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TRANSCRIPTIONAL ACTIVITY OF GENE ENCODING SUBUNITS R1 AND R2 OF INTERFERON GAMMA RECEPTOR IN PERIPHERAL BLOOD MONONUCLEAR CELLS IN PATIENTS WITH SLOW CORONARY FLOW

TRANSKRIPCIONA AKTIVNOST PODJEDINICA R1 I R2 KOJE KODIRAJU GENE ZA RECEPTOR ZA INTERFERON GAMA U MONONUKLEARNIM ĆELIJAMA PERIFERNE KRVI PACIJENATA SA SPORIM KORONARNIM PROTOKOM

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Summary

Background: Slow coronary flow (SCF) is a coronary artery disorder characterized with delayed opacification of epicardial coronary arteries without obstructive coronary disease. The pathophysiological mechanisms of SCF remain unclear. One of the possible mechanisms that may participate in the pathology of SCF is endothelial dysfunction related to the inflammatory process. Interferon gamma (IFN-γ) is an inflammatory cytokine that acts through its specific receptor composed of two subunits, IFN-γR1 and IFN-γR2. Transcriptional activity of the gene encoding these subunits influences IFN-γ activity. This study aimed to investigate the gene expression of IFN-γ receptor subunits in peripheral blood mononuclear cells (PBMC) from patients with SCF.

Methods: The study was performed with 30 patients (22 male/8 female) aged 35–76 (52.8±11.7 years) with SCF and 15 sex- (11 male/4 female), Body Max Index (BMI)- and age-matched (54.73±9.42 years) healthy subjects. Total mRNA was extracted from PBMC and was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The relative expression values ($2^{-\Delta\Delta Ct}$) between control and case groups were determined and the Mann-Whitney *U* test was used for statistical analysis.

Results: There was a significant increase in the gene expression of IFN-γR1 in PBMC from SCF patients *vs*. con-

Kratak sadržaj

Uvod: Spor koronarni protok (SKP) kao poremećaj koronarnih arterija odlikuje odložena opacifikacija epikardijalnih koronarnih arterija bez opstruktivne koronarne bolesti. Patofiziološki mehanizmi SKP još su nerazjašnjeni. Jedan od mehanizama koji potencijalno učestvuju u patologiji SKP je endotelna disfunkcija povezana sa inflamatornim procesom. Interferon gama (IFN-γ) je inflamatorni citokin koji deluje preko svog specifičnog receptora sastavljenog od dve podjedinice, IFN-γR1 i IFN-γR2. Transkripciona aktivnost gena koji kodira ove dve podjedinice utiče na aktivnost IFN-γ. Cilj ove studije bio je da se istraži genska ekspresija podjedinica receptora IFN-γ u mononuklearnim ćelijama periferne krvi (MĆPK) pacijenata sa SKP.

Metode: Studijom je obuhvaćeno 30 pacijenata (23 muškarca / 7 žena) starosti 35–76 (52,8±11,7) godina sa SKP i 15 (11 muškaraca / 4 žene) zdravih subjekata odgovarajućeg pola, indeksa telesne mase (ITM) i starosti (54,73 ±9,42 godina). Ukupna mRNK je ekstrahovana iz MĆPK i određena pomoću qRT-PCR. Određene su relativne vrednosti ekspresije između kontrolne i grupe pacijenata a Man-Vitnijev *U* test je upotrebljen za statističku analizu.

Rezultati: Postojao je značajan porast genske ekspresije IFN-γR1 u MĆPK pacijenata sa SKP u poređenju s kontrolama (P<0,0001), ali razlike između genske ekspresije

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trols (P< 0.0001); but the differences in IFN-γR2 gene expression were statistically insignificant between patient and control groups (P= 0.853).
Conclusions: It can be concluded that IFN-γR1 gene expression may influence the function of microvasculature and thereby contribute to the pathophysiology of SCF.

Keywords: slow coronary flow, interferon gamma receptor, gene expression, inflammation, coronary artery disease

IFN-γR2 pacijenata i kontrolnih subjekata nisu bile statistički značajne (P=0,853).
Zaključak: Može se zaključiti da genska ekspresija IFN-γR1 može uticati na funkciju mikrovaskulature i time doprineti patofiziologiji SKP.

