Increased plasma concentrations of interleukin 35 in patients with coronary artery disease

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Abstract

Introduction: Atherosclerosis leading to coronary artery disease (CAD) is a chronic inflammatory condition. Interleukin 35 (IL-35) released by regulatory T cells (Tregs) has been found to be associated with CAD in the Chinese population. However, nothing is known about the relation between IL-35 concentrations and cholesterol levels. The aim of the study was to assess the levels of IL-35 in CAD patients and healthy subjects from a Caucasian population, and to analyze the relationship between IL-35 and the levels of total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, left ventricular ejection fraction (LVEF), sex and postmenopausal status.

Material and methods: Thirty-one patients with CAD and 30 healthy controls were included in the study. Levels of plasma IL-35 were analyzed by ELISA. The LVEF was assessed by transthoracic echocardiographic examination. Plasma levels of cholesterol fractions and C-reactive protein (CRP) were assessed by immunoenzymatic methods.

Results: The CAD patients had higher levels of IL-35 as compared to healthy controls (58.1 ±16.6 pg/ml vs. 5.35 ±3.35 pg/ml; \( p < 0.001 \)). IL-35 levels negatively correlated with total and LDL cholesterol concentrations (\( R = –0.31, p < 0.01 \)) and positively correlated with HDL cholesterol in men (\( R = 0.53, p < 0.01 \)). In women, IL-35 levels negatively correlated with LVEF (\( R = –0.29, p < 0.05 \)) and positively with the duration of postmenopausal status (\( R = 0.55, p < 0.01 \)).

Conclusions: These results suggest a possible association between high levels of IL-35 and CAD.

Key words: interleukin-35, coronary artery disease, atherosclerosis, cholesterol, menopause, left ventricular ejection fraction.

Introduction

Atherosclerosis leading to the development of coronary artery disease (CAD) is a chronic inflammatory condition [1]. Many immune disorders are observed in atherosclerotic plaques, such as increased synthesis of the Th1-type cytokines interferon (IFN)-\( \gamma \) and interleukin (IL)-12, which predominate over Th2 cell mediators [2, 3]. As an imbalance between T helper 1 (Th1)- and T helper 2 (Th2)-type cytokines does not fully account for the immune dysfunctions observed in atherogenesis, the immunomodulatory cytokines of other Th cell subsets may be potentially significant. According to Lin et al., patients with CAD demonstrate significantly lower plasma
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C-reactive protein (CRP) levels were assessed in all patients, and only those with a CRP below 10 mg/l were included in the study (Table I B). The informed consent was received from each patient included in the study. The clinical status of CAD patients is described in Table I A.

**Collecting and processing of blood samples**

Fasting blood samples were obtained by venous puncture, collected in sodium-heparin vacuum tubes and processed within 45 min by centrifugation for 20 min at 2200 g. The supernatant was stored at −80°C. All samples were thawed only once.

**Interleukin-35 assessment in ELISA**

Plasma IL-35 levels were assessed by enzyme-linked immunosorbent assay (ELISA) using a ELISA ST-360 ELISA microplate reader (450 nm and 630 nm) according to the manufacturer’s protocol (USCN Life Science Inc, E92008Hu). The range of IL-35 ELISA was 15.6–1000 pg/ml.

| Parameter | CAD (n = 31) | Healthy (n = 30) |
|-----------|-------------|-----------------|
| Age [years] | 63.8 ±1.3 | 58.2 ±0.7** |
| Gender (female/male) | 12/19 | 15/15 |
| Hypertension, n (%) | 29 (93) | 0 (0) |
| DM, n (%) | 8 (25) | 0 (0) |
| Obesity, n (%) | 9 (29) | 0 (0) |
| Nicotinism, n (%) | 6 (19) | 0 (0) |

| Parameter | CAD (n = 31) | Healthy (n = 30) |
|-----------|-------------|-----------------|
| TCH [mg/dl] | 166.1 ±9.3 | 215.6 ±5.9*** |
| LDL [mg/dl] | 96.4 ±7.5 | 129.1 ±6.1*** |
| HDL [mg/dl] | 50.1 ±3.0 | 63.4 ±2.9*** |
| TG [mg/dl] | 115.3 ±8.9 | 115.6 ±9.5 |
| CRP [mg/l] | 3.18 ±0.56 | 2.1 ±0.4 |
| LVEF (%) | 50.0 ±2.3 | 63.0 ±0.6*** |

