IL-32 Serum Levels in Coronary Artery Disease Patient and its Relationship with IL-6 and TNF-α Serum Levels

Mina Mohammad-Rezaei  
Shahrekord University of Medical Science

Reza Ahmadi  
Shahrekord University of Medical Science

Ali Rafiei  
Shahrekord University of Medical Science

Arsalan Khaledifar  
Shahrekord University of Medical Science

Shohila Fatahi  
Shahrekord University of Medical Science

Azadeh Samiei-Sefat  
Shahrekord University of Medical Science

Shohreh Emami  
Shahrekord University of Medical Science

Nader Bagheri (n.bagheri1985@gmail.com)  
Shahrekord University of Medical Science  https://orcid.org/0000-0001-5196-5513

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Abstract

Coronary Artery Disease (CAD) is a chronic inflammatory disease caused by atherosclerosis and arteries become clogged due to plaque formation, fat accumulation, and various sorts of immune cells. IL-32 is a new proinflammatory cytokine, which enhances inflammation through inducing different inflammatory cytokines. The purpose of current research was to assess IL-32 serum levels in coronary artery disease subjects and its relationship with serum levels of IL-6 and TNF-α. Forty-two subjects diagnosed with CAD and thirty-nine control subjects were enrolled in the research. Serum levels of IL-6, TNF-α, and IL-32 were measured using the enzyme-linked immunosorbent assay (ELISA). IL-32, TNF-α, and IL-6 serum levels were significantly higher by 2.7, 3.48, and 3.2-fold in the CAD subjects than in control subjects, respectively. Moreover, no significant difference was found in TNF-α, IL-6 and IL-32 serum levels with the clogged arteries number in the CAD group. TNF-α and IL-32 serum levels in the CAD subjects with cardiac arterial stenosis in one major vessel were significantly increased than CAD subjects with cardiac arterial stenosis in more than one major vessels. ROC curve analysis revealed that serum levels of IL-32, TNF-α, and IL-6 showed good abilities in predicting CAD. Also, Multiple logistic regression analyses suggested that TNF-α, IL-6, and IL-32, serum levels of LDL and ox-LDL were independently related to the presence of CAD, while HDL serum levels were not. TNF-α, IL-32, and IL-6 showed an increase in CAD group and serum levels of these cytokines showed good abilities in predicting CAD. Our data suggested the involvement of TNF-α and IL-32 in the early stage of CAD.

Introduction

Coronary Artery Disease (CAD) is one of the most common causes of death globally (1). CAD is a chronic inflammatory disease as a result of atherosclerosis. Arteries in patients with CAD become clogged due to plaque formation, fat accumulation, and various sorts of immune cells that this process is called atherosclerosis (2). Epidemiological studies have reported numerous critical environmental and genetic risk factors like male gender, elevated blood pressure, elderly, increased low-density lipoprotein cholesterol (LDL-C), obesity, lifestyle factors such as lack of exercise, unhealthy diet, and smoking, associated with CAD (2).

Macrophages play a vital role in atherogenesis by producing inflammatory mediators and cytokines and accumulating cholesterol. During the course of atherosclerosis, macrophages take up oxidized low-density lipoprotein (ox-LDL), and the cholesterol derived from ox-LDL is accumulated in these cells. Macrophages pumped accumulated cholesterol by reverse cholesterol transfer (RCT) and defect in this process may lead to transformation of macrophages to foam cells (3). Macrophage necrosis and cholesterol secretion in the environment lead to atherosclerosis (4). Atherosclerosis is distinguished by the recruitment of innate and specific immune cells such as monocytes and lymphocytes to the artery wall. The smallest accumulation of ox-LDL causes the overlying endothelial cells to be stimulated and produce various inflammatory cytokines. These inflammatory cytokines perform several actions including activation and chemotaxis of leukocytes, activation of smooth muscle cells and macrophages, facilitating the transport of lipids into the plaque, increasing the permeability of endothelial cells, and
increasing the entry and transport of lipoproteins (5). IL-32, additionally named tumor necrosis factor (TNF)-α-induced factor and natural killer cell transcript (NK)-4, is a new proinflammatory cytokine, which enhances inflammation by inducing different proinflammatory cytokines (e.g., TNF-α, IL-6, IL-8, and IL-1β) (6, 7). Recent studies demonstrated that IL-32 has a vital function in inflammatory diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and type 2 diabetes mellitus (T2DM) (8–10). The association of TNF-α, IL-6 and, IL-32 has not yet been ascertained. Therefore, the object of the current research was to assess IL-32 serum levels in the coronary artery disease group and its relationship with serum levels of IL-6 and TNF-α.

