Correlation between expression levels of IL-37, GM-CSF, and CRP in peripheral blood and atherosclerosis and plaque stability

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Abstract
The study aimed to study the correlation between expression levels of interleukin-37 (IL-37), granulocyte macrophage colony-stimulating factor (GM-CSF), and C-reactive protein (CRP) in peripheral blood and the status of atherosclerosis (AS) and plaque stability and to confirm the clinical significance of these inflammatory factors in the pathogenesis of AS. A total of 64 AS patients (case group) were selected and divided into unstable plaque group (group A, 28 cases) and stable plaque group (group B, 36 cases) according to the color ultrasonography results of arterial vessels. At the same time, 30 healthy subjects were classified into the control group. General information of the enrolled subjects was collected, including levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), CRP, and homocysteine (Hcy). The expression levels of IL-37 and GM-CSF in the serum of peripheral blood samples collected from these subjects were measured by enzyme-linked immunosorbent assay (ELISA). There was no significant difference between the case group and the control group in the levels of TC, TG, HDL, and LDL (P > 0.05). However, the expression level of Hcy in the case group was significantly higher than that in the control group (P < 0.05). Compared with the control group, the expression levels of IL-37, GM-CSF, and CRP in the case group were significantly increased (P < 0.05). In addition, compared with group B, the expression level of GM-CSF in group A was significantly increased (P < 0.05), while no significant difference was detected between group A and group B in the expression levels of IL-37 and CRP (P > 0.05). In conclusion, inflammatory factors IL-37, GM-CSF, CRP, and Hcy were all involved in the pathogenesis of AS, and the increased levels of GM-CSF were closely related to the progress of unstable plaques. These results may aid the early diagnosis/treatment of AS.

Keywords
atherosclerosis, CRP, GM-CSF, inflammatory, IL-37

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Introduction
Atherosclerosis (AS) is a chronic inflammatory disease involving inflammatory cytokines and multiple types of cells (including vascular endothelial cells (ECs), mononuclear macrophages, mast cells, T lymphocytes, and vascular smooth muscle cells). Among them, EC damage is a key initiating step in the pathogenesis of AS. In addition, the cells most closely related to the pathogenesis of AS are monocyte–macrophages and T lymphocytes, whose interaction jointly promotes cell-mediated
immune responses. In the pathological process of AS, multiple inflammatory factors are secreted due to the dysfunction of vascular ECs. At the same time, adhesion molecules are expressed on the surface of ECs to attract white blood cells. When white blood cells interact with ECs, they secrete more inflammatory mediators and ingest oxidized low-density lipoprotein (LDL) to form foam cells. Subsequently, the development of AS lesions leads to the formation of fatty streaks, fibrous plaques, and atheromatous plaques. As a newly discovered immunosuppressive factor with significant anti-inflammatory effects, interleukin-37 (IL-37) can be expressed in various immune cells. It has been found that IL-37 is highly expressed in foam cells of human coronary arteries and AS plaques in the neck, suggesting that IL-37 is involved in the occurrence and development of AS. Secreted by inflammatory cells in plaques, granulocyte macrophage colony-stimulating factor (GM-CSF) can promote the regeneration of blood vessels, leading to plaque instability and promotion of AS lesion development. The inflammatory response against C-reactive protein (CRP) is closely related to the development of AS, especially in unstable plaques, in which the inflammatory activity of CRP becomes higher. In this study, the expression levels of inflammatory factors in the carotid artery of AS patients, such as IL-37, GM-CSF, and CRP, were measured to explore the relationship between these inflammatory factors and AS/plaque stability. In this study, expression levels of IL-37, GM-CSF, CRP, and other inflammatory factors in the serum of carotid artery cells were determined to explore the relationship between these inflammatory factors and AS and plaque stability, hence verifying the clinical significance of these inflammatory factors.

