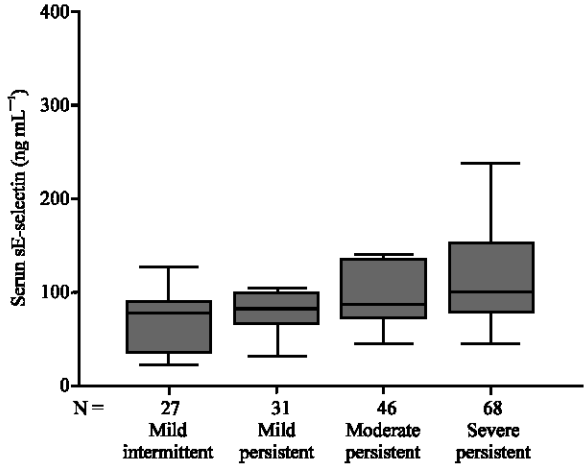

Fig. 1: The levels of sE-selectin in asthmatic patients

E-selectin polymorphism and severity of asthma: Asthmatic patients were classified into four categories of severity based on that has previously been recommended (National Asthma Education and Prevention Program.). The asthma severity in our population is mild intermittent asthma 15.7%, mild persistent 18%, moderate persistent 26.7% and severe persistent 39.5% of asthmatic adults. As the Table 4 shows the level of sE-selectin shows a stepwise increase with the progression of severity status of asthma ($p = 0.001$). Otherwise, higher level of sE-slectin was related to a stepwise increased in severity of asthma (Fig. 1). The mean age of the patients with severe persistent asthma was higher than that of those with mild intermediate asthma ($p = 0.001$), but there was no significant difference between the other groups. Otherwise, the lung function was also significantly decreased from mild intermediate to severe persistent asthma. As the mean baseline of percent predicted FEV1 was not significantly differed between mild intermediate and mild persistent asthma groups ($p = 0.2$), but it decreased in the moderate and severe persistent asthmatics ($p<0.0001$) compared with that of the mild intermediate group. In addition the frequencies of 128Arg allele carriage were significantly different in the mild intermediate, mild persistent, moderate persistent and severe persistent asthma groups (18.5, 9.7, 34.8 and 50%, respectively).

DISCUSSION

In this case-control study of asthmatics of varying severity we found that the levels of serum E-selectin were elevated on severity status of asthma. On the others hand, stepwise increase of sE-selectin levels were observed from intermittent to severe persistent asthma. In addition, our results have shown that there is an association between clinical expression of asthma and E-selectin gene Ser128Arg polymorphism. Arg allele was over-represented in asthmatic patients in comparison to controls. Thus, this allele might confer susceptibility against the development of asthma. This finding is in agreement with a study has been showed that asthma is characterized by airway hyper-responsiveness and inflammation of the lung, accompanied by the accumulation of lymphocytes and eosinophils (Hakansson *et al*., 1995).

On the basis of the strong association of S128R with asthma, we were interested in whether a correlation exists between the concentration of sE-selectin in serum and S128R genotypes. We found that Arg allele accounts for 18% (Table 2) in the asthmatics group, which is significantly higher than in normal controls (4%, $p<0.05$). It indicates that Arg allele is associated with early onset asthma and may be a risk factor for asthma. The role of adhesion molecules in asthma is supported by several studies showing an increased expression of adhesion molecules in bronchial epithelial and endothelial cells from asthmatics (Gosset *et al*., 1995; Vignola *et al*., 2000). The association of the E-selectin genotype and phenotype (sE-selectin levels) and analysis of the association with a clinical outcome is the novel aspect of the present study.

Increased expression of soluble E-selectin is associated with a number of inflammatory conditions including atherosclerosis (Li *et al*., 2005). In addition, the increased expression was also observed in non-insulin-dependent diabetes (Bannan and Grant, 1998).

