IL-23 gene expression in PBMCs of patients with coronary artery disease

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Abstract. *Objective:* Both adaptive and innate immune systems are involved in coronary artery disease (CAD). The aim of this study was to evaluate TH17 cytokines expression profiles in un-stimulated peripheral blood lymphocytes (PBMCs) of patients with coronary artery disease.

Methods: Expression profiles of IL-17, IL-23, and TGF- β 1 were determined in individuals with and without CAD using Real-time PCR.

Results: A significant decrease in IL-23 gene expression in un-stimulated PBMCs of patients with CAD compared to those without CAD was found ($p = 0.003$, OR = 0.045, 95% CI: 0.006–0.355).

Conclusion: Our data reinforce the potential role of the IL-23 as a critical regulatory molecule that bridges the innate and adaptive arms of the immune system in the complex mechanisms associated with the development of atherosclerosis.

Keywords: Cytokine, gene, expression, CAD, IL-23

1. Introduction

Atherosclerosis is a chronic inflammatory disease of vessel walls with involvement of both innate and adaptive immune system [1]. However, current information regarding the underlying mechanisms of the disease is controversial. Several lines of evidence based on animal models and humans studies suggest that immune cells directly participate in development of the atherosclerotic plaques [1,2]. Considerable evidence supports the involvement of adaptive immune system in development and progression of coronary artery disease (CAD) [3]. Th1 cytokines with IFN- γ as the

prototype of this group are widely accepted as a key regulator of immune mechanisms in atherogenesis [1]. The role of Th2 pathway in atherosclerotic process is still controversial depending on the experimental model studied and the stage and/or site of the lesion. Recent data have focused on the imbalance between Th1 and Th2 cytokines and the role of microenvironment in initiation and progression of atherosclerosis in coronary arteries [4,5]. Th-17 and Treg cells have emerged as important regulators of inflammatory reactions in CAD. The contribution of these cell types as possible participants in atherosclerotic lesion development is indicating the complex mechanisms and the variety of cell types involved in disease process [6–8].

Th17 cells secrete IL-17A, IL-17F, IL-21, IL-22 and IL-23. Th17 or so called Th3 cytokines bridge innate and adaptive immune responses and have also regulatory effects on Th1- and Th2-cells cytokine production and functions. Convincing data support the idea of Treg and B cells contribution during atherogenesis [6–8].

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Table 1
Primer sequences for real-time PCR quantification

Gene	Primer pair sequences	Amplicon size
HPRT F	5'-CCTGGCGTCGTGATTAGTGAT-3'	131 bp
HPRT R	5'-AGACGTTTCAGTCCTGTCCATAA-3'	
TGF- β 1 F	5'-CGACTACTACGCCAAGGA-3'	150 bp
TGF- β 1 R	5'-GAGAGCAACACGGGTTC-3'	
IL-23 F	5'-GGACAACAGTCAGTTCTGCTT-3'	115 bp
IL-23 R	5'-CACAGGGCTATCAGGGAGC-3'	
IL-17 F	5'-CATAACCGGAATACCAATACCAAT-3'	104 bp
IL-17 R	5'-GGATATCTCTCAGGGTCTCATT-3'	

In the present study we determined the expression profile of IL-23, IL-17 and TGF- β 1 in un-stimulated peripheral blood lymphocytes (PBMCs) of patients with CAD (CAD+). We compared cytokine profile results with those observed in un-stimulated PBMCs from individuals without CAD (CAD-).