**Materials and methods**

**Subjects**

From November 2017 to September 2018, a total of 64 AS patients confirmed by carotid color ultrasonography at Ningbo Medical Center Lihuili Hospital were selected and classified into case group. Among them, 28 patients had unstable plaques and 36 patients had stable plaques (the plaque integral method was adopted: regardless of the plaque length, the maximum thickness of each isolated plaque on the same side was added to obtain the total score of the carotid artery plaque on that side. The sum of carotid artery plaque integral on both sides was the total plaque integral. Healthy subjects were defined as those with less than 1.1 total plaque scores; the total plaque score of 1.1–5.0 indicated mild carotid AS, and stable plaque patients were considered. When the total plaque score was 5.1–10.0, moderate carotid AS was indicated, and the patients were considered to be unstable plaque patients). During the same period, 30 healthy subjects in the physical examination center were selected as the control group. During the same period, 30 healthy subjects in the physical examination center were selected as the control group, and color ultrasonography of carotid artery was performed. Informed consent was obtained from all patients, and this study was approved by the ethics committee of Ningbo Medical Center Lihuili Hospital.

Inclusion criteria are as follows: asymptomatic AS and AS with stable or unstable plaques, regardless of the severity of vascular luminal stenosis; smoking history of less than 5 years (according to World Health Organization’s (WHO) standard, one person who smokes more than one cigarette per day for over 1 year is defined as a smoker); aged 40–80 years, regardless of gender; and admitted to the hospital within 7 days of onset.

Exclusion criteria are as follows: (1) patients younger than 40 years or older than 80 years; (2) patients with infection in the respiratory tract, digestive tract, or urinary system, or those with signs of other chronic infectious diseases within 1 month prior to enrollment; (3) patients with chronic hepatitis, cirrhosis, and severe liver and kidney insufficiency; (4) patients with history of surgery or trauma within 1 month prior to enrollment; (5) patients with acute cerebral infarction, acute myocardial infarction, necrosis of limb embolism, and other acute or chronic histological and organ ischemic diseases; (6) patients with history of diabetes, blood diseases, chronic inflammatory diseases, autoimmune diseases, and other diseases.

**Main reagents and equipment used in the experiment**

Main reagents used in this study are as follows: IL-37 ELISA kit (R&D systems, USA), GM-CSF ELISA kit (R&D systems, USA), double
sandwich enzyme-linked immunosorbent assay (ELISA) kit (Sigma, USA), the first antibody working fluid, horseradish peroxidase, dilute sulfuric acid, and so on.

Main equipment used in this study are as follows: TGL-168 centrifuge, DENLEY DRAGON Wellscan MK 3 plate reader, Wellwash 4 MK2 microplate washer, PYX-DHS constant temperature incubator, XW-80A vortexer, pipettes, DHG-9023A dryrer, IU22 diasonograph, cobas e411 automatic immunoassay analyzer, 7600 automatic biochemical detector, and PHILIPS Brilliance 64-row multislice spiral CT.

**Collection of specimens**

The fasting venous blood samples of all the selected subjects were taken within 24h, and 2 mL of the samples was reserved followed by centrifugation for 10 min at 3000 r/min. The serum was separated, and 0.5 mL was taken and stored at −80°C. On the day of analysis, the specimens were thawed at room temperature, mixed on an oscillator for 10 min, sonicated for 1 h, and centrifuged at 4°C for 10 min (at 3000 r/min). Subsequently, the relevant biochemical indexes and CRP values of all samples were measured.

**Measurement of IL-37**

Each well was added with 100 µL of the standard substance or the test sample, and the reaction plate was thoroughly mixed and then incubated at 37°C for 40 min. The reaction plate was thoroughly washed 4–6 times with a washing liquid and dried with a piece of filter paper. A total of 50 µL of distilled water and 50 µL of primary antibody working fluid were added to each well (except the blank control). The reaction plate was then thoroughly mixed and incubated at 37°C for 20 min. After washing the plate, 100 µL of the enzyme-labeled antibody working solution was added to each well. The reaction plate was further incubated at 37°C for 10 min. After washing the plate, 100 µL of the substrate working solution was added to each well, and the plate was incubated in the dark at 37°C for 15 min. Finally, 100 µL of stop buffer was added to each well and mixed to terminate the reaction. The optical density (OD) value in each well was measured at 450 nm within 30 min using a microplate reader.