Changes in the concentration of soluble selectins in plasma usually reflect altered cell surface turnover and proteolytic cleavage and therefore, these changes are often used as markers of a role for the selectins in asthma and allergy (Venge *et al*., 1994; Dogu *et al*., 2002). There was some reports that plasma levels of soluble sE-selectin in homozygous Arg128Arg and heterozygous Ser128Arg subjects were significantly higher than in wild-type Ser128Ser (Mlekusch *et al*., 2004) and the 128Arg allele enhanced thrombin generation and fibrin formation significantly (Yoshida *et al*., 2003).

In the present study, the levels of sE-selectin related to clinical asthma severity. Thus, higher sE-selectin levels were related to decrease in airway conductance and disease activity. This finding is accordance with accumulation of inflammatory cells in the airways of asthmatics (Amin *et al*., 2000).

The Ser128Arg polymorphism lies in the EGF domain of E-selectin that is conserved among all selectin. Although the principal ligand contact points of the selectins lie within the lectin domain (Somers *et al*., 2000), domain swaps between E-selectin have suggested that the EGF-domain can modulate the binding properties of the lectin domain to surface-immobilized ligand without affecting the equilibrium binding properties toward soluble ligand (Kansas *et al*., 1994). The nucleotide substitution of E-selectin EGF domain has been shown to profoundly affect the ligand binding affinity. The polymorphism studied is located within a gene which is crucial for the recruitment of leukocyte in to airway endothelium, thus an association of the Arg allele of Ser128Arg single nucleotide polymorphism with asthma could be reasonable. The relationship between the E-selectin Ser128Arg polymorphism and asthma may reflect an amplified inflammatory response resulting from the action of altered selectin molecules containing the Ser128Arg mutation. The substitution of arginine for serine (561A to 561C nucleotide) has been shown to dramatically decrease binding specificity while increasing affinity for additional ligands (Wenzel and Spear, 1994), resulting in an increase in cellular adhesion of two-to threefold. This mutation alters selectin binding specifically (Revelle and Beck, 1996) leading to a gain of function under flow conditions, possibly amplifying the number of leukocytes that roll and subsequently arrest on endothelium (Yoshida *et al*., 2003). This tethering mechanism could theoretically amplify the number of leukocytes interacting with mutated airway endothelial cells during bronchial asthma. The E-selectin 128Arg allele may thus increase leukocyte adherence to activated airway endothelium, thereby contributing to the progression of bronchial asthma. Upregulation of endothelial adhesion molecules facilitates the interaction with leukocytes and platelets (Tsakiris *et al*., 1999). E-selectin is of particular interest in this context, because it is expressed on activated endothelial cells and therefore induced localized inflammation at the airway tract. When E-selectin is shed from the endothelial surface it can be detected as soluble sE-selectin in the plasma (Rauchhaus *et al*., 2002). It has been shown that the level of sE-selectin increase in the severe asthmatic patients compared with mild/moderate asthma (Hamzaoui *et al*., 2001).

It was hypothesized that frequent genetic variants may contribute significantly to the genetic risk for common and complex phenotypes (Risch, 1996) and that therefore, high prevalence of the 128Arg allele also provides a strong biological rationale for the involvement of this polymorphism in the regulation of secretion of serum sE-selection levels in asthmatics. However, the presence of the 128Arg allele does not seem to be correlated with higher levels of circulating sE-selectin levels. Polymorphism is in the coding region of the gene. Polymorphisms in this region do not normally affect gene expression levels and consistent with Rauchhaus *et al*. (Rauchhaus *et al*., 2002) we did not find an association between sE-selectin levels and the S128R polymorphism.

It is noteworthy that while the wild type E-selection recruits specifically activated Th-1 lymphocytes (Austrup *et al*., 1997) which produce proinflammatory cytokines and chemokines (Galimberti and Searpini, 2004), the presence of the S8128R polymorphism extends the range lymphocytes recruited by E-selection, including Th2 and B lymphocytes (Rao RM 2002). This subset of T cells produces Interleukin 4 (IL-4) IL-5 and IL-13, leading to recruitment of eosinophils and enhances production of immunoglobulin (particularly IgG and IgE) from activated B cells and increase recruitment of eosinophils and other leukocytes to in flamed airway endothelium. On the other hand, children were shown to have elevated serum levels of E-selectin during acute asthma exacerbation (Tang *et al*., 2002).

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