INFLAMMATORY MARKERS AND INTERLEUKIN-6 (-174 G/C) GENE POLYMORPHISM IN PERIPHERAL VASCULAR DISEASE

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ABSTRACT

Objective: *Peripheral vascular disease (PVD) is an atherosclerotic and prothrombotic process leading cause of mortality. Atherosclerosis is recognized as a chronic inflammatory disease and starts as a protective response in damage to the endothelium and smooth muscle cells of the wall of the artery. The aim of this study was to investigate the association of interleukin-6 (-174 G/C) polymorphism with pro- and anti-inflammatory cytokines such as Interleukin-6 (IL-6), Interleukin-8 (IL-8), and Interleukin-1 Receptor antagonist (IL-1Ra) in PVD.*

Material and Method: *A hundred-two patients with PVD and 65 healthy individuals as control group were included in this study. All subjects were genotyped for IL-6 (-174 G/C) using Restriction Fragment Length Polymorphism (RFLP).*

Results: *The plasma concentrations of hs-CRP, IL-6, IL-8, IL-1Ra were higher in patients with PVD than those of the controls. The frequency of GG genotype of IL-6 was more frequent than the other genotypes of IL-6 in patients with PVD and control group.*

Conclusion: *We suggest that the increased plasma levels of IL-6, IL-8, IL-1Ra and GG genotype of IL-6 seem to be a risk factor for the development of PVD.*

Keywords: *Atherosclerosis, inflammation, endothelium, polymorphism* **Nobel Med 2017; 13(1): 21-25**

PERİFERİK VASKÜLER HASTALIKTA İNTERLÖKİN-6 (-174 G/C) GEN POLİMORFİZMİ VE İNFLAMATUVAR BELİRTEÇLER

ÖZET

Amaç: Periferik Vasküler Hastalık (PVH) mortaliteye yol açan aterosklerotik ve protrombotik bir süreçtir. Ateroskleroz arter duvarının endotel ve düz kas hücrelerinin hasarına koruyucu cevap olarak başlayan kronik inflamatuvar bir hastalık olarak tanınmaktadır. Çalışmamızın amacı PVH'da İnterlökin-6 (-174 G/C) polimorfizmi ile İnterlökin-6 (IL-6), İnterlökin-8 (IL-8) ve İnterlökin-1 Reseptör antagonisti (IL-1Ra) gibi pro- ve anti- inflamatuvar sitokinler ile ilişkisini araştırmaktır.

Materyal ve Metot: PVH'ı olan 102 hasta ile kontrol grubu olarak da 65 sağlıklı yetişkin çalışmaya dahil edilmiştiır. Tüm kontrol ve hasta grubunda IL-6 (-174 G/C) genotiplemesi, Restriksiyon Parça Uzunluğu Polimorfizmi (RPUP) yöntemiyle yapıldı.

Bulgular: Plazma hs-CRP, IL-6, IL-8, IL-1Ra konsantrasyonları kontrol grubu ile karşılaştırıldığında, PVH grubunda anlamlı olarak yüksek bulundu. IL-6 GG genotip sıklığı diğer genotiplere göre PVH'da ve kontrol grubunda daha yüksek olarak saptandı.

Sonuç: IL-6 GG genotipi ve artmış plazma IL-6, IL-8, IL1Ra düzeylerinin PVH gelişiminde risk faktörü olabileceğini düşünmekteyiz.

Anahtar kelimeler: Ateroskleroz, inflamasyon, endotel, polimorfizm. **Nobel Med 2017; 13(1): 21-25**

INTRODUCTION

Peripheral vascular disease (PVD) is a marker of systemic atherosclerosis that affects particularly arteries of the lower extremities in PVD.