**GM-CSF detection**

Each well was added with 100 µL of the standard substance or the test sample, and the reaction plate was thoroughly mixed and then incubated at 37°C for 40 min. The reaction plate was thoroughly washed 4–6 times with a washing liquid and dried with a piece of filter paper. A total of 50 µL of distilled water and 50 µL of primary antibody working fluid were added to each well (except the blank control). Subsequently, the reaction plate was thoroughly washed again 4–6 times with the washing liquid and dried with a piece of filter paper. In the next step, 100 µL of the enzyme-labeled antibody working solution was added to each well. The reaction plate was further incubated at 37°C for 10 min. After the reaction plate was again thoroughly washed 4–6 times with the washing liquid and dried with a piece of filter paper, 100 µL of the substrate working solution was added to each well, and the plate was incubated in the dark at 37°C for 15 min. Finally, 100 µL of stop buffer was added to each well and mixed to terminate the reaction. The OD value in each well was measured at 450 nm within 30 min using a microplate reader.

**Detection of CRP, Hcy, and TG CHOHDLLDL (double-antibody sandwich ELISA kit)**

One hundred microliters of standard or sample to be tested was added to each well, and the reaction plate was thoroughly mixed and placed at 37°C for 40 min. The reaction plate was thoroughly washed 4–6 times with a washing liquid and dried on a filter paper. A total of 50 µL of distilled water and 50 µL of primary antibody working fluid were added to each well (except blank). The CRP monoclonal antibody was combined with the enzyme sample, then the monoclonal antibody was reacted with the sample and the standard substance to antagonize CRP, and the biotinylated CRP was added to make the immune complex appear, which gathered and adhered on the surface of the plate. The biotin protein streptavidin labeled by horseradish peroxidase was combined with biotin, and the substrate tetramethylbenzidine (TMB) was added. The enzyme turned blue, and dilute sulfuric acid was added. When it turned blue, dilute sulfuric acid was added to the terminal solution, and when it turned yellow, addition was stopped. The
absorbance was measured at a wavelength of 450nm under a microplate reader within 30 min.

The level of serum homocysteine (Hcy) was determined by the circulating enzyme method. The concentration of TG CHOLDLDDL (total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), and LDL) was detected with an automatic biochemical analyzer.

Statistical method

SPSS 22.0 software was used for statistical processing. The comparison of mean values obtained from small sample pools in two groups was carried out using the t-test after the normality distribution of the data was confirmed. The measurement data were expressed as (mean ± s). The data not obeying normal distribution or heterogeneity of variance were tested by the rank sum test. Counting data were compared by analysis of variance (ANOVA). P < 0.05 was considered to be statistically significant.

Results

Comparison of general data and relevant biochemical indicators between case and control groups

As shown in Table 1, there was no significant difference in age and gender of case group and control group (P > 0.05). There was no significant difference between the two groups in the levels of TC, TG, HDL, or LDL (P > 0.05). However, the level of Hcy expression was significantly increased in the case group (P < 0.05).

Comparison of expression levels of inflammatory factors between case group and control group

It can be concluded from Table 2 that the levels of IL-37, GM-CSF, and CRP in the case group were significantly higher than those in the control group, and the difference was statistically significant (P < 0.05).

Comparison of general data and the levels of inflammatory factors among group A, group B, and control group

From Table 3, it can be concluded that there was no significant difference among group A, group B, and control group in terms of the age and gender (P > 0.05). Compared with the control group, the expression levels of IL-37, GM-CSF, and CRP in group A and group B were significantly increased (P < 0.05). Compared with group B, the expression level of GM-CSF in group A was significantly increased (P < 0.05), although no significant difference was found between group A and group B in terms of their expression levels of IL-37 and CRP (P > 0.05).

Table 1. Comparison of general data and relevant biochemical indicators between case group and control group.

|                  | Case group (n = 64) | Control group (n = 30) | P value |
|------------------|---------------------|------------------------|---------|
| Age (years)      | 61.33 ± 10.13       | 57.32 ± 11.02          | 0.234   |
| Gender (male/female) | 48/16               | 21/9                   | 0.716   |
| TC, median (lower quartile, upper quartile) (mmoL/L) | 3.72 (3.41, 4.51)    | 3.90 (3.43, 4.81)      | 0.569   |
| TG, median (lower quartile, upper quartile) (mmoL/L) | 1.13 (0.86, 1.57)    | 1.44 (0.96, 1.56)      | 0.136   |
| LDL (mmoL/L)     | 2.18 ± 0.68         | 2.17 ± 0.73            | 0.985   |
| HDL, median (lower quartile, upper quartile) (mmoL/L) | 1.16 (0.93, 1.42)    | 1.25 (0.89, 1.73)      | 0.427   |
| Hcy, median (lower quartile, upper quartile) (mmoL/L) | 20.42 (16.48, 24.15) | 8.73 (6.54, 11.38)     | <0.001  |

Hcy: homocysteine; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TC: total cholesterol; TG: triglyceride.