Ključne reči: spor koronarni protok, receptor za interferon gama, genska ekspresija, inflamacija, koronarna arterijska bolest

Introduction

Slow coronary flow (SCF) has been defined as an angiographic finding characterized by delayed opacification of the epicardial coronary arteries in the absence of obstructive coronary disease which was first described by Tambe et al. in 1972 (1). Incidence of this common angiographic finding has been reported to be 1–7% in patients undergoing coronary angiography (2, 3), 40% in patients with normal arteries (4), 16% in cardiac syndrome X (CSX) patients (5) and 4% in patients undergoing angiography for rapid assessment of unstable angina (6). It has been reported more frequently in young men, most commonly smokers or with the history of smoking (2).

Only a limited number of studies have focused on SCF since the first description; therefore, the precise pathophysiological mechanisms and the clinical importance of SCF have not yet been extensively understood (7). Several proposed mechanisms for this phenomenon include: small-vessel disease, microvascular vasomotor dysfunction, diffuse atherosclerosis, endothelial dysfunction (3, 8, 9), platelet dysfunction (10), and imbalance of vasoconstrictor factors (11, 12). Occlusive disease of the small coronary arteries, a form of early-phase atherosclerosis, has also been proposed as a cause (13). According to recent studies, inflammation is another important etiologic factor (14, 15). Increased levels of inflammatory cytokines in SCF patients may be an indicator of endothelial activation and inflammation, which may lead to the SCF phenomenon (14).

Interferon gamma (IFN-γ), a type II IFN family cytokine, is an anti-viral agent produced by many immune cell types (16). It is an important cytokine with a multitude of functions. Besides its immunomodulatory and inflammatory activities (17), this cytokine is involved in atherogenesis, apoptosis (17, 18), nociceptive pathways (19), endothelial dysfunction (20) and progression of atherosclerosis (21).

IFN-γ acts through its specific receptor composed of two subunits, IFN-γR1 and IFN-γR2. Transcriptional activity of the gene encoding of these subunits influences IFN-γ activity. Since one of the possible mechanisms that may participate in the pathology of SCF is endothelial dysfunction related to inflammatory process and there is no evidence regarding the possible role of IFN-γ in SCF, the present study aimed to investigate the gene expression of IFN-γ receptor subunits in peripheral blood mononuclear cells (PBMC) from patients with SCF.

Materials and Methods

Study population

The study includes 30 SCF patients aged 35–76 (52.8±11.70 years) and 15 sex-, body mass index (BMI)- and age-matched (54.73±9.42 years) healthy subjects as controls. Entry criteria were typical anginal chest pain, positive treadmill test, normal angiogram and thrombolysis in myocardial infarction (TIMI) frame count (quantitative way of assessing coronary artery flow) greater than 23 frames (22). Patients with known coronary or peripheral vascular disease, ectatic coronary arteries, non-ischemic dilated cardiomyopathy, renal and hepatic dysfunction, evidence of ongoing infection or inflammation, hematological disorders, known malignancy and diabetes mellitus were excluded from the study. A questionnaire was administrated to obtain general information from each patients including age, sex, BMI, systolic and diastolic blood pressures. The study was approved by the Medical Ethics Committee (ethical approval code: IRumsu.rec.1393.26) at Urmia University of Medical Sciences, Urmia, Iran, and all subjects gave written informed consent.

Study protocol

Ten milliliter whole blood samples were collected from the basilic vein into tubes containing EDTA and the separation of PBMC from whole blood was accomplished through density gradient centrifugation using Ficoll. Five ml of Ficoll–Hypaque (Baharafshan, Iran) was stratified under 20 mL of peripheral blood and phosphate-buffered saline (PBS) mixture, and then the sample was centrifuged at 800 g for 20 min at room temperature. PBMC were collected from buffy coat layer and were washed twice with PBS. Total RNA was extracted from PBMC, using RNX-Plus Solution (CinaGen Co.,Tehran, Iran) and the purity of RNA extracts was qualitatively evaluated by electrophoresis in 1% agarose gel stained with ethidium bromide and quantified by measuring absorption at 260 nm and 280 nm, then calculating the 260A/280A ratio (260A/280A must be greater than 1.8) in a biophotometer (Ependorf AG, Germany). Primers for amplification of IFN-γ receptor's subunits and β-actin as an endogenous control were as following, depicted in *Table I* (GenFanavaran Co., Tehran, Iran) (23).

Table I Primer sequences used for qRT-PCR.