It is also associated with a marked increase in the risk of ischemic events.[1,2] Several studies have shown that inflammatory processes contribute to the development of PVD.[3] It has been suggested that circulating markers of systemic inflammation could be able to predict future cardiovascular disease such as myocardial infarction (MI), stroke (CVA) and PVD.[4] These markers include C-reactive protein (CRP), proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), IL-1 receptor antagonist (IL-1Ra) that are known potential biomarkers for cardiovascular diseases.[5-7] IL-6 (-174 G/C) polymorphism has also recently been suggested to influence the development of PVD.[7,8]

IL-8 is a proinflammatory cytokine involved in atherosclerosis.[9] It has been suggested that increased IL-8 expression leads to formation and progression of atherosclerotic plaques in PVD.[10,11]

IL-1Ra, a natural antagonist of IL-1 has anti-inflammatory properties. Expressions of IL-1 and IL-1Ra play a role the development and progression of atherosclerosis. Moreover, it has been proposed that IL-1Ra levels increase in arterial ischemia of lower extremities.[12]

In the light of these findings, we aimed to investigate the levels of hsCRP and cytokines such as IL-6, IL-8 and IL-1Ra and also genetic variants of IL-6 (-174 G/C) in PVD.

MATERIAL AND METHOD

A hundred-two Turkish patients (mean age: 62.78 ± 11.54 years) who were admitted to the Department of General Surgery, Istanbul Faculty of Medicine, Istanbul University were included in the study. The majority of the patients had concomitant diseases, as shown in Table 1. Any patients with co-existing renal impairment, pernicious anemia, hypothyroidism, malignancy, abdominal aortic aneurysm and patients taking medications such as thiazide diuretics were excluded from the study. The control group consisted of 65 unrelated healthy adults (mean age: 59.66 ± 10.36 years) recruited from our General Surgery Department, who did not have any findings suggestive of a vascular disease. Exclusion criteria for the control group included obvious symptoms or signs of peripheral vascular disease, ankle-brachial index lower than 0.9 on clinical assessment. In order to create a control group, without any peripheral vascular disease, the patients who were assigned to this group neither had any accompanying disease such as hyperlipidemia and hypertension. A written consent was obtained from each subject.

Blood samples were taken from the ante-bracheal vein after a 12-hour fasting. Venous blood samples collected in vacuum tubes were centrifuged. Serum was separated immediately and stored -20°C until studied for determination of cytokines. Serum hs-CRP levels were immediately determined. Serum cytokine levels were determined by ELISA kits (Biosource, California, USA). Samples for HbA1c determination were collected in vacuum tubes containing lithium heparin and studied immediately by using autoanalyzer (DPP modular systems, Roche).

Blood samples were collected in vacuum tubes containing disodium EDTA for determination of IL-6 polymorphism. Genomic DNA was isolated from peripheral leukocytes as described by Miller *et al.*[13] Isolated DNA samples were stored at -70°C until analysis for determination of IL-6 polymorphism. The isolated DNA was subjected to polymerase chain reaction (PCR) for determination of IL-6 genotype. The PCR amplification mixture contained each of primer 5'-TGACTTCAGCTTTACTCTTTGT-3' (forward) and 5'-CTGATTGGAAACCTTATTAGG-3' (reverse) primers. IL-6 genotyping was performed by polymerase chain reaction (PCR) and restriction digestion. PCR products were digested with Sfan 1 restriction enzyme. Digested DNA was transferred to 3% agarose gel stainned with ethidium bromide for detection of three genotypes (GG, GC, CC).[14] Separated bands were visualized under UV lamb. The genotypes were read by two independent individuals.

Statistical Analysis

Statistical analyses were performed using the SPSS software package, version 15.0. Distributions of genotypes and alleles were compared by chi-square test and continous variables were evaluated using Student t-test. Statistical analyses were also carried out by Kruskal Wallis test for effect of IL-6 genotype on serum IL-6 levels in controls and patients. Power analysis was performed using http://biostat.mc.vanderbilt.edu/wiki/bin/view/Main/PowerSampleSize. *p* value less than 0.05 was accepted statistically significant.