**Material and methods**

**Patients**

The study included 61 adult subjects: a group of 31 coronary artery disease patients (12 females and 19 males, age 63.8 ±1.3), and a control group of 30 healthy volunteers (15 females and 15 males, age 58.2 ±0.7) (Table I A). The inclusion criterion was the presence of coronary artery disease, defined as at least 75% stenosis of one of the coronary arteries diagnosed by angiography of the coronary arteries by a cardiologist. The exclusion criteria were as follows: autoimmune diseases, allergy, immunodeficiency, neoplastic disease, immune response modifying drugs, such as glucocorticoids, cytostatics, monoclonal antibodies, tuberculosis, AIDS and HIV infection, viral hepatitis and fever. The criteria for exclusion from the control group were the presence of any symptoms of coronary artery disease in the history and ECG, and any other acute and chronic diseases. Plasma C-reactive protein (CRP) levels were assessed in all patients.
Plasma C-reactive protein assessment

Plasma CRP levels were assessed by immunoturbidimetry, amplified with latex molecules (Co-bas c, Roche Diagnostics, USA) using a Roche/Hitachi Cobas 6000 in the Wladyslaw Bieganski Hospital Laboratory, Lodz, Poland.

Plasma total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides assessment

Plasma cholesterol and triglyceride levels were assessed enzymatically, while plasma HDL cholesterol concentration was assessed immunoenzymatically. A Cobas 6000 (Cobas Integra, Roche Diagnostics) instrument was used for all measurements in the Wladyslaw Bieganski Hospital Laboratory, Lodz, Poland.

Left ventricular ejection fraction assessment

Transthoracic echocardiographic examination was performed with MyLab 25 Gold, Esaote. Linear measurements were made according to the European Society of Echocardiography [12]. The LV volumes used to estimate ejection fraction (LVEF) were determined using the modified biplane Simpson’s method. All echocardiographic measurements were performed by a cardiologist with subspecialty training in echocardiography. Reproducibility of echocardiographic measurements (interobserver and intraobserver bias) was assessed in the patients as a coefficient of variance from two sets of measurements made by two observers.

Statistical analysis

The results are presented as mean ± SEM for variables with a normal distribution of values. The distribution of particular variables was verified by the Shapiro-Wilk W test, whereas the Levene test was performed to test homogeneity of variances. If the results demonstrated normal distribution and homogeneous variance, the significance of differences between 2 groups was estimated using the Student t-test for independent trials. However, if any of these criteria were not fulfilled, a Mann-Whitney U test was used for analysis of the differences between the 2 groups. Spearman’s correlation was used to calculate correlations between IL-35 levels and other measured parameters. In all tests, a value of p < 0.05 was considered to be statistically significant.

Results

Plasma levels of IL-35 and CRP in coronary artery disease patients

Plasma concentrations of IL-35 were significantly higher in patients with CAD (n = 31) as compared to healthy donors (n = 30) (58.1 ±16.6 pg/ml vs. 5.35 ±3.35 pg/ml; p < 0.001) (Figure 1). Moreover, out of 31 CAD patients, IL-35 was detectable in the sera of 21 (68%) individuals, whereas 5 of 30 (16%) healthy controls had detectible levels of IL-35. This indicates that the levels of IL-35 in most of the healthy subjects was very low. In order to exclude the presence of systemic acute or chronic inflammation, which may interfere with IL-35 levels and cause the difference in IL-35 concentrations between groups, patients with CRP below 10 mg/l were included in the study. There were no differences in CRP levels between CAD patients and healthy subjects (Table I B).