**Subjects And Methods**

**Subjects**

A total of 84 subjects, undergoing coronary angiography at Hajar University Hospital from October 2019 to December 2019, were included in the study. The cardiologist performed the final diagnosis of CAD by angiography. All patients had varying degrees of clogged arteries and healthy control was not found in this study. For this reason, subjects with arterial stenosis up 50% are in the CAD subjects (n=42), at least in one of the main coronary arteries, and patients with arterial stenosis <30% are in the control subjects (n=42). People with a history of chronic diseases (liver disease, kidney disease, and stroke), infectious diseases, cancer, allergies, and blood diseases were excluded. In the control group, individuals with a history of atherosclerotic plaque or angina were excluded. All subjects provided informed written approval to cooperate in this study, and the ethics committee of this study approved Shahrekord University of Medical Sciences.

**Clinical and laboratorial evaluation**

A complete physical examination was performed for all, also biochemical analysis of the blood. Patients' demographic information like drug treatment, family background, height, weight, smoking, systolic blood pressure (SBP), diastolic blood pressure (DBP), and body mass index (BMI) were also recorded by the researcher. Resting blood pressure was measured using standard instructions. Body mass index (BMI) was assessed with data on height and weight and weight/height 2 (kg/m2) formula. Serum was isolated, and the levels of biochemical parameters including blood glucose (BG), sodium, triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), blood urea nitrogen (BUN), potassium, high-density lipoprotein-cholesterol (HDL-C), creatine phosphokinase myocardial band (CPK-MB), creatinine, and troponin I in serum were measured using standard enzymatic and spectrophotometric techniques (Pars Azmoun Company's kit).

**TNF-α, IL-6, and IL-32 enzyme-linked immunosorbent assay (ELISA)**
IL-32 is evaluated by an ELISA kit (ZellBio GmbH; Germany) with intra-assay Coefficients of Variability (CV) < 10% and Inter-assay CV < 12%. Briefly, add (40μl sample(s) + 10μl IL-32-Ab), 50μl standards and 50μl Streptavidin-HRP, let them react for 60 minutes at 37°C. Following washing, add 50μl Chromogen solution A and 50μl B. 50μl stop solution was added and then the OD was read via ELISA reader (Dynex DS2, USA) at 450 nm. IL-6 and TNF-α serum levels were evaluated with an ELISA kit (Carmania Pars Gene, Kerman, Iran) and company instructions with intra-assay Coefficients of Variability (CV) < 3% and Inter-assay CV < 8%. Summarily, first added 100 μl of cell supernatant to 96 wells already coated with TNF-α and IL-6 capture antibody. After washing, bound proteins were identified via the addition of human TNF-α and IL-6 detection antibodies and HRP conjugated streptavidin. The TMB substrate was added and then the OD was read at 450 nm. TNF-α, IL-32, and IL-6 serum levels were also measured using standard samples.

Statistical analyses

Statistical analyses were implemented using GraphPad Prism software version 8.4.3 (GraphPad Software, La Jolla, CA, USA) and SPSS Statistics (SPSS Inc., Chicago, IL, USA). Categorical data were tested by an Independent-Samples t-test. Data were reviewed for normality by the Shapiro-Wilk normality test before any statistical analyses. Quantitative data were assessed via independent-samples t-test (between two groups). A receiver operating characteristic (ROC) curve analysis was used and area under curve (AUC) was assessed to determine the predictive value of each independent variable for CAD. Then, the multivariate logistic regression analysis was used for determining the correlation of TNF-α, IL-6, and IL-32, LDL and ox-LDL with CAD subjects. To examine the association among parameters for parametric data used Pearson correlation analysis. P ≤ 0.05 was assumed significant.

Results

Anthropometric characteristics and biochemical parameters of the study subjects

Anthropometric measurements and biochemical parameters of the study subjects are revealed in Table 1. No significant difference was found in the age, potassium, gender, HDL-C, BMI, SBP, DBP, TG, BUN, sodium, and creatinine among the participants. In contrast, BG, CPK-MB, TC, LDL-C, and Troponin I levels showed a significant increase in the CAD subjects in compare with control subjects (mean of control group Trop I = 93.38 ng/ml, mean of CAD subjects Trop I mean = 11753 ng/ml).
Table 1
Anthropometric characteristics and biochemical parameters of the study subjects.