RESULTS

Table 1 shows the clinical characteristics of patients with PVD and controls. Of the 102 patients with PVD were consisted of 28% females (Female/Male: 29/73) and the control group were 45% females (Female/Male: 29/36) (p<0.05). There were no significant differences in ages between patients and control groups ($p>0.05$).

The mean plasma concentrations of glucose ($p<0.001$), cholesterol ($p<0.001$), triglycerides ($p<0.001$), HDL-cholesterol ($p<0.001$), LDL-cholesterol ($p<0.001$), and HbA1c ($p<0.001$), the ratio of cholesterol/HDL-cholesterol ($p<0.001$), hs-CRP ($p<0.001$), IL-6 ($p<0.001$), IL-8 ($p<0.001$), IL-1Ra ($p<0.001$) were higher in patients with PVD than those of the controls (Table 2).

The distributions of the genotypes and alleles of the study groups were consistent with the Hardy-Weinberg equilibrium. The genotype and allele distributions of IL-6 (-174 G/C) in the patients with PVD were not significantly different compared to those of the controls ($p>0.05$). The frequencies of GG, GC and CC distributions of IL-6 genotype were 46.1%, 31.4%, 22.5% in patients with PVD and were 48.4%, 37.1% and 14.5% in control group, respectively (Table 3). The frequency of GG genotype was more frequent than the other genotypes of IL-6 in patients with PVD and controls. The frequency of CC genotype was elevated in patient group as compared with controls but the difference was not significant. Although the levels of hs-CRP and IL-6 were higher in subjects with GG genotype than subjects with GC or CC genotype, the differences were not significant.

Table 1. Clinical characteristics of the patients and controls

Clinical feature	PVD patients (n= 102)	Controls (n= 65)	*p* value*
Sex (F/M) n(%)	29(28)/73 (72)	29(45)/36(55)	<0.05
Age (years)	62.78 ± 11.54	59.66 ± 10.36	>0.05
BMI (kg/m^2)	27.27 ± 1.75	25.73 ± 2.31	<0.05
Hypertension n(%)	84 (79.2%)	0	<0.001
Diabetes mellitus n(%)	87 (82.1%)	0	<0.001

*: $p<0.05$ as significancy level, **BMI:** body mass index, **PVD:** peripheral vascular diseasefemale

Table 2. Biochemical parameters and cytokine levels in patients with PVD and controls

Parameters	PVD (n= 102)	Controls (n= 65)	*p* value*
Glucose (mmol/l)	8.62±3.64	5.48±0.71	<0.001
HbA$_1$c (mmol/mol)	47.84±17.12	37.20±4.96	<0.001
Cholesterol (mmol/l)	4.12±1.17	4.92±1.02	<0.001
Triglycerides (mmol/l)	1.89±1.46	1.33±0.61	<0.001
HDL-chol (mmol/l)	0.86±0.25	1.29±0.36	<0.001
LDL-chol (mmol/l)	2.36±0.94	3.09±0.80	<0.001
Cholesterol/HDL-chol ratio	4.79±4.6	3.81±2.8	<0.001
hs-CRP (mg/l)	71.13±70.86	2.19±2.96	<0.001
IL-6 (pg/ml)	122.86±109.30	18.19±8.25	<0.001
IL-8 (pg/ml)	99.78±111.74	47.22±88.70	<0.001
IL1-Ra (pg/ml)	100.34±43.22	42.23±22.99	<0.001

PVD: Peripheral vascular disease, **HDL-chol:** HDL-cholesterol, **LDL-chol:** LDL-cholesterol,
*: $p<0.05$ as significancy level

DISCUSSION

Inflammatory processes at atherosclerotic plaques are an important factor for the progression and clinical outcome of atherosclerotic disease.[12] Several studies have shown that inflammatory processes contribute to the development of PVD.[5,7] Cytokines, key regulatory glycoproteins in inflammatory processes are associated with atherogenesis and modulate plaque morphology and stabilization.[15] IL-6, a multifunctional proinflammatory cytokine, regulates humoral and cellular responses and