Total cholesterol, LDL, HDL and triglyceride levels

The levels of total cholesterol, LDL and HDL cholesterol and triglycerides, among the strongest risk factors of CAD, were assessed in the patients included in the study. Our results reveal that the total cholesterol and LDL concentrations in the sera were lower in the CAD patients (n = 31) than the healthy subjects (n = 30) (166.1 ±9.3 mg/dl vs. 215.6 ±5.9 mg/dl; p < 0.001; and 96.4 ±7.5 mg/dl vs. 129.1 ±6.1 mg/dl; p < 0.001, respectively). Lower levels of both total cholesterol (TCH) and LDL in CAD patients occurred due to statin therapy. The CAD patients (n = 31) were also found to have lower levels of HDL (50.1 ±3.0 mg/dl vs. 63.4 ±2.9 mg/dl; p < 0.001). No difference was observed in TG levels between the analyzed groups (115.3 ±8.9 mg/dl vs. 115.6 ±9.5 mg/dl; p > 0.05) (Table I B).

Associations between plasma IL-35 levels and total cholesterol, HDL and LDL cholesterol and triglyceride concentrations

Next, we analyzed the relation between IL-35 plasma levels and CAD risk factors. Negative correlations were observed between IL-35 level and...
both the total cholesterol and LDL cholesterol concentrations in the whole group of patients included in the study \((R = -0.43, p < 0.0001, \text{ and } R = -0.31, p < 0.01, \text{ respectively}; n = 61)\). Conversely, a trend, however, non-significant statistically, was observed between IL-35 levels and HDL cholesterol concentrations in all patients \((R = 0.23, p = 0.06, n = 61)\). However, while further analysis revealed that this correlation was negative in the group of women \((R = -0.34, p = 0.07, n = 61)\), this correlation was found to be strongly positive in men \((R = 0.53, p = 0.001)\) (Table III). No association was found between IL-35 levels and triglyceride concentrations \((n = 61)\) (Table II).

**The association between LVEF and plasma IL-35 levels**

The LVEF was still significantly lower in CAD patients \((n = 31)\) than in healthy controls \((n = 30)\) \((50.0 \pm 2.3\% \text{ vs. } 63.0 \pm 0.6\%; p < 0.001)\) (Table I B). Interestingly, a weak negative correlation was observed between IL-35 levels and LVEF in all subjects \((R = -0.29, p < 0.05, n = 61)\), whereas further analysis revealed that while this correlation was stronger in the female subjects \((R = -0.48, p = 0.01, n = 27)\), no association between these parameters was observed in the male subjects \((R = 0.2, p > 0.05, n = 34)\) (Table III).

**Associations between plasma IL-35 levels, age of the patients and duration of postmenopausal status in women**

A positive correlation was observed between IL-35 levels and age of patients included in the study \((R = 0.26, p < 0.05, n = 61)\) (Table II). However, while this positive correlation was found to be much stronger in the women \((R = 0.67, p = 0.001, n = 27)\), no association between these two parameters was found in the men \((R = 0.2, p > 0.05, n = 34)\) (Table III). Further analysis revealed a positive correlation between the IL-35 levels and the duration of postmenopausal status in women \((R = 0.55, p < 0.01, n = 27)\). Additionally, in the group of postmenopausal women \((n = 15)\) IL-35 plasma concentrations were found to be significantly higher as compared to premenopausal women \((n = 12)\) \((59.4 \pm 18.7 \text{ pg/ml vs. } 3.9 \pm 3.0 \text{ pg/ml}; p < 0.01)\) (Figure 2). Mean duration of post-menopausal status was 13.54 ± 1.6 years \((n = 15)\).

**Table II. Association between plasma IL-35 levels and CAD risk factors, CRP, LVEF, age and duration of postmenopausal status in whole group of patients included in the study \((n = 61)\)**

| IL-35 [pg/ml] vs. | R Spearman’s | P-value |
|------------------|--------------|---------|
| TCH [mg/dl]      | -0.43        | < 0.001 |
| LDL [mg/dl]      | -0.31        | < 0.01  |
| HDL [mg/dl]      | 0.23         | 0.06    |
| TG [mg/dl]       | -0.10        | > 0.05  |
| CRP [mg/l]       | -0.05        | > 0.05  |
| LVEF (%)         | -0.29        | < 0.05  |
| Age [years]      | 0.26         | < 0.05  |