| Variables                  | Control (n = 42) | Case (n = 42) | P-value  |
|----------------------------|------------------|---------------|----------|
| Age (year)                 | 59.76 ± 12.38    | 64.69 ± 12.46 | 0.065    |
| BMI (kg/m2)                | 25.58 ± 3.35     | 25.56 ± 3.48  | 0.981    |
| Gender [male (%)]          | 51.28            | 48.78         | 0.853    |
| SBP (mmHg)                 | 122.62 ± 14.09   | 126.74 ± 15.49| 0.231    |
| DBP (mmHg)                 | 75.35 ± 19.20    | 77.02 ± 16.49 | 0.579    |
| BG (mg/dL)                 | 107.71 ± 28.95   | 125.6 ± 27.06 | 0.004    |
| TC (mg/dL)                 | 139.88 ± 41.54   | 168.17 ± 39.73| 0.02     |
| TG (mg/dL)                 | 145.48 ± 78.33   | 153.43 ± 107.31| 0.613   |
| HDL (mg/dL)                | 57.19 ± 10.91    | 55.95 ± 16.28 | 0.778    |
| LDL (mg/dL)                | 56.02 ± 38.59    | 81.53 ± 35.46 | 0.003    |
| CPK- MB (µg/l)             | 9.74 ± 4.33      | 17.64 ± 9.81  | <0.0001  |
| Trop I (ng/l)              | 93.38 ± 73.07    | 11753 ± 17555.72| <0.0001| |
| BUN (mg/dl)                | 18.31 ± 0.77     | 20.17 ± 0.93  | 0.131    |
| Creatinine (mg/dl)         | 0.898 ± 0.02     | 1.029 ± 0.03  | 0.08     |
| Sodium (mEq/L)             | 139.58 ± 0.58    | 140.95 ± 0.53 | 0.212    |
| Potassium (mEq/L)          | 4.01 ± 0.06      | 4.14 ± 0.07   | 0.178    |

BG, blood glucose; BMI, body mass index; BUN, Blood urea nitrogen; CPK- MB, Creatine phosphokinase-MB; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, Low density lipoprotein; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; Trop I, Troponin I.
IL-6, TNF-α, and IL-32 serum levels in the control and CAD group

TNF-α, IL-6, and IL-32 serum levels in the CAD subjects were significantly increased by 2.55, 3.32, and 3 -fold than the control subjects, respectively (Fig. 4A-B: TNF-α; \( P = 0.0029 \), IL-6; \( P < 0.0001 \), and IL-32; \( P < 0.0001 \)).

The utility of TNF-α, IL-6, and IL-32 serum levels in predicting CAD

The potential of TNF-α, IL-6, and IL-32 serum levels for diagnosis of CAD was examined by ROC curve analysis. Our data showed an almost good ability for differentiation among disease status and controls using TNF-α (Area = 0.716 (0.602–0.829), \( P = 0.001 \)), IL-6 (Area = 0.905 (0.838–0.971), \( P < 0.0001 \)), and IL-32 (Area = 0.783 (0.681–0.886), \( P < 0.0001 \)) (Fig. 2A-C).

Association between TNF-α, IL-6, and IL-32 serum levels with the clogged arteries number in the CAD group

CAD-positive subjects were divided into two groups according to the cardiologists’ medical report: CAD-positive subjects with cardiac arterial stenosis in one major vessel (group A) and CAD-positive subjects with cardiac arterial stenosis in more than one major vessels (group B). TNF-α and IL-32 serum levels in the CAD subjects suffering from cardiac arterial stenosis in one major vessel were significantly increased by 2.94 and 1.7-fold than CAD subjects with cardiac arterial stenosis in more than one major vessels, respectively (Fig. 3A and 3D: TNF-α; \( P = 0.009 \) and IL-32; \( P < 0.024 \)). But there was no significant
difference in IL-6 serum levels with the number of clogged arteries in the CAD subjects (Fig. 3B: \( P = 0.499 \)).

**Independence of TNF-\( \alpha \), IL-6, and IL-32, LDL and ox-LDL serum levels in predicting CAD**

Multivariate logistic regression analysis can be used to estimate odds ratios for TNF-\( \alpha \), IL-6, and IL-32 serum levels in predicting CAD. Multiple logistic regression analyses suggested that TNF-\( \alpha \), IL-6, and IL-32, LDL and ox-LDL serum levels were independently related to the presence of CAD, while HDL serum levels were not. Our multivariate logistic regression model confirmed that TNF-\( \alpha \) (OR = 0.988, 95% CI 0.977–0.998, \( P = 0.027 \)), IL-6 (OR = 1.107, 95% CI 1.052–1.189, \( P = 0.002 \)), IL-32 (OR = 1.087, 95% CI 1.028–1.169, \( P = 0.009 \)), LDL (OR = 0.948, 95% CI 0.891–0.989, \( P = 0.038 \)), and ox-LDL (OR = 1.113, 95% CI 1.041–1.222, \( P = 0.008 \)) were proved to be independent predictors of CAD.