Table 3. IL-6 (-174 G/C) genotypes and allele frequencies in PVD and controls

	PVD (n= 102) (n%)	Controls (n= 65) (n%)	*p* value
Genotypes			
IL-6			
GG	47(46.1)	33 (48.4)	NS
GC	32(31.4)	23 (37.1)	NS
CC	23 (22.5)	9 (14.5)	NS
Alleles			
G	126 (0.62)	89 (0.68)	NS
C	78 (0.38)	41 (0.32)	NS

NS: Nonsignificant, **PVD:** peripheral vascular disease

plays a central role in inflammation and tissue injury.[5,16] It also stimulates the production of acute-phase proteins by the liver. These proteins include CRP, serum amyloid A (SAA) and fibrinogen.[17,18]

Elevated levels of IL-6 and CRP used as markers of inflammation, have been found to predict the future risk developing MI and mortality in patients with coronary artery disease (CAD).[19,20] IL-6 like CRP, was found at the sites of atherosclerotic plaques.[21]

In our study, serum IL-6 and hs-CRP levels were higher in patients with PVD than those of the controls. Also, we found the ratio of cholesterol/HDL-cholesterol significantly increased in patients group than that of controls. Our results were consistent with the results of the previous studies. Vainas *et al*. and McDermott *et al*. proved that the level of CRP, along with total/HDL cholesterol ratio was the strongest predictor of PVD. [21,22] In the Rotterdam Study, it has been suggested that CRP could be used to predict the development of PVD and evaluate its severity.[23-25]

It has also been shown that the levels of IL-6, IL-1Ra, fibrinogen and C-reactive protein in patients with PVD are increased as compared to those without PVD.[26]

We found that the distribution of IL-6 (-174 G/C) genotypes between patients with PVD and controls was not significant in this study. The distribution of GG genotype was more frequent than the other genotypes in patients with PVD and control group. This finding may be attributed to the small number of subjects in our study. On the contrary of our study, Flex *et al*. and Libra *et al*. have shown that the GG genotype was significantly more frequent in patients with PVD.[8,9] In addition to these, Flex *et al*. suggested that there is a strong association between IL-6 gene polymorphism and PVD.[9] Tuygun *et al*. shown the distributions of IL-6 gene polymorphism were significantly different in peripheral arterial disease (PAD) with four or more risk factors such as DM, hypertension, CAD, dyslipidemia, family history of PAD and CAD than having fewer risk factors in patient group.[27] On the other hand, Danielsson *et al*. also suggested that high IL-6 levels are correlated with advanced PVD, but they found no association between the -174 IL-6 gene promoter polymorphism and PVD.[6] According to us, the frequency of GG genotype was compatible with the results of previous studies.

In the present study, we found that IL-1Ra, IL-8 levels were significantly higher in patients with PVD compared to those of the controls. It has been shown that IL-1Ra levels were correlated with the occurrence and stage of the disease in patients with PVD.[12] In addition, IL-1Ra has been shown to be up-regulated in atherosclerosis. Therefore, it is considered that IL-1Ra is to be a reliable marker of the activation of anti-inflammatory mechanism.[12,23]

Therefore, it is important to determine systemic markers which may reflect the inflammatory activity in the plaques. According to these results, we think that elevated IL-1Ra levels have anti-inflammatory effects by inhibiting the increased levels of pro-inflammatory cytokines in PVD. Moreover, Signorelli *et al*. reported that significantly higher levels of IL-8 were found in patients with PVD compared to those of the controls.[10] IL-8 is a neutrophil chemoattractant found in macrophage-rich atherosclerotic plaques.[15]

In the light of these findings, we suggest that inflammatory markers such as hs-CRP, IL-6, IL-8, IL-1Ra increase in patients with PVD. In addition to these, the distribution of GG genotype was more frequent than those of the other genotypes in PVD and control group when IL-6 (-174G/C) polymorphism is evaluated. However further studies are needed to determine the association between the distribution of IL-6 genotypes and PVD.

*The authors declare that there are no conflicts of interest.

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