Primers	Sequence	Ta
IFN-γ R1	5′-ATACCGAAGACAATCCAGGAAAAGTGGAACA-3′ (forward) 5′-GCGATGCTGCCAGGTTCAGACTGGTTACTA-3′ (reverse)	68 °C
IFN-γ R2	5′-CAAGGACAGCTCACCAAAGGATGACG-3′ (forward) 5′-CAGCTCCGATGGCTTGATCTCTTCCA-3′ (reverse)	68 °C
β-actin	5′-TCACCCACATGTGCCCATCTACGA-3′ (forward) 5′-CAGCGGACCCGCTCATTGCCAATGG-3′ (reverse)	68 °C

Ta: annealing temperature

Table II Demographic and clinical characteristics of SCF patients and control group†

Parameter	Control	SCF	P value
Age (years)	54.73±9.42	52.8±11.70	0.583
Sex (female/male)	4/11	8/22	0.060
BMI (kg/m^2)	27.74±3.47	26.93±4.46	0.543
Systolic BP (mmHg)	139.27±11.46	128.07±15.87	0.019*
Diastolic BP (mmHg)	89.60±9.70	78.53±13.11	0.006*
Heart rate (number)	76±9	74±7	0.321
WBC (×1000 mm^3)	6.44±1.17	7.52±2.68	0.289
Smoking (%)	0	60.71	0.0002*
Family history of CHD (%)	0	26.66	0.027*
Aspirin (%)	–	70	–
Statins (%)	–	63.33	–
Beta-blockers (%)	–	66.66	–

† Results expressed as mean ± standard deviation. BP: Blood pressure, BMI: Body Mass Index, WBC: White Blood Cell, SCF: Slow Coronary Flow, CHD: Coronary Heart Disease

Complementary DNA (cDNA) were synthesized by using the BioRT cDNA first strand synthesis kit (Bioflux-Bioer, Hangzhou, China) according to manufacturer's instructions. In order to determine the gene expression of IFN-γR1 and IFN-γR2, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the SYBR Green RT-PCR kit (Bioneer, Accu Power 2X Green Star^{TM} qPCR Master mix, Deajeon, Korea). PCR conditions were as follows: 94 °C for 15 min; 45 cycles: 94 °C for 15 s, 68 °C for 30 s and 72 °C for 30 s. All reactions were carried out in duplicate. Relative gene expression was determined by ΔΔCt method between patients and controls (24). Data are presented as the fold change in gene expression normalized to β-actin as an endogenous reference.

Statistical analysis

The data were analyzed by Statistical Package for Social Sciences (SPSS) 22 software. In order to check the normality of the distribution, Kolmogorov-Smirnov test was performed. In case of a normal distribution, the *t*-student test was used; otherwise, the Mann-Whitney *U* test was performed. All values were expressed as means ± standard deviation (mean ± SD). Differences were considered to be significant at P value less than 0.05.

Results

Demographic and clinical characteristics of both groups are depicted in *Table II*.

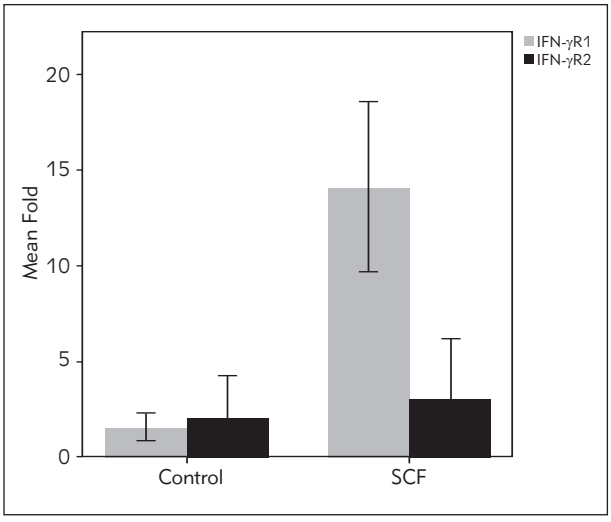

Figure 1 IFN-γR1 and IFN-γR2 mean fold changes in control and SCF groups.

In SCF and control groups, the mean differences in systolic blood pressure and diastolic blood pressure were statistically significant. There was an increase in mean WBC count in SCFs *vs*. controls, but it was not significant. This means leukocyte count had no effect on differences in expression of the studied gene.