2. Material and methods

2.1. Subjects

We assessed a series of patients who underwent coronary artery angiography because of chest pain at Cath Lab Center of Dr. Shariati Hospital, Tehran, Iran. The procedures were done and interpreted by trained cardiologists. We recruited 25 consecutive individuals that were defined as having CAD according to the coronary angiography and had significant stenosis (decrease of the internal diameter of more than 50%) in all 3 main coronary arteries (CAD+). In addition, 25 consecutive individuals with normal coronary angiographic studies (considered as CAD-) were all included in the study. The study was approved by Ethics committee of Tehran University of Medical Sciences (TUMS). Personal/demographic questionnaire was completed also for all patients and all subjects gave written informed consent before participation in this study. The whole procedures of angiography and interpretation of results were done by trained cardiologists. Significant stenosis was defined as a decrease of the internal diameter of more than 50% in all 3 main coronary arteries. We recorded the hypertension, diabetes mellitus, hyperlipidemia, smoking status and family history of other cardiovascular disorders such as myocardial infarction (MI) or premature coronary heart diseases (premature CHD) in first degree relatives for all the participants. Subjects with a history of taking medication for hypertension or those with an average blood pressure of $\geq 140/90$ mmHg were identified as having hypertension. Diagnosis of diabetes mellitus and dyslipidemia were

performed according to the American Diabetes Association (ADA) criteria [1] and National Cholesterol Education Program Adult Treatment Panel III [9], respectively.

2.2. Isolation of RNA and synthesis of cDNA

Total cellular RNA was extracted from 5 cc of each individual's fresh peripheral blood collecting in heparin-containing tubes. RNA extraction was then carried out as described by Tripure reagents (Roche) manufacturer's instructions. RNA pellets were dissolved in 30 μ l DEPC water and were stored at -70°C . The purity and concentration of RNA were determined by measuring OD260/280 ratio and OD260, respectively on a Nano-Drop spectrophotometer (NanoDrop Thermo Scientific 2000). Preparations with a ratio of OD260/280 that was lower than 1.6 were discarded. For cDNA synthesis, Expand Reverse Transcriptase (Roche) was used as the manufacturer recommends.

2.3. Quantitative real-time PCR

To investigate cytokine mRNA expression level, quantitative real time PCR was conducted using commercially available kit SYBR Premix Ex Taq II (Takara, Japan) and an ABI stepOneTM Sequence (Applied Biosystems, CA, USA). The reaction mixture carried out in 48-well plates with the final volume of 20 μ l consisting 10 μ l of 2 $\times$ SYBR Premix Ex Taq, 1 μ l of forward and reverse primers (1 μM), 7.2 μ l of di-ionized water, 0.2 ROX and 1.5 μ l of 10-fold diluted cDNA product. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene was used as internal control for normalization. PCR reactions were carried out in duplicates using specific oligonucleotide primers for amplification. The sequences of primer pairs used are given in Table 1. Real-time PCR was performed with an initial denaturation step of 10 s at 95°C , 40 cycles at 95°C for 5 s and 60°C for 30 s.

Fig. 1. Cytokine gene expression in patients with CAD+ versus CAD- individuals. There was a significant decrease in IL-23 gene expression CAD+ versus CAD- ($P = 0.003$).

Table 2
Baseline characteristics in Iranian subjects with and without CAD

Variable	CAD+	CAD-	p value
sex (male) (%)	71%	68%	0.5
age* (yr)	60 ± 8	59 ± 11	0.9
Current smokers N(%)	17%	10%	0.4
Hypertension N(%)	75%	47%	0.05
Diabetes mellitus N(%)	57%	10%	0.001
Dyslipidemia	36%	16%	0.07
TChol*	183 ± 42	184 ± 44	0.9
TG*	185 ± 77	148 ± 47	0.07
LDL*	108 ± 36	129 ± 42	0.1
HDL*	40 ± 10	41 ± 8	0.6
Past MI (%)	61%	0%	< 0.000

TChol: Total Cholesterol, TG: Triglyceride, LDL: Low Density Lipoprotein, HDL: High Density Lipoprotein, Past MI: past history of myocardial infarction, *Variables are described based mean ± standard deviation.

2.4. Statistical analysis

Gene expression data were normalized against HPRT as reference gene. Data Analysis was performed using the $2^{-\Delta\Delta CT}$ method. The significance of differences for gene expression between control and test groups

was established by Mann-Whitney Test. Test of normality for distribution of variables was performed using Shapiro-Wilk test. Analysis was performed by SPSS version 15 and $P \leq 0.05$ was considered as significant statistical analysis.