**Table III. Association between plasma IL-35 levels and CAD risk factors, CRP, LVEF, age and duration of postmenopausal status in women \((n = 27)\) and men \((n = 34)\)**

| IL-35 [pg/ml] vs. | Female \((n = 27)\) | Male \((n = 34)\) |
|------------------|----------------------|-------------------|
| HDL [mg/dl]      | R Spearman’s | P-value | R Spearman’s | P-value |
|                  | -0.34       | 0.07     | 0.53        | < 0.01  |
| LVEF (%)         | -0.48       | 0.01     | 0.2         | > 0.05  |
| Age [years]      | 0.67        | 0.001    | 0.14        | > 0.05  |
| Post-menopause   | 0.55        | < 0.01   |              |         |

**Discussion**

The present study demonstrates that patients with CAD have significantly higher plasma concentrations of IL-35 as compared to healthy individuals. This result contradicts those recently published by Lin et al., who report that IL-35 levels were significantly lower in stable angina patients as compared to individuals with chest pain syndrome not associated with CAD \[4\].

According to Lin et al., decreased IL-35 levels might be negatively associated with the inflammatory status of CAD patients \[4\]. Notably, in this study, subjects with stable angina not only had significantly higher CRP levels, reflecting the current inflammatory condition, than the control group, but they also displayed lower concentrations of IL-35. In our study, however, the levels of CRP were

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As lipid disorders are among the most important risk factors of atherosclerosis and CAD development, the association between IL-35 and lipid levels was also assessed. A negative correlation was noted between IL-35 levels and both total cholesterol and LDL cholesterol concentrations in the whole group of patients, implying the existence of a certain relationship between these factors. However, it should be noted that these correlations, although statistically significant, are not strong. Interestingly, oxidized cholesterol has already been shown to also decrease the production of IL-10 [13], an anti-inflammatory cytokine. Moreover, treatment of hypercholesterolemic individuals with atorvastatin was evidenced to decrease LDL cholesterol concentrations and increase serum levels of IL-10 [14]. Notably, in our study, patients with CAD displayed lower levels of total cholesterol and LDL cholesterol as compared to the healthy group. Lower levels of both total cholesterol and LDL cholesterol in CAD patients could result from the statin therapy, which, according to the current guidelines, is a key and obligatory component of the pharmacological secondary prevention after myocardial infarction. Therefore, one cannot prejudge about the possible significance of the correlation between LDL cholesterol and IL-35.

There are almost no data in the literature on the effect of statins on IL-35 generation. Only atorvastatin has been shown to increase IL-35 levels in combination with methylprednisolone in the cerebrospinal fluid in multiple sclerosis patients as compared to methylprednisolone monotherapy [15]. As the majority of CAD patients included in the study were on statin therapy, one cannot exclude that it may have affected IL-35 levels. Further studies are needed.

High-density lipoprotein cholesterol levels were found to be significantly lower in CAD patients than in the healthy group. Additionally, a positive, however, non-significant trend of correlation was observed between IL-35 and HDL cholesterol levels (p = 0.06). Surprisingly, further analysis revealed that in the group of women, a positive, however, non-significant trend of correlation was negative (p = 0.07), whereas in men, it was found to be strongly positive (p = 0.001). Although there was no difference in IL-35 levels between women and men, HDL cholesterol concentrations were significantly higher in women (data not shown). Therefore, it could be speculated that HDL, contrary to LDL cholesterol, may positively regulate IL-35 synthesis in men.

Further analysis of the association between plasma IL-35 levels and the age of patients revealed certain differences related to gender. Firstly, the positive correlation between the IL-35 concentrations and age in women suggests that older male individuals tend to have higher IL-35 levels. We also observed that IL-35 plasma concentra-

![Figure 2. Plasma IL-35 concentrations in postmenopausal (postMENO, n = 12) and premenopausal (preMENO, n = 15) women; statistical significance ***p < 0.01](image-url)
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In conclusion, CAD patients have higher levels of IL-35 as compared to healthy controls. Interleukin-35 levels negatively correlate with both total cholesterol and LDL cholesterol concentrations, positively correlate with HDL in men and negatively correlate with LVEF in all patients. Additionally, IL-35 levels are higher in postmenopausal women and may be related to the duration of the postmenopausal status.

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Conflict of interest

The authors declare no conflict of interest.

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