Expression of gene encoding IFN-γ receptor subunits was higher in SCF patients *vs*. controls (*Figure 1*). There was a significant increase in gene expression of IFN-γR1 in PBMC from SCF patients *vs*. control individuals (14.09±11.43 *vs*. 1.6±1.31; P<0.0001), but the differences in IFN-γR2 gene expression were statistically insignificant between patients and controls groups (3.08±8.09 vs. 2.08±3.93; P=0.853). In this study, Spearman analysis between IFN-γR1 and IFN-γR2 expression revealed that there was a positive correlation at the 0.001 level (r=0.306, P=0.046). In multiple logistic regression analysis, gene expression of IFN-γR1 strongly (OR: 1.761, P=0.018, CI=1.104–2.809) predicted SCF.

Discussion

Althogh several possible mechanisms have been proposed for the SCF phenomenon, its exact etiology remains unclear (7). Histopathological (3) and intravascular ultrasound studies (25) suggest a pathophysiologically relevant interaction between endothelial dysfunction (26), diffuse atherosclerosis and the SCF phenomenon despite angiographically normal coronary arteries (25). In recent years, mechanism-oriented studies have shown inflammation plays an important role in the initiation, development as well as evolution of atherosclerosis (27, 28). Inflammation is a major contributing factor in many cardiovascular events and its association with the clinical setting of coronary artery disease has been demonstrated (29). Recent studies have reported a possible role of inflammatory mechanisms in the pathology of SCF (14, 15). Turhan et al. showed increased plasma levels of soluble adhesion molecules in patients with SCF (15) indicating increased levels of inflammatory cytokines in these patients may be possible markers for endothelial activation and inflammation that may lead to SCF (14). Thereby, it can be expected that differences in the expression of inflammatory cytokines and their receptors exist in these patients. Among different inflammatory cytokines, IFN-γ is considered to have a significant role in the progression of atherosclerosis (18, 21). By stimulating cytokine production, recruiting inflammatory cells to the site of injury through increased expression of chemokines and adhesion molecules and regulating the rate of proliferation, differentiation and apoptosis, IFN-γ has a potential impact on the process of atherosclerosis development (21). It has been shown to increase CD40 expression on macrophages, endothelial cells and smooth muscle cells (30) and therefore can potentially promote further CD40/CD40 Ligand mediated inflammation in atherosclerosis (18). Increased serum level of CD40, an indirect marker of CD40/CD40L, was demonstrated in SCF (7). IFN-γ triggers the formation and release of reactive oxygen species which leads to oxidative stress and endothelial dysfunction (31). Oxidation of oxidation-sensitive substance like B-vitamins (eg. folic acid and B12), essential cofactors in homocysteine (Hcys)-methionine metabolism, cause hyperhomocysteinemia (32) and cellular immune activation found in coronary heart disease (31) as well as SCF (8). All the mentioned actions support IFN-γ role in inflammation, endothelial dysfunction and eventually atherosclerosis. The half-life of circulating IFN-γ is short, and systemic measurements cannot reliably assess its activity (33). IFN-γ stimulates the production of neopterin, a by-product of the guanosine triphosphate-biopterin pathway, by activated macrophages and monocytes (33). It is known that neopterin level predicts adverse prognosis in coronary artery disease (34). Elevated serum level of neopterin as a systemic marker of IFN-γ was shown in SCF patients by Varol et al. (35).

Preinflammatory condition in the vessels is associated with activation of circulating mononuclear cells in coronary artery disease (CAD) patients which is reflected by IFN-γ (23). According to Fernandes et al. marked activation of T lymphocytes and increased expression of IFN-γ exist in CAD patients (36). Oleveria et al. reported that PBMC are capable of producing a high rate of chemokines and cytokines such as IFN-γ involved in the regulation of lymphocytes and monocytes migration and remaining in atherosclerotic lesions in CAD patients (37).

Recent studies have reported increased mononuclears activation leads to elevated generation of

superoxide and production of proinflammatory cytokines (23). Dabek et al. showed that several fold increase in the expression of IFN-γ receptor subunits may be responsible for the prooxidative state in CSX via higher responsiveness to this cytokine and by affecting the function of microvasculature may participate in the pathology of CSX (23).

In our study, both subunits were over-expressed, but the gene expression of IFN-γR1 was significant. To our knowledge, this is the first report demonstrating the relationship of SCF with gene expression of IFN-γ receptor subunits.

Since the pathological mechanisms of slow flow phenomenon remain controversial, the information on the role of IFN-γ in order to prove the hypothesis of the role of inflammation in this disease can be helpful. According to our results, it can be concluded that IFN-γR1 gene expression may influence the function of microvasculature and thereby contribute to the pathophysiology of SCF. Further studies on a larger scale are needed to elucidate the etiology of SCF.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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