3. Results

The mean age ± standard deviation (SD) was 60 ± 8 years in CAD+ (71% men) and 59 ± 11 years (68% men) in CAD- subjects, respectively. Although body mass index was similar in both groups (27 ± 2 in CAD+ and 27.5 ± 2 in CAD-), CAD+ patients had classic cardiovascular risk factors more commonly than CAD- individuals. Table 2.

3.1. Cytokine gene expression in patients with CAD (CAD+) compared to individuals without CAD (CAD-)

Figure 1 shows cytokine gene expression in CAD+ patients and CAD- individuals. Quantitative real-time

PCR analyses disclosed TGF- β 1 gene expression was similar in both groups. It was also the case for IL-17 gene expression. Nevertheless, a significant decrease in IL-23 gene expression in un-stimulated PBMCs of patients with CAD+ compared to those without CAD was found ($p = 0.003$, OR = 0.045, 95% CI: 0.006–0.355). After adjustment for classical risk factors we found that the association between CAD and IL-23 remained still significant and was independent of Diabetes mellitus, Hypertension, age and sex ($p = 0.026$, OR = 0.002, 95% CI: 0.001–0.484).

4. Discussion

Several lines of evidence driven from numerous functional genomic studies support the fact that immune cells especially T cells play a critical role during lesion development in atherosclerosis [10]. IL-23-IL-17 axis has been recently linked to the pathogenesis of atherosclerosis [10]. However, current data regarding the exact role of Th17 cells in various stages of atherosclerosis has been controversial and requires further elucidation [10–13]. In this study, we have found a significant down-regulation of IL-23 gene expression in un-stimulated PBMCs of patients with CAD. IL-23 is actively involved in expansion of Th17 cells [14]. Th1-driven response is closely involved in autoimmunity and pathogenesis of several chronic inflammatory disorders including atherosclerosis [15]. As Th17 is involved in activation and derivation of Th1 responses, therefore it might be speculated that Th17 cells have a pro-atherogenic function. In contrast several previous reports have found a protective role of IL-17 in atherogenesis indicating a complex regulatory function of Th17 cells in various inflammatory conditions [10, 11]. It has recently been shown that IL-23 can act as a negative regulator of IL-12 induced IFN- γ production and Th1 immunity independent of other Th17 cytokines [16]. This might imply that IL-23 is involved in pathogenesis of CAD by other mechanisms rather than Th17 cell expansion and regulation, which must be further investigated.

TGF- β 1 is suggested to be a promising anti-inflammatory cytokines which contribute in CAD pathogenesis. TGF- β 1 is produced by various cell types including Treg cells and inhibit proliferation, activation and differentiation of T cells towards Th1 and Th2 and regulates the expression of other cytokines from Th1-, Th2- and Th17-cells. Some studies suggested that inhibition of TGF- β 1 signaling pathway is asso-

ciated with increased differentiation of T-cells towards Th1 and Th2 phenotypes accelerating the atherosclerosis processes [17–19]. Our results did not show any significant change in TGF- β 1 expression in CAD+ and CAD- subjects which might be again due to the role of other confounding factors and, therefore, requires to be further elucidated.

It is possible that the different and often contradictory results obtained in previous studies regarding the role of inflammatory cytokines in CAD may be partly due to the different experiment set-up including differences in diagnostic criteria, gender, age, diet and most importantly presence of other clinical phenotypes including obesity and diabetes. All these cytokines are involved in most inflammatory processes having pro- or anti-inflammatory nature in various conditions. Some of these cytokines belong to a family of cytokines with similar signal transduction receptor that can potentially alter the results of studies in experimental models.

In conclusion, our data reinforce the potential role of the IL-23 as a critical regulatory marker that bridges the innate and adaptive arms of the immune system in the complex mechanisms associated with the development of atherosclerosis.

Conflict of interest

The authors declare no conflict of interest.

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