**Correlation between IL-32 serum levels with TNF-\( \alpha \) and IL-6 in CAD subjects**

IL-32 serum levels did not show a correlation with TNF-\( \alpha \) and IL-6 serum levels in CAD subjects (Fig. 4A-B). Also, serum levels of IL-6 had not a correlation with TNF-\( \alpha \) serum levels in CAD subjects (Fig. 4C).

**Discussion**

Atherosclerosis is a chronic inflammatory disease discriminated by endothelial cell dysfunction and plaque formation and immune cells have a vital function in the process of atherosclerosis (11). Cytokines are expressed by various cells implicated in atherosclerosis's pathogenesis and act on multiple purposes, with numerous effects, and are mostly responsible for clogged arteries (12). In this study, we found that TNF-\( \alpha \), IL-6, and IL-32 serum levels were significantly increased in the CAD subjects compared to the control group. The results of a study by Yang. et al. confirmed that IL-32 plasma levels were significantly higher in CAD group and positively correlated with the severity of CAD. The principal source of IL-32 is macrophages and CD4\(^+\) T lymphocytes, but they are also secreted by endothelial cells and smooth muscle cells. (13). Gotsman et al. reported that IL-6 and TNF-\( \alpha \) serum levels were significantly higher in CAD group and independently related to the severity of CAD (14). Another study reported that treatment with anti-IL-6 receptor antibody was beneficial in restricting atherosclerosis provoked by dyslipidemia and inflammation (15). A novel study observed that IL-6 and TNF-\( \alpha \) serum levels were significantly higher in CAD group and had a positive associated with the stenosis severity of CAD (16). In contrast, our results showed that serum levels of TNF-\( \alpha \) and IL-32 in the CAD subjects suffering from cardiac arterial stenosis in one major vessel were significantly increased than CAD subjects with cardiac arterial stenosis in more than one major vessel. But no statistically significant difference was found in IL-6 serum levels with the number of clogged arteries in the CAD subjects. We suggest two reasons for these results: a) TNF-\( \alpha \) and
IL-32 are proinflammatory cytokines and increased in the early disease. b) CAD subjects with cardiac arterial stenosis in one major vessel are in the early disease and CAD subjects with cardiac arterial stenosis in more than one major vessel are in the stage of chronic disease. IL-32 modulates inflammatory pathways for generating several proinflammatory cytokines like TNF-α, IL-6, and IL-1β which contribute to the pathogenesis of both atherosclerosis and inflammatory diseases (17). Several investigations have demonstrated that IL-32 plasma levels had a positive correlation with IFN-γ and IL-17 in CAD group and IL-17 and IFN-γ plasma levels were positively correlated with the severity of CAD (13). IL-17 and IFN-γ are the main cytokines of Th17 and Th1 cells, respectively. IL-32 may be implicated in CAD by regulation of Th1 or Th17 differentiation (13). The results of other studies reported that IL-32 regulates downstream inflammatory mediators like IL-6, TNF-α, IL-1β, CCL2/5, and MMP1/9/13 that is an essential mechanism partaking in the pathogenesis of CAD (17, 18). In contrast, our results demonstrated that IL-32 serum levels did not show a correlation with serum levels of TNF-α and IL-6 in CAD subjects. Moreover, serum levels of IL-6 did not show a correlation with serum levels of TNF-α in CAD subjects.

Previous studies have also reported that IL-32 regulation in human primary liver cells, HepG2, and THP-1 cells influence ABCG1, ABCA1, apoA1, and LXRα mRNA expression. This study showed a significant role for IL-32 in cholesterol homeostasis (19). Lei et al. reported that cholesteryl esters content increased with TNF-α treatment and decreased with ACAT inhibitor. This study indicated that TNF-α, by the NF-κB pathway, improves the formation of lipid-filled cells and raises cholesteryl esters accumulation through ACAT. Data of this research confirm the hypothesis that TNF-α is pro-atherosclerotic during the early phase of lesion development (20). A limitation of the present study was that all patients had varying degrees of clogged arteries and healthy control was not found in this study, and further studies needed to establish the role of IL-32 in the pathogenesis of CAD.

In conclusion, the increase of TNF-α, IL-32, and IL-6 in CAD group and serum levels of these cytokines showed good abilities in predicting CAD. These results suggested the participation of TNF-α and IL-32 in the early stage of CAD. Nonetheless, more investigations are needed to assess the potential causal correlation between IL-32 and the pathogenesis of CAD.

Declarations

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Ethical approval

This study was approved by the ethical board of Shahrekord University of medical sciences with number: IR.SKUMS.REC.1398.199.
Conflict of interest

All authors approved this manuscript. No competing interests declared.

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