Table 2. Expression levels of inflammatory factors in case group and control group.

|                  | Case group (n = 64) | Control group (n = 30) | P value |
|------------------|---------------------|------------------------|---------|
| CRP, median (lower quartile, upper quartile) (mmoL/L) | 34.16 (8.96, 103.92) | 3.42 (2.13, 4.55)      | <0.001  |
| IL-37, median (lower quartile, upper quartile) (μg/L) | 201.83 (158.47, 268.52) | 125.44 (88.16, 157.92) | <0.001  |
| GM-CSF, median (lower quartile, upper quartile) (ng/mL) | 8.77 (4.47, 36.17)   | 4.33 (3.35, 6.76)      | 0.002   |

CRP: C-reactive protein; GM-CSF: granulocyte macrophage colony-stimulating factor; IL-37: interleukin-37.
**Table 3.** Comparison of expression levels of inflammatory factor among group A, group B, and control group.

|                      | Group A (n = 28) | Group B (n = 36) | Control group (n = 30) | P value |
|----------------------|-----------------|-----------------|------------------------|---------|
| Age (years)          | 62.14 ± 11.02   | 59.84 ± 10.64   | 57.32 ± 11.02          | 0.163   |
| Gender (male/female) | 23/5            | 29/7            | 21/9                   | 0.927   |
| CRP, median (lower quartile, upper quartile) (mnoL/L) | 88.35 (23.02, 125.14) | 27.94 (4.85, 65.29) | 3.42 (2.13, 4.55) | <0.001 |
| IL-37, median (lower quartile, upper quartile) (μg/L) | 202.15 (181.54, 312.65) | 203.86 (145.29, 265.17) | 125.44 (88.16, 157.92) | <0.001 |
| GM-CSF, median (lower quartile, upper quartile) (ng/mL) | 68.91 (15.92, 96.15) | 5.26 (4.14, 11.19) | 4.33 (3.35, 6.76) | <0.001 |

CRP: C-reactive protein; GM-CSF: granulocyte macrophage colony-stimulating factor; IL-37: interleukin-37.

**Discussion**

AS is a chronic inflammatory disorder involving both innate and adaptive immunity.\(^9\) The results of this study showed that there was no significant difference in the levels of serum lipid between case group and control group (\(P > 0.05\)). Abnormal lipid metabolism promotes the formation of atheromatous plaques during the onset of AS to induce EC damages, one of the main causes underlying the pathogenesis of AS. However, no significant difference was detected between the two groups in terms of their levels of blood lipids (TC, TG, LDL, and HDL). Such results may be related to the diet taken by the patients and their use of lipid-regulating drugs.

IL-37, a novel inflammatory suppressor of AS, plays a role against AS at the cellular and molecular levels. It has been shown that increasing IL-37 can protect AS.\(^7\) GM-CSF, as an inflammatory factor, can be secreted by macrophages, smooth muscle cells (SMCs), and ECs in plaques.\(^10\) It has been shown that the increased expression level of IL-37 is correlated with the occurrence of AS. The expression level of IL-37 in the case group was significantly higher than that in the control group (\(P < 0.05\)), and the expression level of IL-37 in the control group was low. GM-CSF is involved in angiogenesis in AS plaques, which is closely related to the stability of AS plaques and disease progression. In this study, the GM-CSF level in the case group was significantly higher than that in the control group (\(P < 0.05\)). Meanwhile, this study found that the level of GM-CSF in group A was significantly higher than that in group B. This suggests that IL-37 may play an anti-inflammatory role by activating anti-inflammatory pathways, affecting gene transcription, and inhibiting inflammatory cells from producing pro-inflammatory factors. GM-CSF may cause plaque instability by promoting the formation of new blood vessels, which may lead to an increase in anti-inflammatory and pro-inflammatory cytokines in patients.

CRP can be synthesized by liver cells under the stimulation of TNF-\(\alpha\), IL-1b, IL-6, and other cytokines. CRP can activate inflammatory cells such as monocytes and granulocytes and induce inflammatory cells to secrete large amounts of cytokines, which further damage vascular endothelium.\(^11\) The mechanism of Hcy-induced formation of AS has not been fully elucidated, and numerous studies have confirmed that endothelial injury and vascular smooth muscle hyperplasia are the key links.\(^12\) It has been indicated that the inflammatory response of CRP is closely related to the development process of AS, especially in unstable plaques, the inflammatory activity level of CRP is more obvious, and the inflammatory activity level of AS terminates with the stability of plaque calcification.\(^13,14\) Hcy was involved in the occurrence of AS, and IL-37, GM-CSF, and CRP were all increased in the case group, among which the level of GM-CSF in group A was significantly higher than that in group B. These data can further predict that inflammation is involved in the whole process of the occurrence of AS, and inflammation can stimulate angiogenesis and the formation of unstable plaques.

In summary, there are still some deficiencies in this research. These data further confirm that inflammation is involved in the whole process of AS, and inflammation can stimulate angiogenesis and formation of unstable plaques. However, it remains to be further investigated. Our study only provides a research direction about the occurrence of AS, which is a very realistic research topic.
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References
1. Tousoulis D, Oikonomou E, Economou EK et al. (2016) Inflammatory cytokines in atherosclerosis: Current therapeutic approaches. European Heart Journal 37(22): 1723.
2. Ren H, Zhou X, Luan Z et al. (2013) The relationship between carotid atherosclerosis, inflammatory cytokines, and oxidative stress in middle-aged and elderly hemodialysis patients. International Journal of Nephrology 2013: 835465.
3. Xu XR, Zou ZY, Xiao X et al. (2013) Effects of lutein supplement on serum inflammatory cytokines, ApoE and lipid profiles in early atherosclerosis population. Journal of Atherosclerosis and Thrombosis 20(2): 170–177.
4. Pires AS, Souza VC, Paula RS et al. (2015) Pro-inflammatory cytokines correlate with classical risk factors for atherosclerosis in the admixed Brazilian older women. Archives of Gerontology and Geriatrics 60(1): 142–146.
5. Chiva-Blanch G, Urpi-Sarda M, Llorach R et al. (2012) Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: A randomized clinical trial. The American Journal of Clinical Nutrition 95(2): 326–334.
6. Frieri M (2012) Accelerated atherosclerosis in systemic lupus erythematosus: Role of proinflammatory cytokines and therapeutic approaches. Current Allergy and Asthma Reports 12(1): 25–32.
7. Liu J, Lin J, He S et al. (2018) Transgenic overexpression of IL-37 protects against atherosclerosis and strengthens plaque stability. Cellular Physiology and Biochemistry: International Journal of Experimental 45(3): 1034–1050.
8. Mahadik SR, Deo SS and Mehtalia SD (2008) Association of adiposity, inflammation and atherosclerosis: The role of adipocytokines and CRP in Asian Indian subjects. Metabolic Syndrome and Related Disorders 6(2): 121–128.
9. Shiomi M, Yamada S, Ito T et al. (2009) Correlation of visceral fat accumulation with plasma CRP and atherosclerosis in metabolic syndrome-prone WHHLMI rabbits. Atherosclerosis 9(1): 245–246.
10. Ji Q, Meng K, Yu K et al. (2017) Exogenous interleukin 37 ameliorates atherosclerosis via inducing the Treg response in ApoE-deficient mice. Scientific Reports 7(1): 3310.
11. Ditiatkovski M, Toh BH and Bobik A (2006) GM-CSF deficiency reduces macrophage PPAR-γ expression and aggravates atherosclerosis in ApoE-deficient mice. Arteriosclerosis Thrombosis and Vascular Biology 26(10): 2337–2344.
12. Ying R, Li SW, Chen JY et al. (2018) Endoplasmic reticulum stress in perivascular adipose tissue promotes destabilization of atherosclerotic plaque by regulating GM-CSF paracrine. Journal of Translational Medicine 16(1): 105.
13. Sun H, Koike T, Ichikawa T et al. (2005) C-reactive protein in atherosclerotic lesions: Its origin and pathophysiological significance. The American Journal of Pathology 167(4): 1139–1148.
14. Burke AP, Tracy RP, Kolodgie F et al. (2002) Elevated C-reactive protein values and atherosclerosis in sudden coronary death: Association with different pathologies. Circulation 105(17): 2019–2023.